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Original Article

IN VIVO TOXICITY STUDIES OF BIOSYNTHESIZED SILVER NANOPARTICLES USING *BRASSICA OLERACEAE* IN ZEBRA FISH MODEL

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ABSTRACT

Objective: Nanotechnology opens new applications in many fields including medicine, material science and various technologies. The aim of the current study is to synthesise nanoparticles of *Brassica oleraceae* by green chemistry approach which provides advancement over physical and chemical methods.

Methods: Silver nanoparticles of *Brassica oleraceae* were synthesized by conventional and microwave assisted methods. Further characterization was done using UV-Visible, FT IR, Scanning electron and Transmission electron microscopy. The synthesized silver nano particles of *Brassica oleraceae* were tested for cytotoxicity in Vero and Human epithelial carcinoma cells (HEp -2). The induction and characterization of apoptosis in treated cells were studied using Acridine orange and DAPI staining procedures. *In vivo* toxicity of silver nanoparticles of *Brassica oleraceae* was studied in the developmental stages of Zebra fish embryos. The antioxidant potential and level of oxidative stress induced by studying various enzymes in xenobiotic metabolism such as GST, GSH, GPx (inducers of xenobiotic metabolism) in Vero and HEp -2 cells.

Results: The nanoparticles of *Brassica oleraceae* were able to induce apoptosis in HEp-2 cells and as a potential inducer of Xenobiotic metabolism. Toxicity of the nanoparticles was assessed by studying the rapid diffusion of nanoparticles which is evidently observed in the developing embryos of Zebra fish.

Conclusion: The synthesized nanoparticles of *Brassica oleraceae* oleraceae were found to be stable and less toxic in normal cells when compared to cancer cells

Keywords: Red cabbage, Nanoparticles, *Brassica oleraceae,* Conventional method, Microwave assisted method.

INTRODUCTION

Nanoscience is a field that is budding with impact from different fields of biology. It is the creation and utilization of materials, devices, and systems through the control of matter on the nanometer-length scale, i. e. at the level of atoms, molecules and supra molecular structures. In recent years, nanotechnology has been assessed and implemented in different areas of medicine specially on cancer management and therapeutics with the hope that it will lead to major advances in cancer diagnosis and treatment in future years [1]. Nanotechnology distribution within the body is based on various parameters such as their relatively small size resulting in longer retention time in the cancer tissues These technologies have been applied to improve drug delivery and to overcome the problem of drug delivery in cancer cells. Cancer is the second leading cause of death globally [2].

Dietary chemo preventive compounds offer great potential in the fight against cancer by inhibiting the carcinogenesis process through the regulation of various cell defensive and cell-death machineries. Among the different plant groups crucifers occupies good role as an anticancer agent due to their enriched content of glucosinolates [3,4]. The rich red color of red cabbage (*Brassica oleraceae var rubra)* is due to the presence of anthocyanin a class of pigments which contribute to the antioxidant potential of the plant. Synthesis and assembly of nanoparticles using biological entities would benefit from the development of clean, nontoxic and environmentally acceptable "green chemistry" procedure, probably involving organisms ranging from bacteria to fungi and even plants. Synthesis of nanoparticles from plants could be advantageous over other environmentally biological processes by eliminating the elaborate process of maintaining cell cultures and is gaining importance due to its simplicity and eco-friendliness [5,6]. Silver nanoparticles are also being considered for use in the current generation biological application involving bioimaging probes and cancer treatment.

In the present study the silver nanoparticles of *Brassica oleraceae* was done by conventional and microwave assisted methods The characterization of synthesized silver NPs were done using UV-Visible spectroscopy, SEM, TEM, X-RAY Diffraction and FTIR. Further the cytotoxicity and induction of apoptosis of synthesized nanoparticles was studied using Acridine orange and DAPI staining. The antioxidant potential and as a detoxifiers (by induction of phase II xenobiotic metabolism) was studied using various phase 2 detoxification enzymes such as GST, GPx and catalase. The invivo toxicity assessment was studied on Zebra fish embryos.

MATERIALS AND METHODS

Samples and chemicals

*Brassica oleracea*e var rubra, Dulbecco's Minimum Essential Medium (DMEM), Minimum Essential Medium (MEM), Streptomycin, Penicillin G, Tryphan Blue, MTT, Silver nitrate, Crystal violet, DAPI (4,6-diamindino 2-phenylindole) dyes were obtained from Himedia. Foetal Bovine serum obtained from Himedia. HEp-2 and VERO cells were obtained from (National Centre for Cell Science, Pune and Madras veterinary College, Chennai).

Synthesis of silver nanoparticles from red cabbage

Red cabbage leaves were taken and cut into small pieces and air dried for seven days. The dried leaves were powdered and mixed with water and centrifuged for 10 mins at 10,000 rpm and filtered in a whatmann filter paper. The crude extract was used as a reducing agent. Silver nano particles of Red cabbage were prepared using silver nitrate solution. It was prepared by conventional and microwave assisted methods. The Red cabbage extract was taken in two sterile centrifuge tubes and stirred for 10mts at 37° C. For the conventional method the crude extract is mixed with Silver Nitrate solution and this crude extract was reduced by silver nitrate solution into silver nanoparticles. The solution is kept aside in dark room in room temperature for several days for the synthesis of

silver nanoparticles. The synthesized particles were confirmed by the colour change from pale purple colour to dark brown colour. Thus silver nanoparticles were synthesized by reducing the silver nitrate solution. In the case of the microwave method crude extract was mixed with 1 mM* Silver Nitrate solution and this crude extract reduces silver nitrate solution into silver nanoparticles. The solution was treated with laboratory scale microwave irradiation with several short burst in a cyclic mode (on 15s, off 15 s) to prevent overheating and aggregation of metals for 15 minutes for the synthesis of silver nanoparticles [7].

Characterization of synthesized silver nanoparticles

The synthesized nanoparticles from both conventional (CM) and microwave assisted (MAM) methods were characterized by UV-Visible spectra analysis, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Fourier Transform Infra Red (FT IR) and X ray Diffraction Analysis (XRD). T.h.e synthesised silver nano particles from two sources are designated as conventionl (BOAgP -1) and Microwave assisted (BOAgP-2). T.h.e synthesised particles after characterisation were assessed for its cytotoxicity and induction of apoptosis by DAPI and acridine orange staining

UV-Visible spectrometer analysis

The reduction of pure Ag^+ions was monitored by measuring the UV-Vis spectrum of the reaction medium after diluting the sample with distilled water. Then the sample was scanned in the UV range (200-400 nm*) range. The absorption measurements were done using a Varian Cary 50 UV-Vis spectrophotometer.

SEM and TEM analysis

Scanning Electron Microscopy (SEM) analysis was done to characterize the synthesized nanoparticles. Thin sample films were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid and were allowed to dry under a mercury lamp for 5 mins. Transmission Electron Microscopy was done by placing drops of the silver nanoparticles solutions on carbon coated TEM grids. The films on the TEM grids were allowed to dry in air for 2 mins [8].

XRD analysis

X-ray diffraction measurements were performed in X-ray powder diffractometry. This method is used for the determination of crystallinity of the synthesized silver nanoparticles. XRD data was analyzed to determine the proportion of the different minerals present using Scherrer's equation

$$D = \frac{K\lambda}{\beta^{1/2}\cos\theta}$$

$$D=K\lambda\beta 1/2\cos\theta$$

The equation uses the reference peak width at angle θ, where λ is the X-ray wavelength (1.5418 Å), $\beta^{1/2}$ is the width of the XRD peak at half height and K is a shape factor.

FTIR analysis

FTIR spectrometer simultaneously collects spectral data in a wide spectral range. This confers a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time.

***In vitro* studies**

Cell line maintenance and growth conditions

Vero cells and HEp-2 cells were seeded in culture flasks. Lyophilized Silver nanoparticles from two sources such as synthesized silver nanoparticles (BOAgP1 and BOAgP-2). were dissolved in DMSO (in the ratio of 5mg/ ml) and added to the cells. Cells in the exponential phase of growth were incubated for 24hr at 37^0C with 5% CO_2 incubator. Non treated cells were used as control.

Cytotoxicity assay – MTT method

HEp-2 and Vero cells were cultured in 96 well micro plates ($1x10^5$cells /well in 100 µl complete medium) for 12 h and then incubated with different concentration of Silver NPs synthesized silver nanoparticles (BOAgP-1 and BOAgP-2). The plate was incubated at 37°C in a CO_2 incubator for 48 hrs*. After incubation 10 µl of MTT (5 mg/ml) solution was added to each well and incubated for 4 hrs. The formazan formed was solubilized in 100µl in DMSO and the OD was recorded at 540 nm in ELISA plate reader. Growth inhibition was expressed as percentage [9].

Crystal violet staining

Cells are cultured and treated with silver nano particles (BOAgP-1 and BOAgP-2). A.f.ter 24 hrs cells were washed and fixed in 70% ethanol. Then crystal violet is added and observed under light microscopy to study the cell morphology.

Structural analysis and DAPI staining

DAPI staining was performed to determine the level of apoptosis in the cells. The cells ($5x10^3$) were seeded and treated with the silver nanoparticles for 24h. Untreated cells and the cells treated with DMSO (1%U/V) and H_2O_2 (0.0014%U/V) were used as controls. Treated cells and controls were rinsed with phosphate buffer saline (PBS); fixed with ice-cold 10% Trichloroacetic acid. After 15 mins it was further washed with cold 70, 80, 90% and absolute ethanol. The cells were permeablilised with Triton-x (10% U/V) and after 5 mins, stained with DAPI (4,6-diamindino 2-phenylindole) for 10 mins. To reduce the background, the stained cells were washed with PBS and were cover slipped with 90% glycerol. The stained cells observed under a fluorescence microscope [10].

Acridine orange staining

The treated cells ($1x10^6$) were washed in PBS and centrifuged for 5 mins. The cell pellet was resuspended in PBS and the cells were fixed by transferring the cell suspension in paraformaldehyde in PBS on ice and incubated for 15 min on ice and centrifuged for 5 mins and the cell pellet were resuspended in PBS again centrifuged and transferred to ethanol on ice. After 4 hrs* incubation, it was centrifuged for 5 mins and the cell pellet was resuspended in PBS followed by addition of RNAse A and incubated at 37°C for 30 mins. After centrifugation for 5 mins. The cell pellet was resuspended in PBS followed by addition of Acridine orange staining solution. Cells were observed under fluorescence microscope with an appropriate filter set up.

Antimicrobial activity

Antibacterial activity of the synthesized silver nanoparticles (BOAgP-1 and BOAgP-2). were done on various viz., *Escherichia coli, Salmonella typhii, Vibrio cholera, Staphylococcus aureus and Pseudomonas aeruginosa* by agar well diffusion assay method. The bacterial test organisms were grown in nutrient broth for 24 hrs. Muller Hinton agar plate was prepared and the different microbial suspensions were inoculated by spread plate technique at 37^0C for 24 hrs. The plates were examined for evidence of clear zones of inhibition [11].

Assay of antioxidant enzymes

Vero and HEp-2 cells were plated ($2x10^6$) and treated with 100 µg of synthesized silver nanoparticles (BOAgP-1 and BOAgP-2) of *Brassica oleraceae* for 24 hrs. Cells were collected from the culture flasks using cell scrapper. Cells were centrifuged to get cell pellet and the pellet was sonicated in cold phosphate buffer. Sonicated cell suspension was centrifuged at 3000 rpm for 10 min at 4°C. Supernatant was collected and was used for analysis for various antioxidant enzymes.

Assay of catalase

Catalase was assayed according to the method [12]. To 1.2 ml of phosphate buffer 0.5 ml of cell homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of hydrogen peroxide solution. The decrease in absorbance was measured at 240 nm in intervals of 3 minutes. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide.

Assay of Glutathione peroxidase

Glutathione peroxidase was assayed according to the method. The final incubation mixture consisted of 0.2 ml enzyme source, 0.2 ml of

EDTA, 1 ml of sodium azide and 0.2 ml of tris HCl buffer were added and mixed well. The solution was placed in a 37°C water bath and the reaction was started by the rapid addition of 0.2 ml of glutathione followed by 0.1 ml of H_2O_2 in distilled water. Samples were taken exactly 1 minute after addition of H_2O_2 and the reaction was stopped rapidly by the addition of 0.5 ml of 10% TCA. Non-enzymatic oxidation of GSH was measured in a blank containing the above reagents with buffer substituted for enzyme source. The residual GSH was then measured by the reaction with DTNB [13.14]

Assay of Glutathione-S-transferase

Glutathione S-transferase was assayed by the method [15]. T.h.e reaction mixture containing 1.0 ml buffer, 0.1 ml of CDNB and 0.1 ml of cell homogenate was made up to 2.5 ml with distilled water. The reaction mixture was pre incubated at 37°C for 5 min. 0.1 ml of GSH was added and the change in OD was measured at 340 nm for 3 min at 30 seconds interval.

Assay of reduced glutathione

Supernatant was collected and was used for the estimation of glutathione content. 100 - 400 µL of glutathione standard solution was pipetted to test tubes and the final volume was made upto 1 ml. 3 ml of phosphate buffer was added to all the tubes and mixed well. 0.5 ml of DTNB reagent was added to all the tubes and was incubated at room temperature for 5 minutes. Absorbance was taken at 412 nm within 10 minutes. 50 µL of cell suspension was treated as above and the absorbance was taken at 412 nm*. Blank tubes having all the reagents except glutathione solution and cell suspension was also included [16, 17].

***In vitro* stability study**

The synthesized silver nanoparticles (BOAgP-1 and BOAgP-2) were kept in the room temperature for 60 days and centrifuged at 5,000 rpm for 10 min at 37^{0}C to check the stability. These silver particles did not show any cloudiness or contamination and also proving the stability by observing the pH, colour of the solution which are also not changed.

***In vivo* toxicity analysis**

Zebra fish is the most popular vertebrate model systems and are found in freshwater lakes, streams and ponds. It is one of the most sensitive organisms used in ecotoxicity tests. Toxicity studies were conducted with the help of different concentrations of silver nanoparticles (BOAgP-1 and BOAgP-2) to determine the toxic effects seen in the zebra fish embryos. Ten randomly selected zebra fish embryos were placed in glass petriplates with egg water. Different concentrations of silver nano particles serially diluted and added to the petriplates containing zebra fish embryos to ensure a constant concentration in the beakers. They were covered with a aluminium foil and shifted in to a shaker. The petriplates were shooked constantly at 140 rpm throughout the 48-h exposure time. Shaking was chosen to minimize sedimentation of particles. After 48 h of exposure the immobilization and mortality of the individuals in each petriplates were assessed in stereo microscope. The experiment carried out in triplicates [18].

Statistical Analysis

The statistical analysis was done among the experimental groups with control and normal groups using SPSS Software Version 16 USA). T.h.e one way Annova was done for expressing experimental significance was accepted at a level of $p < 0.05$.

RESULTS AND DISCUSSION

The synthesis, characterization and application of biologically synthesized nanomaterials have become an important branch of nanotechnology. The silver nanoparticles were synthesized from *Brassica oleracea*e using conventional and microwave assisted methods (BOAgP-1 and BOAgP-2) (Fig.1) We studied the effect of silver nanoparticles of Red cabbabge with different methods such as conventional and microwave assisted methods. In this study the bio-reduction of aqueous Ag^+ ions by the leaf extract of the plant *Brassica oleraceae* by both the method were studied. T.h.e formation of silver nanoparticles by reduction of the aqueous silver ions during exposure of *Brassica oleraceae* extract was assessed by UV-Vis spectroscopy. The AgNP particles were confirmed by the colour change from pale purple colour to dark brown colour. The surface plasmon band in the silver nanoparticles solution remains close to 400 nm throughout the reaction period suggesting that the particles were dispersed in the aqueous solution with no evidence for aggregation. The peak is shifted from 380 nm to 420 nm* after the nanoparticles of *Brassica oleraceae* indicating the formation of Nanoparticles (fig 2). T.h.e SEM micrographs ofAgNps (fig 3) obtained in the filtrate showed silver nanoparticle in solution with an average size of about 21 -81 nm for both the types. TEM images recorded from the drop-coated film of the $AgNO_3$ synthesized by treating with silver nitrate solution. The particle size histogram of silver particles produced by red cabbage shows that the particles range in 10-25 nm and possess an average size of 22 nm for both the methods (fig.3.). The TEM was used to evaluate the hydrodynamic size of phytochemical coated silver nanoparticles from red cabbage. The violet red color observed in SEM and TEM determination of stable samples showed a well dispersed nanoparticles with silver by both the methods.

Fig. 1: Synthesis of silver nanoparticles of Brassica oleraceae by a) Conventional b) Microwave method

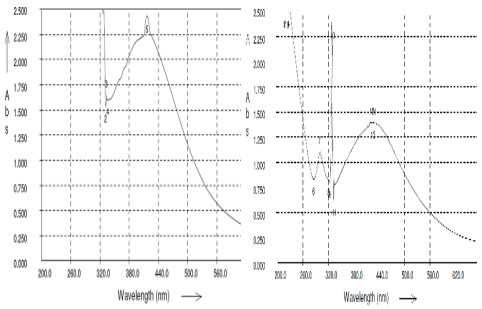

Fig. 2: UV absorption spectra of silver nanoparticles by (a) Conventional method (b) Microwave AssistedMethod

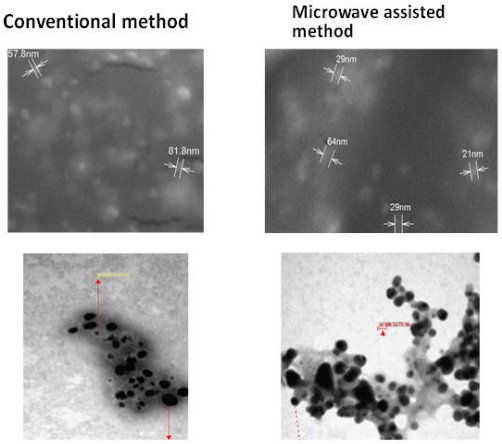

Fig. 3: SEM and TEM Micrographs of silver Nanoparticles of Brassica oleraceae

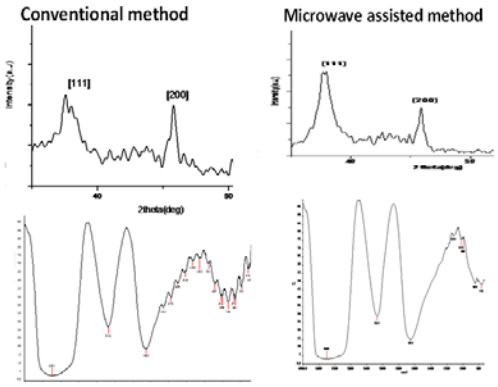

Fig. 4: XRD analysis of silver nano particles of Brassica oleraceae

The particle size of synthesised silver nanoparticles was characterised by XRD analysis. Analysis through X-Ray diffraction confirmed the presence of elemental silver signal of silver and it also provided additional information beyond basic identification. XRD patterns of the synthesised silver nanoparticles using the extract of *Brassica oleracea*e is shown in the fig. 4. The X ray diffraction peaks at 2θ=38 and 46 indexed as 111,200 planes of silver in is observed in conventional method and microwave assisted methods. The particle size of the silver was found to be 24 nm.

The FTIR spectra obtained from the synthesized nanoparticles samples showed absorbance bands observed in the region of 450-4000 cm^{*-1} are 497, 514, 591, 661, 675, 738, 802, 818, 887, 954, 1053, 1132, 1216, 1297, 1376, 1455, 1645, 2144, 3391 cm^{*-1}. The absorption bands for Microwave Assisted Method observed in the region of 450-4000 cm^{*-1} are 526, 681, 769, 846, 995, 1021, 1118, 1651, 2141, 3398 cm^{-1}. T.h.ese absorption bands are known associated with the stretching vibrations for C-Cl (alkyl)– C-Br (alkyl),  C C-C O, -C C- [(in ring aromatic], -C-C- [(in ring aromatic], C-O(esters, ethers) and C-O (polyols),  respectively. The total disappearance of this band after the bio reduction may be due to the fact that the polyols are mainly responsible for the reduction of Ag ions, whereby they themselves get oxidised to unsaturated carbonyl groups leading to a broad peak at 2660 cm^{*-1} Conventional Method and 2660 cm^{*-1}in Microwave Assisted Method (for reduction of Ag)(fig 4). XRD revealed that no elemental particles were present other than silver nanoparticles. FTIR results showed the functional groups which are responsible for the reduction of silver nanoparticles. The cytotoxic effect of silver nano particles of *Brassica oleracea*e were performed on HEp -2 cell in comparison to vero cells in a dose dependant manner.

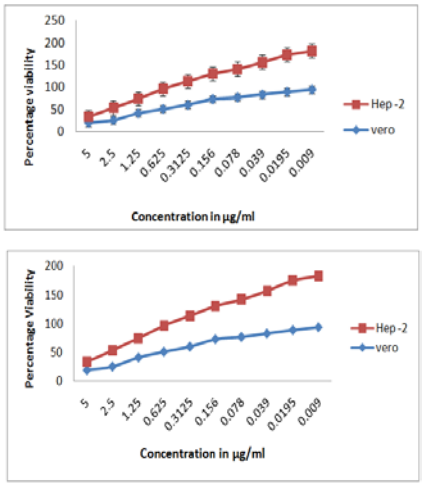

Fig. 5: Cytotoxicity assay of silver nano particles of Red cabbage by c)conventional and d) microwave assisted methods

The assay measures the reduction of dye by mitochondrial dehydrogenase and assumes the cell viability is proportional to the production of purple formazan which is measured spectrophotometrically. The percentage viability (IC_{50} value) of both the particles BOAgP-1 and BOAgP- 2 for HEp -2 and Vero cells were found to be 63 µg and 32 µg and 64 µg and 30 µg.

The values clearly indicate there is not much difference in the percentage viability of AgNPs prepared by different methods. Based on the morphological changes and nature of cell death in treated HEp-2 cells there may be induction of apoptosis which is further confirmed by staining using acridine orange and DAPI staining. The apoptosis can be assessed by measuring the apoptotic bodies or cell fragments. Integrity of mitochondrial membrane potential is considered as an identical feature of healthy cells. Researchers have reported that crucifers would induce apoptosis in treated various cancer cells such as HEp-2, MCF-7 etc. Cells marked with the clear formation of membrane blebbing and chromatin condensation related to apoptotic DNA damage are considered as markers of apoptosis. To further characterize the apoptosis induction was done by fluorescence microscopy analysis (fig 6). Treatment with different concentration of Silver NPs resulted in changes in nuclear morphology with a gradual increase in the amount of nuclear condensation after treatment with AgNPs. A few cells showed cell membrane blebbing and decreased ruffle formation. No blabbing was observed in control cells. The change in shape of cells from flat to round may suggest the destruction of extracellular matrix thus supporting the induction of apoptosis in treated cells [20, 21].

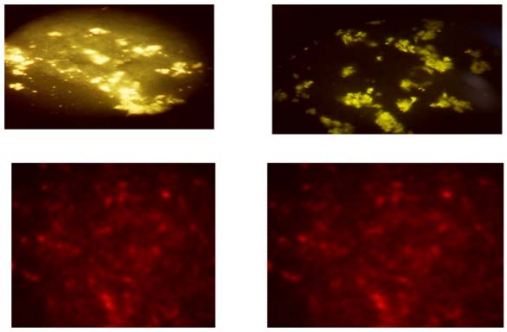

Fig. 6: DAPI and Acridine orange staining of nanaoparticles of Brassica oleraceae

The maximum zone of inhibition of AgNPs (BOAgP-1 and BOAgP-2) was observed against *S. t.y.phii* about 15 mm* (fig. 7). T.h.e mechanism of antibacterial action was presumed to be due to rupture of cell membrane thus creating low molecular weight region in the center of the bacteria to which the bacteria conglomerates resulting in the destruction of cell [22].

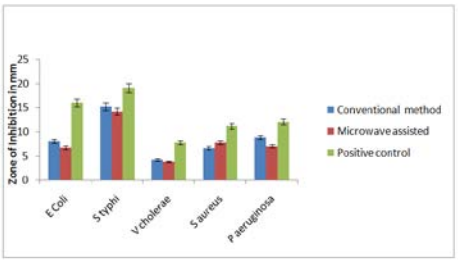

Fig. 7: Antimicrobial activity of silver nano particles of Red cabbage (Conventional and microwave assisted)

Enzymes such as GST, GPx and Catalase are integral parts of the antioxidant system responsible for the elimination of radicals and molecules produced as a consequence of oxidative stress. The GST is a family of phase II detoxification enzyme that catalyses the

conjugation of glutathione (GSH) to a wide variety of electrophilic substances such as drugs and toxins. The activation of the CAT, GST and GPx level in HEp-2 cells were substantially reduced when compared with the normal control group(fig 8). GSH is a ubiquitous sulfhydryl containing molecule in cell that is responsible for maintaining cellular oxidation-reduction homeostasis [32]. A significant depletion of GSH was observed on high concentration of Silver NPs relative to controls of GST activate in cell treated with AgNPs. Depletion in the GSH GST CAT and GPx level were found at 24 hrs* exposure when compared to the positive standard. This indicates a condition of oxidative stress in cells which may arise due to imbalance in the reactive oxygen species formation and antioxidant defense system of cell.

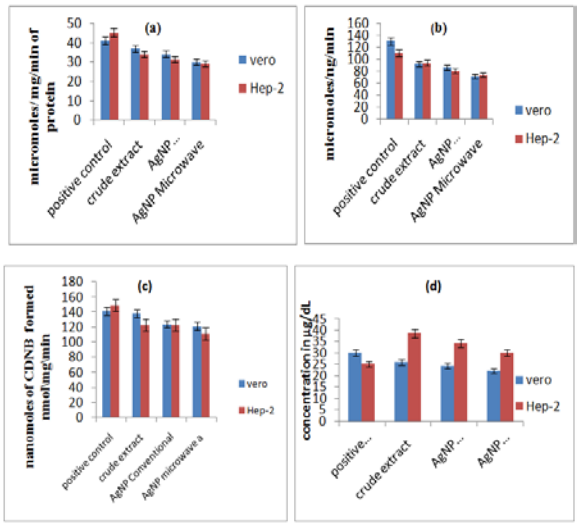

Fig. 8: Effect of silver nano particles of Red cabbage on a)Glutathione peroxidase b) Catalase c) Glutathione S Transferase b) Reduced Glutathione

Stability of synthesized nanoparticles was assessed by keeping the silver nanoparticles at 37°C in dark for several months and checked using UV –Vis spectroscopy. Freshly prepared nanoparticles of Brassica were used as control. A single peak was observed at 400 nm* for both the samples. This shows that the prepared nanoparticles were found to be stable with good shelf life properties.

In vivo toxicity studies were carried out in Zebra fish embryos. Different concentrations of synthesized nanoparticles from both methods were given in the egg medium along with the Zebra fish embryos. T.h.e varying concentrations starts from 1000 µg to 5000 µg /100ml* of the egg water and it is feeded to the embryos and observed their developmental stage pattern with the normal control embryos. Large amounts of dark material were found in the gut tract of fish embryo but not in the control (fig 9).

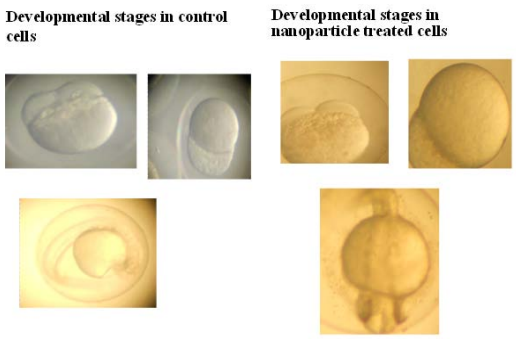

Fig. 9: *In vivo* toxicity analysis of silver nano particles of Brassica oleraceae

It indicates the clear absorption of silver nanoparticles by the embryos,. h.o.wever in the low concentration only ingestion was observed. Many researchers have supported that the silver nanoparticles are filtered from water and tract is filled with it. Aggregate adhere to the outer surface of animal [4, 17]. Large particles however are too difficult to process and thus are more easily ingested. Therefore the uptake and accumulation of nanoparticles is hypothesized to result in the mechanical disruption of feeding appendages and other physiochemical changes and cell death.

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CONFLICT OF INTERESTS

Declared None

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