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Original Research

Synthesis of Silver Nanoparticle from *Allium sativum* as an Eco-Benign Agent for Biological Applications

Mazhar Abbas[1], Tariq Hussain[1], Javed Iqbal[2], Aziz ur Rehman[3], Muhammad Arfan Zaman[3], Kashif Jilani[4]*, Nasir Masood[5], Samiah H. Al-Mijalli[6], Munawar Iqbal[7], Arif Nazir[7]**

[1]Department of Basic Sciences, University of Veterinary and Animal Sciences, Lahore (Jhang-Campus), Pakistan
[2]Deputy Director, Livestock and Dairy Development Department, Punjab, Tehsil Shorkot, District Jhang, Pakistan
[3]Department of Pathobiology, University of Veterinary and Animal Sciences, Lahore, (Jhang-Campus), Pakistan
[4]Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan
[5]Department of Environmental Sciences, COMSATS University Islamabad, Vehari Campus, Punjab, Pakistan
[6]Department of Biology, College of Sciences, Princess Nourah bint Abdulrahman University (PNU), Riyadh 11671, Saudi Arabia
[7]Department of Chemistry, The University of Lahore, Lahore, Pakistan

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Abstract

The study focuses on the fabrication of silver nanoparticles (Ag NPs) using aqueous extracts of *Allium sativum* as reducing agents. Silver ion becomes a good carrier of bioactive compounds of *Allium sativum*, so their biochemical potential such as total phenolic and flavonoids were evaluated. Results showed that as a concentration increases TPC and TFC were also increased. Similar to their secondary metabolite, scavenging potential of Ag NPs of *Allium sativum* was also monitored. Green synthesized Ag NPs exhibited significant antibacterial activity 24±2.6, 22±1.4, 19±2.7 and 20±3.17 against *B. subtilis, S. aureus, E. coli and P. multocida* bacterial strains respectively. Moreover, Ag NPs of garlic did not show any mutagenicity against mutant strain of *S. typhimurium* TA98 & TA100. Brine shrimp lethality assay (BSLA) showed their dose dependent effect. Finally, green synthesized Ag NPs of garlic presented anti-proliferative effect against HEPG2 cell line.

Keywords: synthesis, nanoparticles, eco-friendly, bioremediation, *Allium sativum*

*e-mail: Kashif.jillani@uaf.edu.pk
**e-mail: malikanphd2013@yahoo.com

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Introduction

Plant mediated fabrication of NPs has been increasingly becoming popular owing to its cost-effectiveness and environment friendly nature. Since ancient times, garlic (*Allium sativum*) is considered nutrition and traditional medicine throughout the world. This attribute of garlic is because of so many properties with biological application that have been reported. These properties may include antimicrobial, antioxidant, immunomodulatory, anticancer, anti-inflammatory, hypoglycemic and cardiovascular effects [1-3]. One of the most plenteous class of organosulfur compounds, present in recently sliced or crumpled garlic is found to be responsible for the health benefits associated with garlic consumption. In Europe, oil-macerated garlic product is commonly applied as healthy food but conversely this kind of use is very rare in United States and other countries [2]. There has been a highly increasing interest in natural antioxidants chiefly in nutritional products. For example, vegetables with antioxidant properties have a major role against diseases with oxidative stress. On the similar grounds, garlic holds possible health raising impact owing to high TPC and hance is considered as one of the most useful natural antioxidants [4-6]. Besides, garlic is comprised of different bioactive phytochemicals including phenolics, organosulfur compounds, allyl thiosulfates, flavonoids and vitamins. The most important out of all the components in garlic, the phenolics are mainly responsible for its potential as a health promoter. Additionally, these phenolics are present in huge quantities with their excellent pharmacological properties [7].

The nutritional role of polyphenolic materials and their features have been studied and assessed by multiple researches [8]. For that reason, garlic is considered to rummage reactive oxygen species (ROS). Furthermore, the role of a garlic has been wonderful for the treatment of many diseases especially the most treacherous one i.e., heart disease and cancer. Garlic extract protects oxidative DNA damage due to its significant antioxidant potential [1, 2]. Moreover, it decreases the radiation sensitivity of regular skins in the vicinal parts of tumors [9]. It could be useful for prevention of endothelial disfunction [10]. So it can be said that garlic is extensively used for the protection of oxidative stress, to decrease the danger of long-lasting diseases, avert disease development and treat or stop atherosclerosis and melanoma [3, 11, 12].

The study focuses on fabricating Ag NPs using *Allium sativum* extract for biological applications. The Ag NPs were synthesized using eco-friendly technique to check the cytotoxicity, mutagenicity and antibacterial activities.

Material and Methods

All chemicals and reagents employed in this study were purchased from Sigma Aldrich and Merck and were of analytical grade. The garlic was obtained from the local market, Faisalabad, Pakistan. The cloves of garlic were separated, peeled, sliced and dried. The dried flakes were made to fine powder with the help of grinder. The uniform sized particles were obtained by passing through 80 mesh sieves. The powder was kept sealed jars for further investigations. The aqueous extracts of garlic were prepared by maceration method. We have added 25 g sample in 250 mL flasks in respective aqueous solvents and shaked at 280 rpm, 37ºC for 4 h. Furthermore, the mixture was heated for 20 min at 60 °C. It was cooled down to 25ºC. It was then filtered and centrifuged at 300 rpm for 20 min. The extract was used to produce Ag NPs [13].

For the green synthesis of Ag NPs with *Allium sativum*, 2 mM solution were prepared by dissolving $AgNO_3$ salt in distilled water. Then equal volumes of molar solutions of $AgNO_3$ and *Allium sativum* solutions in 250 mL flask were mixed. The whole solution was lyophilized (freeze drying). Furthermore, the different concentration 10 mg/mL, 20 mg/mL, 30 mg/mL and 40 mg/mL will be used for their antimicrobial, antioxidant and cytotoxicity potential of green synthesized nanoparticles of *Allium sativum* [14].

Total phenolic contents of NPs were determined by Folin-Ciocalteu reagent method as described by Ainsworth and Gillespie, [15]. The total flavonoid contents of different concentration of green nanoparticles were determined following the method reported in Sakanaka et al. [16]. The DPPH assay was carried out as illustrated by Bozin et al. [17]. The absorbance was measured at 517 nm after incubating the sample for 30 min at 25ºC.

$$IC_{50}\ (\%) = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of blank}} \times 100$$

...where blank represents the absorbance of the control reaction (containing all reagents except the test compound) and while sample suggest the same for test compound. The assay was carried out in triplicate.

Antibacterial activity of green synthesized Ag NPs were performed against a panel of microorganism including *P. multocida*, *S. aureus, E. coli and B. subtilis* following the method described in Awwad et al. [18]. The cancer cells were inoculated into Dulbecco's Modified Eagle Medium (DMEM) and nurtured at 37ºC in a 5% CO_2 incubator. After 24 h, the bound cells were examined under a morphological reversal microscope. The cell line was further maintained in a CO_2 incubator and the DMEM was periodically replaced. A healthy cell culture was counted in hemocytometer and 5×10^4 cells/mL was used for MTT (3-[4, 5-dimethylthizol-2-yl]-2, 5-diphnytetrazolium

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bromide) assay. The reduction in MTT is generally because of glycolytic activity in the cell and depends on the presence of NADH and NADPH.

Mutagenicity of green synthesized Ag NPs garlic extract was tested using *S. typhimurium* strains TA98 and TA100 mutant strain. Following the standard recommendation of the Organization for Economic Cooperation and Development (OECD) Testing Guideline 471 [19]. A fresh culture of *S. typhimurium* strains TA98 and TA100 were prepared in nutrient broth and culture containing $2x10^8$–$2x10^9$ (CFUs/mL) cell were used for this study [20].

The brine shrimp assay is a bench top assay used for screening natural products for the presence of bioactive compounds. It was performed by following the method reported in [21]. Different concentration of green synthesized silver nanoparticles of garlic was prepared. The percentage of larvae viability were counted to reduce the baseness. it is repeated three time and average value were documented with mean standard deviation. All the tests were performed in triplicates. The significant differences were determined by one-way ANOVA and the significance level was kept at $p \leq 0.05$.

Results and Discussion

Green synthesized Ag NPs of garlic was prepared and their cytotoxicity, mutagenicity, antioxidant and antibacterial potential was documented. All the results are reported in Table 1.

Different *in vitro* and *in vivo* toxicity assays were performed for synthetic and organic substances used in foods ingredients and medicines. Brine shrimp lethality assay was performed to find out a correlation between toxicity and anticancer research. If some organic or inorganic substance is toxic for brine shrimp, it is likely to be a good candidate for anti-cancer research. From current study, brine shrimps nauplii viability are counted and their nauplii viability are presented in term of percentage, negative or blank control contain only sea water or saline water, with in the specified time period for 24 h, none of them is died. Cyclophosphamide was used as a positive control. Due to their lipophilic nature, it is penetrated into the newly brine shrimp nauplii and all of them were died with one hour and considered as 100 % mortality. Different concentrations (10 mg/mL, 20 mg/mL, 30 mg/mL and 40 mg/mL) of green synthesized Ag NPs of garlic were used against the brine shrimp larvae and it was observed that brine shrimp survival was dose dependent and inversely proportional. As the concentration increases, the larvae survival rate decreases. When brine shrimps were treated with 10 mg/mL of green synthesized silver nanoparticles of garlic 10 mg/mL, after 24 h only 15% larvae died and majority of larvae were surviving. But when concentration increase from 20 to 40 mg/mL the viability of brines shrimps also decreased up to 60%,

Table 1. Brine shrimp lethal activity, in vitro cytotoxicity, mutagenicity and antibacterial activity of green synthesized nanoparticles.

Ag NPs	BSLA viability (%)	MTT viability (%)	TA98	TA100
Negative control for MTT	-	92.36±6.28	-	-
Positive control for MTT	-	16.34±2.54	-	-
10 mg/mL	85±10	76.17±3.41	508±48	586±64
20 mg/mL	70±20	62.74±5.39	526±73	578±128
30 mg/mL	55±15	52.69±3.82	513±61	618±38
40 mg/mL	40±15	38.21±1.74	530±103	649±156
Negative control for BSLA	95±10	-	-	-
Positive control for BSLA	00±0	-	-	-
Blank (Negative control)	-	-	486±64	554±31
Positive control: TA98 ($K_2Cr_2O_7$)	-	-	3260±252	-
Positive control: TA100 (NaN_3)	-	-	-	3941±218
Antibacterial Activity				
Ag Nanoparticles	B. subtilis	*S. aureus*	*E. coli*	*P. multocida*
10 mg/mL	16±0.8	15±1.7	12±1.4	13±0.6
20 mg/mL	18±0.5	17±2.6	15±1.7	16±1.4
30 mg/mL	21±1.6	20±0.9	17±0.8	18±1.3
40 mg/mL	24±2.6	22±1.4	19±2.7	20±3.17
Rifampicin	34±1.25	30±1.38	28±1.83	35±1.62

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it is concluded that some bioactive compounds bind with silver particle and easily cross the cell lining and reduces the viability of larvae. The plants were good candidate for anti-cancer research on the basis of their behavior of toxicity to brine shrimp [1, 12, 22-24].

The MTT cell-proliferation assay is a colorimetric assay for assessing cell metabolic activity. In current study, HepG2 cell line was used to check the cell viability of green synthesized Ag NPs. From results documented in Table 1 shows that negative control group only growth media and HepG2 cells shows maximum cell viability 92.36±6.28 when positive control Cis-Platin was used as (5 µl/mL) only 16.34±2.54 cell was survived and more than 84% are unable to survive. Similar when green synthesized Ag NPs of garlic with different concentration from 10 to 40 mg/mL were used to check their antiproliferative activity from result it is concluded that it is dose dependent as concentration increase cell viability decreases, at 10 mg/mL maximum number of cell were able to metabolize the tetrazolium dye and result shows that up to 76.17±3.41 HepG2 cells were survive, but as concentration increase from 20 to 40 mg/mL cell viability decreases from 62.74±5.39, 52.69±3.82 and 38.21±1.74 respectively. Similar effect of *Allium wallichii* extracts as an anticancer were also reported by Bhandari et al. [25].

In-vitro mutagenicity of any organic and inorganic compounds is widely used using mutant strain of *Salmonella typhimurium* TA98 & TA100. Green synthesized Ag NPs of garlic was screened against TA98 &TA100. From results, it is concluded that using four different concentration of green synthesized AG NPs (10 to 40 mg/mL) did not show any cytotoxicity against tested mutant strains. A number of revertant colony for control or blank only contain selective growth media and fresh culture of *Salmonella tyhimurium* TA98, total number of revertant colony as an average was 486±64, but with 10 mg/mL NPs, total number of revertant colony was documented 508±48 which is non-significant to control group. Similar finding was also recorded for 20 to 40 mg/mL, the number of revertant colony was 526±73, 513±61 and 530±103 respectively. The colonies reversion in case of TA100 were slightly higher than TA98 strain. A total number of revertant from control to Ag NPs with 10 to 40 mg/mL were recorded as 554±31, 586±64, 578±128, 618±38, 649±156 respectively which is also showing non mutagenic potential of Ag NPs. It can be concluded that Ag NPs did not shows any mutagenic potential. So, using advanced purification technique, bioactive compounds may be isolated from garlic and used as an ingredient for the development of drugs.

The antioxidant activity of any plant-based material is associated with the quantity of polyphenols. These phenolics (secondary metabolites) have antioxidant capacity due to their behavior as a reducing agent, hydrogen donors and oxygen quenching ability. Therefore, the nutraceutical worth of any food is assessed by their quantification in the particular Food. The reaction with Folin-Ciocalteu reagent develops blue color solution. The intensity of color designates the intensity of phenolics. The TPC of green synthesized Ag NPs of *Allium sativum* at 10 mg/mL was 40.16±2.63 mg GAE/100g was quantitatively measured by spectrophotometric method. The concentration of Ag NPs of *Allium sativum,* was increased 20 mg/mL, 30 mg/mL and 40 mg/mL, TPC were also increasing 51.83±4.26 mg GAE/100 g, 68.57±1.42 mg GAE/100g and 84.16±3.54 mg GAE/100 g respectively. Our results are in accordance with the already reported findings [26]. It was inferred that garlic is a rich source of phenolics [24, 27].

Flavonoids are good indicators of pharmacological and biochemical effects and are connected to advanced antioxidant activities. Different concentrations of green synthesized Ag NPs of *Allium sativum* were used to screen out the total phenolic contents. At 10 mg/mL concentration of Ag NPs, TPC were quantified as 28.27±4.32 mg CE/100 g. On increasing the concentration to 20 mg/mL, the flavonoids contents were also increased, which is near to double of first concentration 37.35 ± 2.15 mg CE/100 g. Using 30 mg/mL and 40 mg/mL concentration flavonoids contents were also 49.74±2.38 mg CE/100g, 58.34±1.76 mg CE/100 g respectively measured. The present study is also in good agreement with previous work [2]. They evaluated that thermal treatment decreased the flavonoid content in garlic. The extraction of TPC and TFC depends on the solvent type, polarity and other experimental conditions. So, optimization of parameters is quite an important factor for proper extraction. In this regard, methanolic extracts revealed maximum extraction compared to other extracts. Our findings are in accordance with the reported study [28].

A fresh solution of stable free radical 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) was used as a standard. Different concentrations of Ag NPs were employed. We conclude that with the increase in the concentration of Ag NPs, the rummaging activity for free radical was also increased. The maximum value (79.8%) was observed at 40 mg/mL Ag NPs. On the other hand, at 10 mg/mL, it was 44.67%.

Antibacterial activity (Table 1) of green synthesized Ag NPs was evaluated and the plates were incubated at 37ºC for 24 h. The zone of inhibition was measured. Ag NPs, have shown promising antibacterial activity, but these green synthesized NPs showing better bactericidal effects against gram positive strain, *B. subtilis* and *S. aureus* as compared to gram negative strain *E. coli,* and *P. multocida*. Moreover, it is observed that due to excessive uses of synthetic antibiotics, micro-organism had develop rsistance against these synthetic antibiotics. Such type of natural remedy with some modification with inorganic compounds like, silver, gold, copper and many more, may develop to treat many infectious diseases. According to 2018 survey in Pakistan, more than 63% population consists on rural areas. It was conlucded that these green nanoparticle of *Allium*

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sativium inhibit bacterial growth. The silver particle along with *Allium sativum* shows synergistic effect, because silver ion through proteins ion channels easily cross the cell membrane and also help to increase the polarity of membrane to uptake *Allium sativum* particles. Furthermore, it reduces the bacterial load growth and clear zone around the disc was observed and documented by measuring the zone in milimiter (mm).

Conclusions

In this study, Ag NPs were prepared using aqueous extracts of *Allium sativum* as reducing agents. The TPC, TFC and antibacterial activity of NPs were also determined. From results, it was observed that green synthesized nano particles exhibit significant antibacterial activity against a gram positive and gram-negative bacterial strains. On the other hand, Ag NPs did not show mutagenicity against mutant strain of *S. typhimurium* TA98 &TA100. Brine shrimp lethality assay (BSLA) was dose dependent. The Ag NPs have shown promising antibacterial activity and could be considered as a natural remedy for many diseases.

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Conflict of Interest

The Authors declare no conflict of interest.

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