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Synthesis of biogenic silver nanoparticles from polyphenolic *Brassica nigra* and their potential antifungal, antibacterial, antioxidant, and anticancer activities

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Abstract

Green synthesis of silver nanoparticles (AgNPs) has consistently revolutionised the field of nanotechnology, and bio-based AgNPs have emerged as efficient therapeutic tools in biomedical science. Synthesis and characterization of silver nanoparticles from *Brassica nigra* (BN) and evaluation of antifungal, anticancer antibacterial, and antioxidant activities were investigated in this study. The characteristics of BN-AgNPs was studied using various spectroscopic techniques measuring ultraviolet-visible (UV–Vis) spectra, determination of particle size, zeta potential, scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Elemental composition was determined using energy-dispersive spectroscopy (EDX). Formation of AgNPs was confirmed by UV–Vis spectroscopy. Cytotoxic activity was determined at different nanoparticle concentrations on the MCF-7 cell line with the maximum cytotoxic effect observed at 100 μg/ml. BN-AgNPs also showed strong antifungal and antimicrobial activities along with antioxidant properties. The green synthesis of nanoparticles is comparatively simple, eco-friendly and safer than conventional methods and the observed anticancer activity in the current report is suggestive of the biogenic AgNPs as therapeutic agent for cancer therapy.

Introduction

Research on nanoparticles (NPs) has gathered interest among the scientific community in the field of biomedicine, in diagnostics, and cancer therapy. In recent years, noble metal NPs such as gold (AuNPs), silver (AgNPs), palladium (PdNPs), and platinum (PtNPs) had gained significance in nanomedicine (Vinay *et al.,* 2020; Vijilvani *et al.,* 2020). The diversified role of NPs is due to their numerous functions in chemical kinetics, nanoelectronics, cytocompatibility studies, drug delivery, and tissue engineering (Scroccarello *et al.,* 2019; Slepicka *et al.,* 2019). There are various methods including physical and chemical synthesis of NPs (Kiran *et al.,* 2020). Unlikely, these methods imposes higher toxicity risk as various toxic and dangerous chemicals are employed in the synthesis. Furthermore, production rate and biodegradability is slow in these methods (Curtis *et al.,* 2006). The major concern in reducing toxicity is the development of techniques involving nontoxic chemicals for the production of NPs. Hence, eco-friendly and bio-based nanoparticles must be developed (Nishanti *et al.,* 2019; Valsalam *et al.,* 2019). Alternatively, significant attempts have been invested to determine a process employing microbes and plant flora, as nanofactories to produce the metal NPs. Production of NPs from other biological sources is also shown earlier (Mandal *et al.,* 2006; Govindaraju *et al.,* 2010). Yet, formation of NPs from these sources possesses certain drawbacks. Contrarily, green chemistry utilizing plants resources has gained importance as it is simple, quick, economical and environmentally sustainable. The green method synthesis of metal nanoparticles has achieved significant awareness in recent years because these nanoparticles are non-toxic (Pei *et al.,* 2019; Vijayan *et al.,* 2019). In biomedical sciences evaluating the production of pure and environmentally friendly nanoparticles, AgNPs are known to have an incredible potential (Chandrasekhar and Vinay, 2017; Ansar *et al.,* 2018; Adnan *et al.,* 2020; Priya *et al.,* 2020).Silver

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particles are of more interest in their colloidal state as they also possess antibacterial activities (Vinay and Chandrasekhar, 2017)

Cancer is known to affect an enormous population worldwide. The use of chemotherapeutic drugs reduces the cancer rate, yet with the risk of infection (Torres-Martinez *et al.,* 2019; Valsalam *et al.,* 2019; Wang *et al.,* 2020; Lopez Ruiz *et al.,* 2020). Compared to other metals, Ag compounds are non-toxic and have proved over the years an effective treatment of diseases like cancer. Hence, synthesis of silver nanoparticles and its effect on cancerous cells was undertaken in addition to studying its antimicrobial and antioxidant properties. Antibacterial and anticancer effect from various plant sources has been documented previously. Bio-based AgNPs prepared from the ethanol extracts of rose plant exhibited significant antibacterial and anticancer activity in human cancerous cell lines (Manikandan *et al.,* 2015; Vinay *et al.,* 2020). Sidr honey has also been shown to act as an anticancer agent and antimicrobial agent and stimulates the cell growth of some lines (e.g., Hala) and inhibits growth in other cell lines (e.g., HepG2) (Ghramh *et al.,* 2020).

Brassica nigra (BN) has been recognized as super food. The incorporation of cruciferous vegetables into the human diet can have a positive health effect and promote well-being. Scientific studies have declared BN to be one of the healthiest vegetables (Samec *et al.,* 2019). In genera Brassica, the species of nigra has played a significant role in the progression of numerous different species. BN helps in digestion by increasing salivary amylase activity, ameliorating the release of saliva, and boosting the digestive system. The specific activity of BN in the enhancement of digestive activity can be utilized in treating patients suffering from gastroparesis. *Brassica* species have also shown modulation for the treatment of infectious bovine mastitis (Sobrinho Santos *et al.,* 2019). A cluster of biologically active sulfur-containing compounds called glucosinolates are present in cruciferous plants including the Brassicaceae family. These compounds are known to have significant nutritional importance. Moreover, BN is known to possess ant oxidizing properties (Zafar *et al.,* 2017; Amooaghaie *et al.,* 2018). The occurrence of phytochemicals like indoles which possess detoxifying property makes it an ideal candidate plant for the synthesis of AgNPs in the current study. Henceforth, the current study shows synthesis of AgNPs using *B. nigra* (as capping and reducing agent) for evaluation of their antimicrobial, free radical scavenging activity and potential anticancer activity to MCF- 7 cells.

Materials and methods

Collection, processing, and preparation of BN leaf extract

Fresh green leaves of BN (30 g) were purchased and they were washed with double-distilled water to remove dust particles, and subsequently air-dried. To prepare the extract, the leaves were boiled for 20 min in water and stored.

Silver nanoparticles green synthesis and detection of AgNPs

To produce the AgNPs, the leaf extract (10 ml) was mixed with 10^{-3} M Ag-NO_3 aqueous solution for reduction. To detect and characterize AgNPs, absorption spectra was obtained using UV-Vis spectrophotometer at different intervals (1, 7, and 16 h) and monitored for bioreduction of Ag+ ions.

Electron microscopic analysis of silver nanoparticles

Samples were prepared by dropping synthesized silver nanoparticles onto a copper-layered grid. Absorbent papers were used to blot the excess solution and the grid was further dried by exposing it to mercury lamp for 5 min and imaged. Further, size and shape of the BO-AgNPS was analyzed using TEM (JEM-2100F) (Fahad *et al.,* 2019).

BN-AgNPs and cytotoxicity

Human breast cancer cell line MCF-7 were cultured on Eagle minimum essential medium with FBS (10%) at 37°C and 5% CO_2. Air at 95% and relative humidity (100%) was maintained in the incubator. To determine the number of viable cells, hemocytometer was employed. Cells in their growth phase were seeded for attachment in flat bottomed polystyrene plate (flat bottomed) and incubated. Cells were exposed to increasing concentrations of AgNPs and incubated for 48 h. Post incubation, plates were supplemented with MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at concentration of 5 mg/mL in PBS and incubated at room temperature for 180 min. The percentage of viable cells was then estimated by measuring the absorbance of the plate at 570 nm using plate reader.

Assessment of antibacterial activity

The synthesized BN-AgNPs were tested for inhibitory activity against gram-positive and gram-negative bacterial strains (Table 1) which were cultivated using the agar disk diffusion method or the Kirby-Bauer Method (CLSI, 2009). To perform antibacterial activity, bacterial inoculum (approximately 10^8 CFU/ml) was mixed with Mueller

Hinton broth and plated. The discs were soaked separately with 100 µL of double-distilled water, silver nitrate (+control), and the Ag-NP solution. Sample displayed the greatest inhibition zone diameter after 16 h incubation.

Antifungal activity by the agar well diffusion assay

To assess the antifungal activity of BN-AgNPs, two fungal strains, Candida albicans and Candida glabrata, were employed and tested by the agar well diffusion method. Fresh cultures were prepared in MH broth and incubated at 28°C for 48 - 72 h. Following incubation, the cultures were evenly spread with sterile cotton swabs onto MHA plates, and the wells were punched with a sterile borer (5 mm). Fifty microliters of each bio-based BN-Ag-NP, standard NPs (2 mg/ml), and metal salt solutions were added to the wells with a sterile micropipette and incubated for 24 - 48 h at 28°C. After incubation, the inhibition zones were recorded.

Antioxidant properties of BN-AgNPs

DPPH assay

DPPH (0.1 mM) was added to synthesized BN-AgNPs at different concentrations, mixed and incubated for 15 min under dark conditions. The rate of decrease in DPPH levels were assessed by recording absorbance at 517 nm (Souza *et al.,* 2012). Ascorbic acid was used as reference standard to determine the antioxidant capacity of BN-AgNPs.

Nitric oxide and Hydroxyl -scavenging assay

The nitric oxide-scavenging activity was determined following method of Patel and Patel (2011) with absorbance recorded at 546 nm. Purple formazan (nitroblue tetrazolium) formed in reaction involving nicotinamide adenine dinucleotide and BN-AgNPs is an indicator of the superoxide anion radical-scavenging assay (Nishikimi *et al.,* 1972).

Synthesized BN-AgNPs (1 ml) was mixed with mixture containing H_2O_2, salicylic acid and ferrous sulfate and incubated for one hour and absorbance read at 510 nm (Smirnoff and Cumbes, 1989).

Statistical analysis

SPSS 17.0 software was used for the statistical analysis. A one-way analysis of variance was used to test the level of significance. Each experiment was repeated three times, with the mean values recorded.

Results

The bio-based production of AgNPs was demonstrated by the change in color (pale green to dark brown) following the mixing of aqueous solutions of BN leaf extracts and silver nitrate solution. Figure 1 confirms the configuration of the NPs produced at different intervals of time. The SEM micrographs of the BN-AgNPs (Figure 2) obtained in the filtrate indicated spacing between the synthesized AgNPs, which were spherically shaped and well distributed without aggregation. The TEM images of AgNPs (Figure 3a) show shapes of formed nanoparticles which are spherical in nature and size of the nanoparticles synthesised ranged from 10 - 50 nm in diameter. Figure 3b depicts the selected area electron diffraction-SAED pattern of AgNPs confirming the structure of the synthesized nanoparticles.

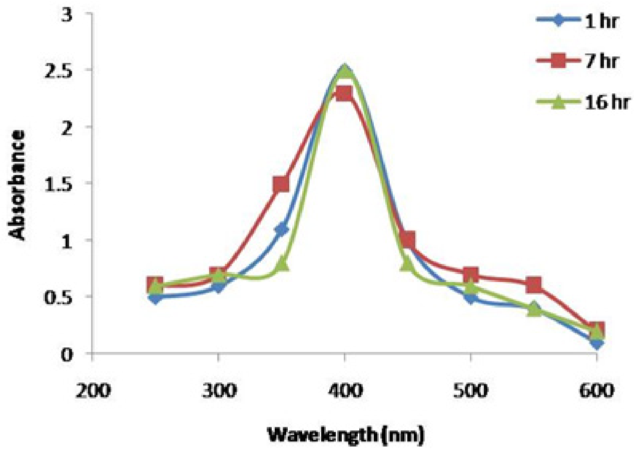

Figure 1: UV-Vis spectra showing absorbance of BN-silver nanoparticles at different time intervals.

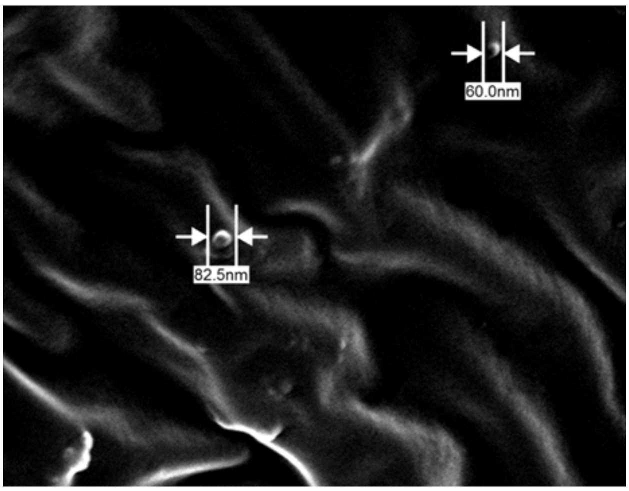

Figure 2: SEM micrograph of the AgNPs prepared with BN-NPs.

AgNPs were also characterized by determining the ZP/electrokinetic potential via the surface electric charge on the NP. This provides information about particle stability. A higher ZP is

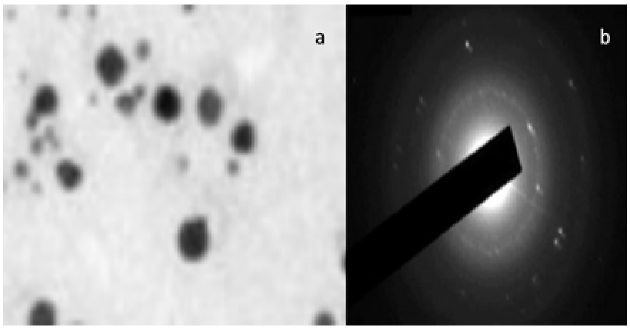

Figure 3:TEM (a) and SAED pattern (b) of Ag nanoparticles prepared from *B. nigra*.

reflective of enhanced electrostatic repulsion and stability. The importance of the ZP value can be correlated with the stability of colloidal dispersions. Particles bearing high −/+ ZP with a lower density and small size tend to deter one another in the suspension, which is related to the stability of the system to resist aggregation. The adherence of the AgNP surface with plant minerals during synthesis is reflected in the image confirming the successful formation of AgNPs from BN.

Cytotoxic and Antimicrobial assay

Cytotoxic activity of synthesized BN-AgNPs was dose dependent; Highest cytotoxic activity was demonstrated at 100 µg/ml (IC_{50} of 55 µg/ml) thus demonstrating the antagonistic effect on the cancerous cells (Figure 4).

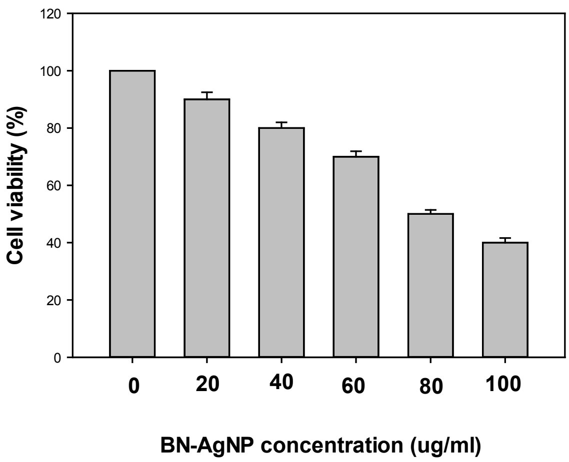

Figure 4: MCF-7 cell line exposed to different concentrations of BN-AgNPs

According to the inhibition areas, the green-synthesized AgNPs were screened for antimicrobial activity against bacterial strains. Approximately nine varied bacterial strains were used and tested for the antibacterial efficiency and two fungal strains were tested for the antifungal efficiency of the synthesized AgNPs. Table 1 shows the results of the disc agar well diffusion assay. The NPs expressed higher antibacterial activity on gram-positive bacteria (ZI ranged from 11 - 13 mm) than on gram-negative bacteria (ZI ranged from 9 - 10 mm). The observed antibacterial activity was dose-dependent and clearly indicated that bio-based BN-AgNPs completely overtake the gram-positive bacteria by showing higher activity than that against gram-negative bacteria. The NPs also expressed high activity against *C. albicans* and *C. glabrata* with ZI values of 13 and 14 mm, respectively.

Table 1: Antibacterial and antifungal activity of BN-AgNPs.

Bacterial and fungal strain	**Zone of Inhibition (mm)**	
	Control	**BN-AgNPs**
Bacteroides fragilis	9	11
Staphylococcus epidermidis	10	12
Staphylococcus aureus	12	13
Enterococcus faecalis	10	11
Streptococcus pneumoniae	9	12
Proteus mirabilis	9	10
Klebsiella pneumoniae	7	9
Escherichia coli	8	10
Pseudomonas aeruginosa	7	9
Candida albicans	11	13
Candida glabrata	13	14

Antioxidant assay

Radicals scavenging capabilities of BN-AgNPs are depicted in Figure 5a - 5d. Figure 5a reflects the DPPH scavenging action of BN-AgNPs. The scavenging activity increased in a dose-dependent manner. Highest antioxidant activity (75%) was demonstrated by BN-AgNP at its higher concentration of 200 µg/ml. Additionally, the IC_{50} values of the BN-AgNPs and ascorbic acid were 47.17 and 44.10 µ g/ml, respectively. Furthermore, results from the nitric oxide assay demonstrated 50–80% of the antioxidant activity with the same concentrations mentioned previously (Figure 5b). A concentration of 200 µg/ml of BN-AgNPs exhibited superoxide radical activity (47 - 75%) and hydroxyl-scavenging activities (42 - 70%) (Figure 5c and 5d).

Discussion

NPs have tremendous applications in biomedical sciences. Nanotechnology involves the design, manipulation, production, and application of materials in the nanometer range (Satpathy *et al.,* 2018).

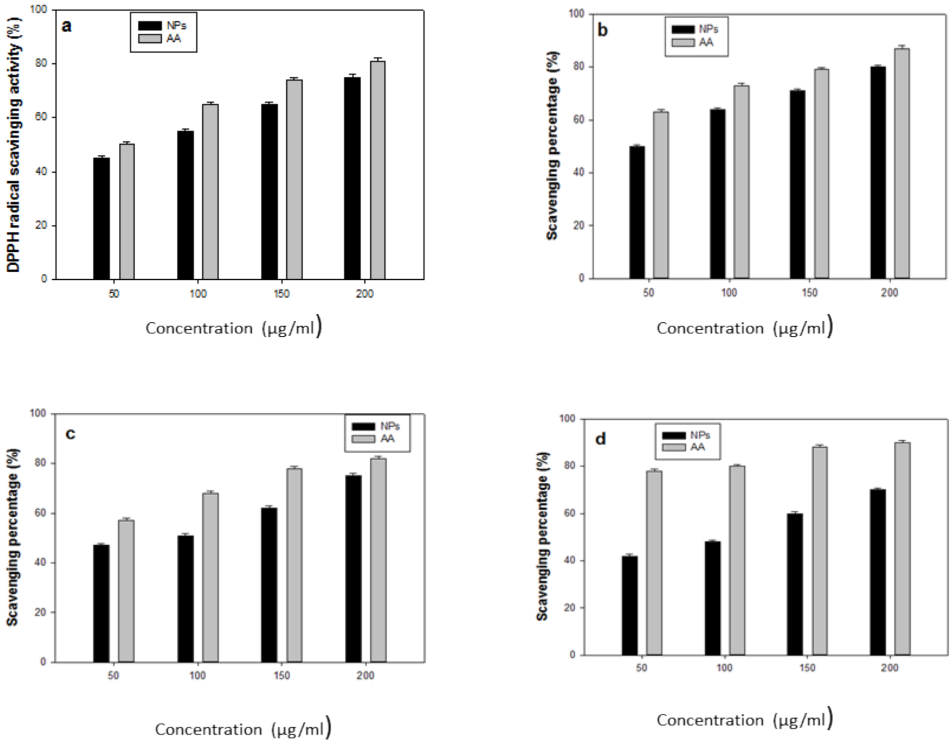

Figure 5: Antioxidant properties of BN-AgNPs; DPPH assay (a); nitric oxide (b); superoxide activity(c); hydroxyl-scavenging activity (d)

The chemistry of NPs and its electronic and optical properties have fascinated researchers globally (Singh *et al.,* 2016). The bio-based production of NPs in this study confirmed that the process is relatively rapid, easy, environmentally friendly, and therapeutically safe for humans. Additionally, the process is comparatively cheaper and has evolved as an alternative to traditional methods (chemical, physical, and microbial methods). Earlier studies have documented the efficiency of phytochemical extracts in the reduction and capping of NPs (Baharara *et al.,* 2014; Bindhu and Umadevi, 2015).

The present study demonstrated the formation of AgNPs following the reduction of silver ions by the BN extract, resulting in a yellowish-brown color solution. The absorption strongly depends on the particle size, dielectric medium and chemical surroundings. Small spherical nano particles (< 20 nm) exhibit a single surface plasmon band (Link and El-Sayed, 2003). The development of smaller size nanoparticles is also indicated by the maximum at 400 nm in UV-visible spectroscopy at a relatively lower wavelength and a broader peak higher wavelength typically indicates an enhancement of particle size. It is generally recognized that UV-Vis spectroscopy could be used to examine size and shape-controlled nanoparticles in aqueous suspension. The size of the synthesized nanoparticles was confirmed by zeta analyzer (DLS). Furthermore, the purity of the prepared AgNPs of BN was evidenced by a sharp silver peak in the EDX analysis. The production of spherical and discrete AgNPs in the 2 - 4 KeV range was in accordance with previous reports (Vijayakumar and Ganesan 2012).Numerous reports have documented the production of green-synthesized NPs (AgNPs and Au-NPs) from plants; however, green synthesis has replaced the requirement of stabilizing and capping agents and has additionally displayed size and structure-based biological activities (Markus *et al.,* 2017; Patil and Kim, 2017; Ponmurugan, 2017; Ramachandran *et al.,* 2017; Wang *et al.,* 2017).

The MTT assay revealed a clear relationship between the dose of BN-capped AgNPs and the assay results; there was enhanced cytotoxicity at higher concentrations of synthesized NPs. The *in vitro* anticancer activity observed are in similarity with earlier reports (Venugopal *et al.,* 2017; Kiran *et al.,* 2020; Ansar *et al.,* 2020). The formation of reactive oxygen species (ROS) and the denaturation of essential molecules cause cellular dysfunction and death (Venugopal *et al.,* 2017).

The AgNPs showed antibacterial activity against a variety of bacteria strains. It could be inferred that the AgNPs with mere attachment to the microbial cells display antimicrobial properties towards bacteria. Previously, the antimicrobial function of various polyphenols and plant extracts in pharmaceuticals and foods was investigated (Abdel-Shafi *et al.,* 2019; Abdalla *et al.,* 2020). Sage, coriander, tea, cloves, and basil all contain phenol compounds that have antimicrobial activity for pathogens (Barbinta-Patrascu *et al.,* 2013; da Silva *et al.,* 2015). Characterization of the enhanced antibacterial effects of AgNPs suggests that NPs will interact with the bacterial growth signaling pathway of putative peptide substrates that is vital for division once within cell. Further, cell death can occur owing to the disruption of ATP synthesis, replication of DNA and or membrane damage by bacteria. Pathogenic bacterial strains exhibited lower resistance against the synthesized AgNPs (Vinay *et al.,* 2019; Tamileswari *et al.,* 2015).

Patients with immunosuppressed diseases such as HIV, cancer, organ transplantation, and patients undergoing prolonged treatment with antifungals have an increased rate of morbidity and mortality due to infections caused by fungal pathogens. Similarly, patients receiving haemodialysis, parenteral nutrition, and chemotherapy who utilise central and peripheral vein catheters are susceptible to fungal infections due to the contamination of these devices with fungal strains, particularly *Candida* species. In the present work, BN-AgNPs showed antifungal activity against *C. alberta* and *C. albicans*.

As can be seen from the scavenging experiments above, the synthesised BN-AgNPs were demonstrated to possess promising antioxidant properties. Oxidative stress in biological systems occurs as a result of an imbalance between the generation of ROS and cellular antioxidant defence. Surplus free radicals have a damaging impact on defensive antioxidant enzymes including superoxide dismutase, catalase, and peroxidase, resulting in cellular damage caused by the oxidation of essential biomolecules, which leads to apoptosis/cell death (Prior *et al.,* 2005). Figure 6 depicts the detoxification of DPPH with AgNPs via single hydrogen transfer reaction. The green-synthesised BN-AgNPs investigated in the present work demonstrated enhanced antimicrobial, anticancer, and antioxidant activities as recent reports (Ashraf *et al.,* 2019; Patra *et al.,* 2019; Kiran *et al.,* 2020). The outcome of the present work would be helpful to clinicians, researchers, and pharmaceutical companies to formulate BN-AgNPs and use them for therapeutic applications against various infections and in malignant therapy.

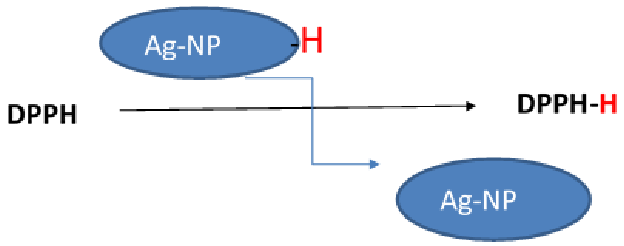

Figure 6: Free radical scavenging action of Ag-NPs on DPPH by single hydrogen transfer

Conclusion

The green synthesis of NPs is advantageous as it is simple, eco-friendly, economical and adaptable to large-scale production. The process excludes the requirements of elevated temperature, toxic chemicals, high pressure, and energy. In conclusion, the synthesis of AgNPs by the green process is of great significance in nanocarrier mediated drug delivery and as promising tool in treatment of various chronic diseases.

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