Exploring the Antioxidant Potential of Silver Nanoparticles Synthesized from Gishta (Annonaceae) Seeds: A Comprehensive Assessment of Free Radical Scavenging Activities

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KEYWORDS
Antioxidant; DPPH; $FeCl_3$; Nitric oxide; Superoxide and Acetogenins.

ABSTRACT:
Antioxidants, often described as "free-radical scavengers," are compounds that can help prevent or slow the damage caused by free radicals to cells. Many plant-based substances are thought to be rich in antioxidants, and one such plant, *Gishta* (a member of the Annonaceae family native to North-East Ethiopia), is commonly used in traditional medicine. However, the antioxidant properties of *Gishta* have not been extensively studied. In the current research, silver nanoparticles were synthesized from *Gishta* seeds using organic solvents, and their antioxidant activities were evaluated through various assays, including DPPH free radical scavenging, FeCl3-based reducing power, nitric oxide free radical scavenging, and superoxide scavenging. Standard reference substances were used for comparison in each test. The results demonstrated that the antioxidant activity of the *Gishta* seed extracts was comparable to that of the standard drugs across all tests. The antioxidant efficacy of the plant can be attributed to flavonoids, alkaloids, and acetogenins present in the seeds. These findings highlight the potential of *Gishta* as a valuable source of antioxidants, which could have therapeutic applications.

1. Introduction

Oxidative stress plays a significant role in the development and progression of various diseases (Jacob RA and Burri BJ, 1996). Reactive oxygen species (ROS), which include free radicals, are linked to neurodegenerative conditions such as Alzheimer's and Parkinson's diseases (Youdim K and Joseph JA, 2001) as well as a range of cancers (Goodwin JS and Brodwick M, 1995). ROS consist of molecules like superoxide anion radicals (O2•−), hydroxyl radicals (OH•), and non-radical species such as hydrogen peroxide (H2O2) and

singlet oxygen (1O2), all of which contribute to cellular damage and accelerate the aging process (Halliwell B and Gutteridge JM, 1989; Gülcin İ et al., 2002). ROS have also been implicated in the development of acute gastric lesions. Furthermore, ROS can damage essential biomolecules, including lipids, proteins, nucleic acids, and carbohydrates, playing a role in aging, cancer, and various diseases (Gülcin İ et al., 2002). Consequently, ROS have been associated with over a hundred conditions, including malaria, heart disease, stroke, arteriosclerosis, diabetes, cancer, and gastric ulcers (Halliwell B, 1991; Hertog MGL et al., 1993; Büyükokuroğlu ME et al., 2001).

To combat this oxidative damage, the human body has several intrinsic defense mechanisms, including enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Exogenous antioxidants, such as vitamins E and C, play a crucial role in protecting the body from oxidative stress, slowing the progression of chronic diseases, and preventing lipid oxidation in food (Yoshikawa T et al., 1997; Mates JM et al., 1999). Antioxidant defense systems, including those found in foods and medicines, are essential in neutralizing the harmful effects of ROS (Mates JM et al., 1999; Ames BN et al., 1993; Bafna PA and Balaraman R, 2004). As a result, the search for new exogenous antioxidants continues. Common synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, and tert-butylhydroquinone are widely used (Sherwin ER et al., 1990). However, concerns about the potential toxicity of BHA and BHT, including liver damage and carcinogenesis, have raised alarms (Sherwin ER et al., 1990; Grice HC, 1986). As a result, there is an increasing focus on developing safer and more effective antioxidants (Gülcin İ et al., 2002; Oktay M et al., 2003). This has led to a shift toward exploring plant-derived antioxidants due to safety concerns associated with synthetic alternatives (Imida K et al., 1983).

Gishta, a plant species known for its short, erect, evergreen tree form and glossy dark green leaves, is of growing interest. It produces large, heart-shaped fruits with white, fleshy edible pulp, typically 15-20 cm in diameter. Every part of the *Gishta* plant is used in traditional medicine (Edwards, S. et al., 2000). Found in rainforests across various regions, it is known by different names, such as thorny custard apple, cherimoya, and Brazilian pawpaw, as well as soursop (in parts of America), cachiman (épineux), shul-ram-fal, hanuman fal, and mullaatha (in India), and Harar and yebere lib (in Ethiopia), where it is referred to as the "heart of cow" (Blackherbals, 2019).

Traditional medicine has long recognized the medicinal value of *Gishta* seeds and leaves in treating cancer and parasitic infections. The seeds are known for their emetic properties, and the oil extracted from them is used to eliminate lice. The flowers possess antispasmodic properties, while the fruit pulp is diuretic and is used to address various ailments. Unripe *Gishta* fruit is considered astringent, making it useful for treating gastrointestinal issues, while ripened fruit serves as an anthelmintic and antiscorbutic.

Additionally, the plant is used to prepare a medicinal beverage to treat gastric problems, and the bark is used to combat diarrhea and dysentery. The therapeutic effects of *Gishta* are largely attributed to bioactive compounds known as acetogenins (Rupprecht, J.K. et al., 1986). Despite its widespread use in traditional medicine, there has been limited research into the antioxidant properties of silver nanoparticles of *Gishta* seed extracts (Saripalli, Harikrishna & Dixit, Prasanna, 2016).

This study aims to synthesize silver nanoparticles (AgNPs) from *Gishta* seed solvent extracts and assess in vitro antioxidant activities.

2. Materials and Methods

2.1 Preparation of Silver Nitrate Solution

To prepare a 1 mM solution of silver nitrate ($AgNO_3$), 0.1699 grams of $AgNO_3$ were dissolved in 1 liter of purified water. The solution was then stored in a dark-colored glass container to protect it from light and prevent auto-oxidation of the silver.

2.2 Plant Material and Sample Collection

In January 2016, *Gishta* fruits were harvested from the Oromo region of Ethiopia, following proper identification and authentication of the plant species under study (Fig. 1a).

2.3 Extract Preparation

The seeds of *Gishta* were processed to prepare the extract through a series of steps. First, the seeds were dried in the shade under aseptic conditions at a temperature of 28 ± 30°C. After drying, the seeds were ground into a fine powder and subjected to Soxhlet extraction using chloroform and methanol, following a standardized procedure (Harikrishna Ramaprasad Saripalli, 2004). The resulting extract was stored at 4°C for one week.

In separate flasks, 1 mM $AgNO_3$ was mixed with the chloroform and methanol extracts, and the total volume was adjusted to 200 ml. These mixtures were then centrifuged at 18,000 rpm for 25 minutes and subsequently heated in a sand bath at 60°C for 10 minutes. Control samples were prepared using each solvent alone, without the seed extract. The incubation process was continued until the solution developed a dark color, indicating the formation of nanoparticles.

To further concentrate the plant extract, the dried material was processed using a flash evaporator for 30 minutes, resulting in a powdered form. The final material obtained was the absolute extract powder, and various concentrations of this powder were prepared by dissolving it in the corresponding organic solvents, as detailed in the table (Harikrishna Ramaprasad Saripalli, 2007).

2.4 Spectral Analysis

A small portion of the sample was dissolved in distilled water and left to suspend for 5 hours. After this period, the suspension was subjected to spectral analysis to assess its properties.

2.5 Study of Characteristics of AgNPs Particles

The physical characteristics of the silver nanoparticles (AgNPs) were examined using standard procedures and a Scanning Electron Microscope (SEM). The re-dispersed nanoparticles were first dried to obtain a powdered form. Ten milligrams of the dried sample were then re-dispersed in ethanol. Thin layers of the sample were applied onto a carbon-supported copper grid and dried under a mercury-vapor lamp.

Chemicals and Instruments:

The following chemicals and instruments were utilized in the study: DPPH (1,1-Diphenyl-2-picryl-hydrazil), 0.1 ml DMSO, ascorbic acid, 1% potassium ferricyanide, 10% trichloroacetic acid, distilled water, 0.1% ferric chloride, sodium nitroprusside, phosphate buffer, methanol, sulfanilic acid reagent (0.33% in 20% glacial acetic acid), naphthyl ethylene diamine dihydrochloride, 50 mM KH_2PO_4-KOH buffer (pH 7.4), 1 mM EDTA, 100 mM hypoxanthine, xanthine oxidase, saline, and NBT (nitro-blue tetrazolium). All chemicals used were of analytical grade. Additionally, standard laboratory glassware, solvent extraction apparatus, and a UV-visible spectrophotometer were employed for the analysis.

In-Vitro Antioxidant Activity

DPPH Free Radical Scavenging Activity

The stable free radical scavenging activity of various solvent extracts of AgNPs from *Gishta* was assessed by monitoring the discoloration of the DPPH solution, following Blois' method

(Blois MS, 1958). Test samples, ranging from 20 to 100 µg/ml, were dissolved in 0.1 ml DMSO and added to 0.1 ml of 0.1 mM DPPH in methanol. The mixture was then vigorously shaken and allowed to stand in the dark for 10 minutes at room temperature (30°C). The absorbance of the DPPH solution was measured at 517 nm using a UV-visible spectrophotometer. Ascorbic acid was used as a positive control. The extent of discoloration was used to determine the scavenging activity of the extract. The DPPH scavenging effect was calculated using the following equation:

DPPH Scavenging Effect (%) = (A0 - A1 / A0) × 100
Where:

- A0A_0A0 is the absorbance of the control reaction (DPPH solution without the solvent extract).
- A1A_1A1 is the absorbance of the DPPH solution in the presence of the solvent extract (Oktay M. et al., 2003; Gülcin İ., *et al*., 2003).

Reducing Power by $FeCl_3$

The reducing power of various solvent extracts of AgNPs from *Gishta* was evaluated using the $FeCl_3$ method. The absorbance of the resulting solution was measured at 700 nm to determine the reducing potential of the extracts.

Nitric Oxide Radical Inhibition Assay

Sodium nitroprusside in an aqueous solution at physiological pH spontaneously generates nitric oxide, which reacts with oxygen to produce nitrite ions. These nitrite ions were quantified using the Griess Illosvoy reaction (Garrat DC, 1964). The free radical scavenging activity (FRSA) or percentage antiradical activity was calculated using the following equation:

% Antiradical Activity = (Control absorbance - Sample absorbance) / Control absorbance × 100

Superoxide Anion Radical Scavenging

Superoxide anion radicals were generated in vitro using hypoxanthine and xanthine oxidase. The superoxide anion scavenging activity was determined by measuring the decrease in absorbance of the reaction mixture. A reduction in absorbance indicated an increase in superoxide anion scavenging activity. The results were expressed as the percentage inhibition of the NBT (nitro-blue tetrazolium) reduction rate, calculated with respect to the reaction mixture that did not contain the solvent extract (saline only).

Image 1 a: Gishta (*Annona spp.*) fruit of Ethiopia

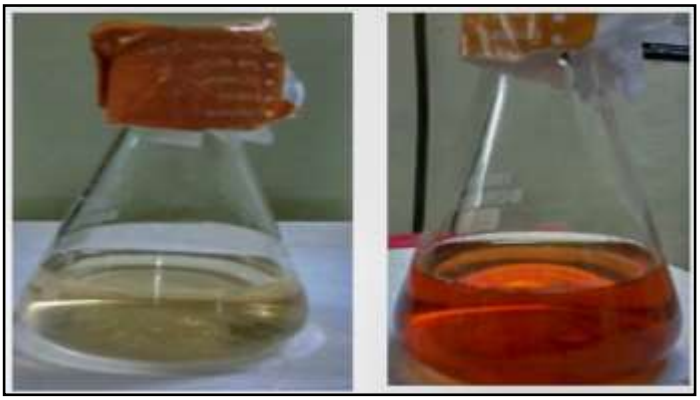


Image 1 b: Change in the color assures AgNPs synthesis

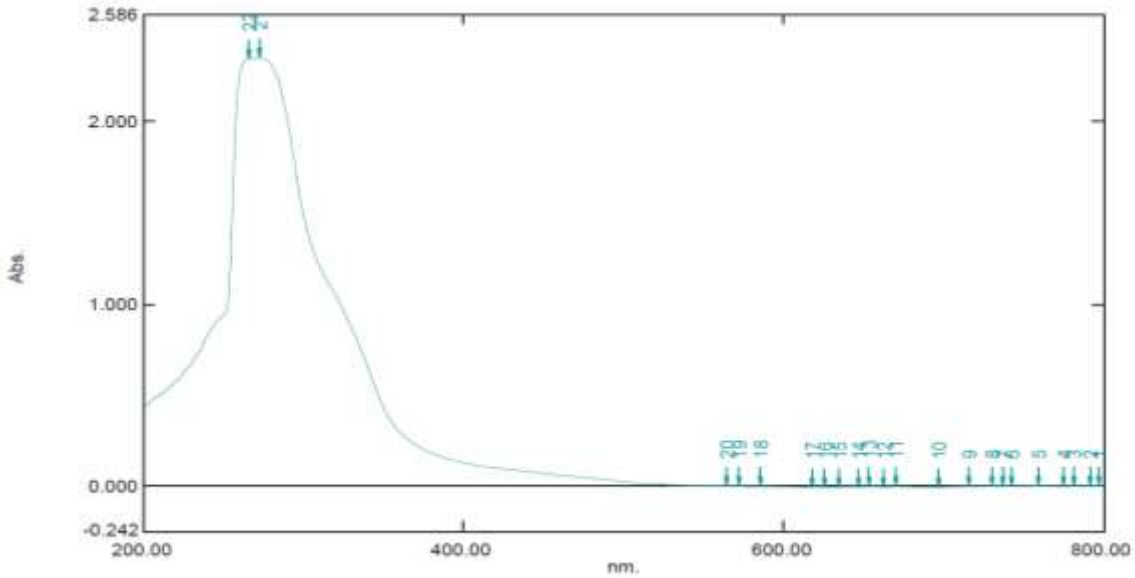
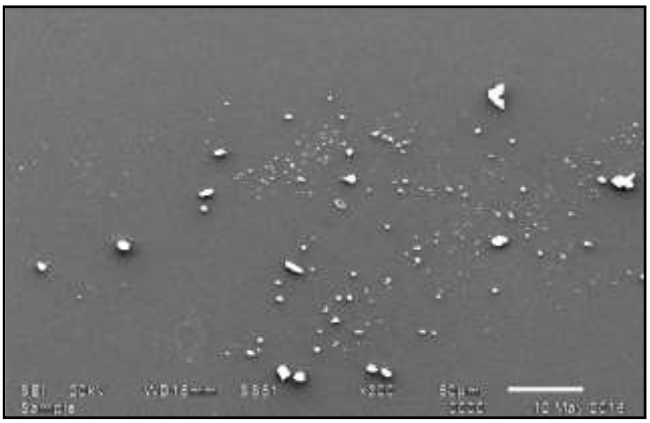


Image 2: Ultra violet and Visible absorption spectrum of Ag nanoparticle of Ghista seeds



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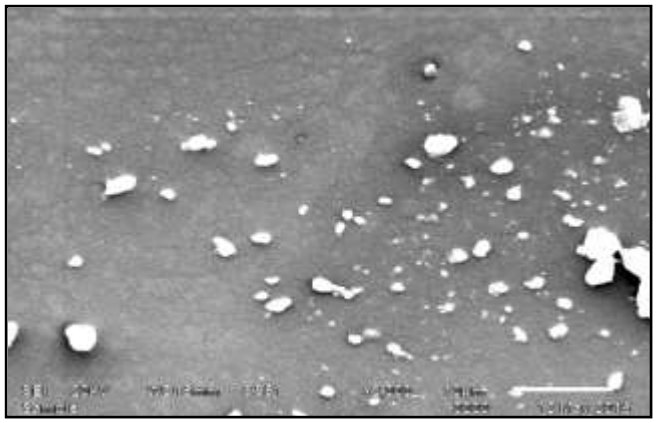

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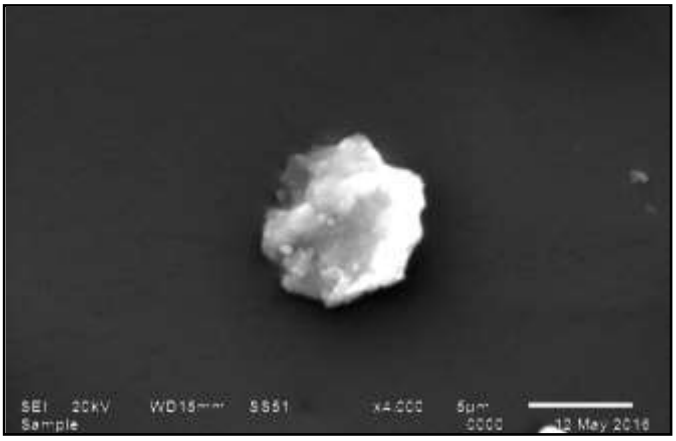

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Image 3: SEM images at different magnifications (a.300X; b.2000X; c.4000X) of silver nanoparticles synthesized from Seeds of Gishta.

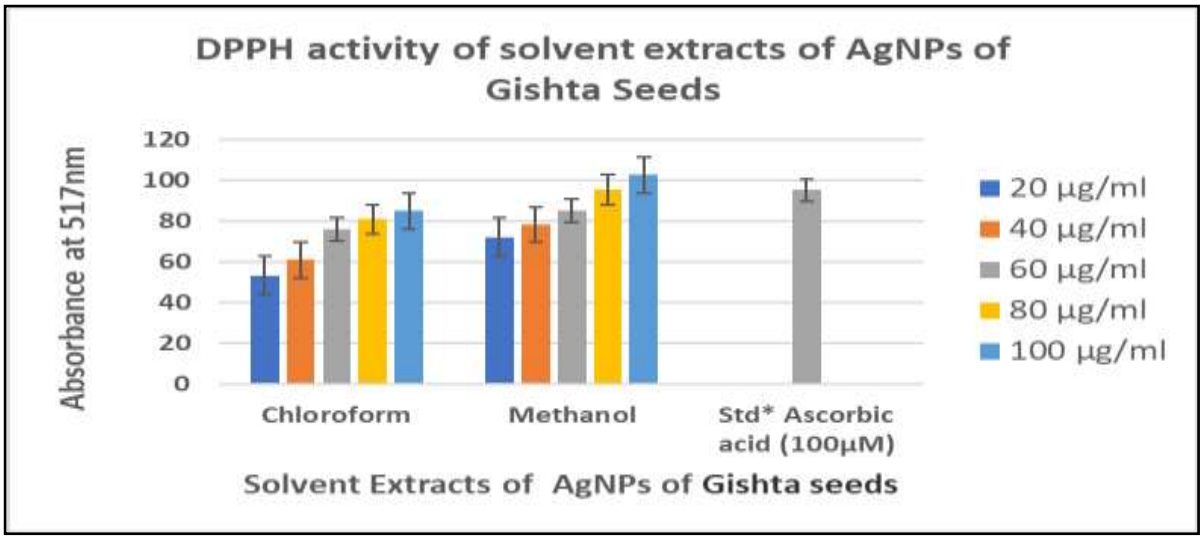
3. Results

The DPPH test was selected for the primary screening of organic solvent extracts of AgNPs from *Gishta* due to its ability to rapidly accommodate multiple samples and its high sensitivity in detecting natural compounds, even at low concentrations. This assay offers valuable insights into how test compounds interact with stable free radicals. DPPH, known for its unpaired electron, exhibits a strong absorption peak at 517 nm in visible spectroscopy, giving it a deep violet color. When a free radical scavenger is present, the unpaired electron pairs off, leading to a stoichiometric decolorization that reflects the number of electrons consumed (Blois M., 1958).The solvent extracts of AgNPs from *Gishta* seeds demonstrated a concentration-dependent DPPH free radical scavenging activity (Table 1). Among the extracts, methanol showed the highest scavenging effectiveness, followed by chloroform (Graph 1). The efficacy of the extracts at various concentrations is outlined in Table 1. Notably, significant DPPH radical scavenging activity was observed at all tested concentrations, highlighting the potent antioxidant potential of the extracts.

Table 1 DPPH activity of solvent extracts of AgNPs of Gishta Seeds

S. No.	Concentration of Test material	% Decrement of Ab (517nm)	
		Chloroform	Methanol
1	20 µg/ml	53.44±2.41	72.31±1.86
2	40 µg/ml	60.93±1.62	78.11±2.41
3	60 µg/ml	76.16±7.15	85.16±1.37
4	80 µg/ml	81.04±4.81	95.51±2.71
5	100 µg/ml	84.95±6.82	102.66±2.48
6	Std*	95.33±1.32	

Std* - Standard Ascorbic acid (100µM).
Results denotes the mean ±S.D. of four experiments in duplicate(s).



Graph 1 DPPH activity of solvent extracts of AgNPs of Gishta Seeds.

Reducing power ($FeCl_3$) of solvent extracts of Gishta seeds

Fe^{3+} to Fe^{2+} transformations were detected in the presence of *Gishta* seed extracts using the Oyaizu method (1986). The antioxidant activity and reducing power of the extracts were found to increase with higher concentrations, as shown in Table 2. The increased absorbance values correspond to a greater reducing power, indicating the enhanced ability of the *Gishta* seed extracts to donate electrons and reduce Fe^{3+} to Fe^{2+}.

Table 2 Reducing power by FeCl3 of solvent extracts of AgNPs of Gishta seeds.

S. No.	Concentration of test material	Absorbance at 700nm		
		Chloroform	Methanol	Std (BHT)*
1	10 µg/ml	0.4535±0.35	0.4744±0.12	0.5171±0.24
2	20 µg/ml	0.5022±0.87	0.5205±0.63	0.5923±0.31
3	30 µg/ml	0.5786±0.24	0.5957±0.32	0.6168±0.28
4	40 µg/ml	0.8242±0.68	0.8752±0.24	0.7635±0.35

5	50 µg/ml	0.8336±0.54	0.8788±0.28	0.8662±0.29

Std* Standard, Butylated hydroxytoluene
Results denotes the mean±S.D. of four experiments in duplicate(s).

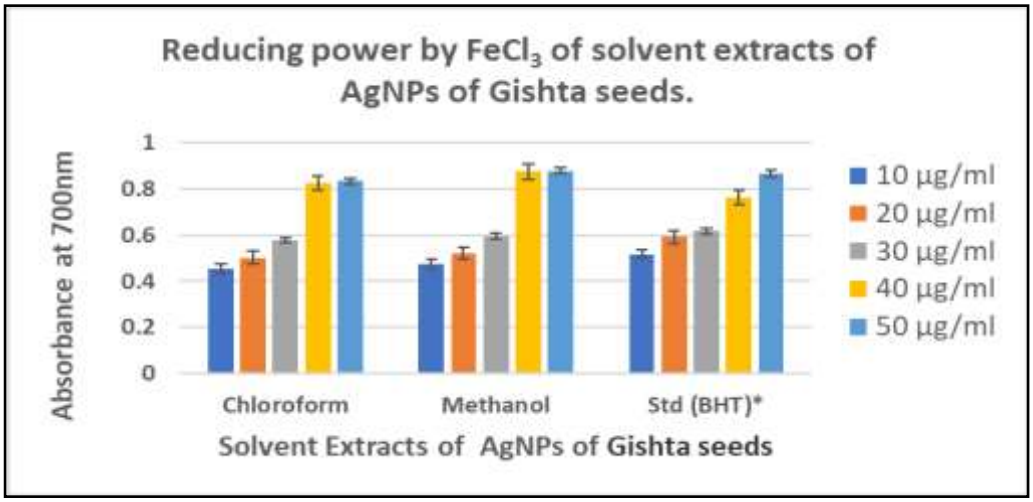
The reducing power of various solvent extracts of AgNPs from *Gishta* seeds was evaluated using the $FeCl_3$ method and demonstrated a concentration-dependent activity. The extracts were tested at increasing concentrations, with Butylated HydroxyToluene (BHT) used as a standard for comparison at corresponding concentrations.
For the chloroform extracts, the activity was observed at the following concentrations:

- 10 µg/ml: 0.4123 ± 0.35
- 20 µg/ml: 0.4565 ± 0.87
- 30 µg/ml: 0.5260 ± 0.24
- 40 µg/ml: 0.7493 ± 0.68
- 50 µg/ml: 0.7578 ± 0.54

The methanol extracts exhibited activity at the following concentrations:

- 10 µg/ml: 0.4313 ± 0.12
- 20 µg/ml: 0.4732 ± 0.63
- 30 µg/ml: 0.5415 ± 0.32
- 40 µg/ml: 0.7956 ± 0.24
- 50 µg/ml: 0.7989 ± 0.28

Although BHT, the standard, showed slightly higher absorbance values than the tested extracts, the absorbance values for the *Gishta* seed extracts increased progressively with higher concentrations. Significant reducing power, as indicated by the $FeCl_3$ method, was observed across all tested concentrations of the different solvent extracts, as illustrated in Graph 2.



Graph 2 Reducing power by $FeCl_3$ of solvent extracts of AgNPs of Gishta seeds.

In-vitro antioxidant efficacy of solvent extracts of Gishta seeds using Nitric oxide method

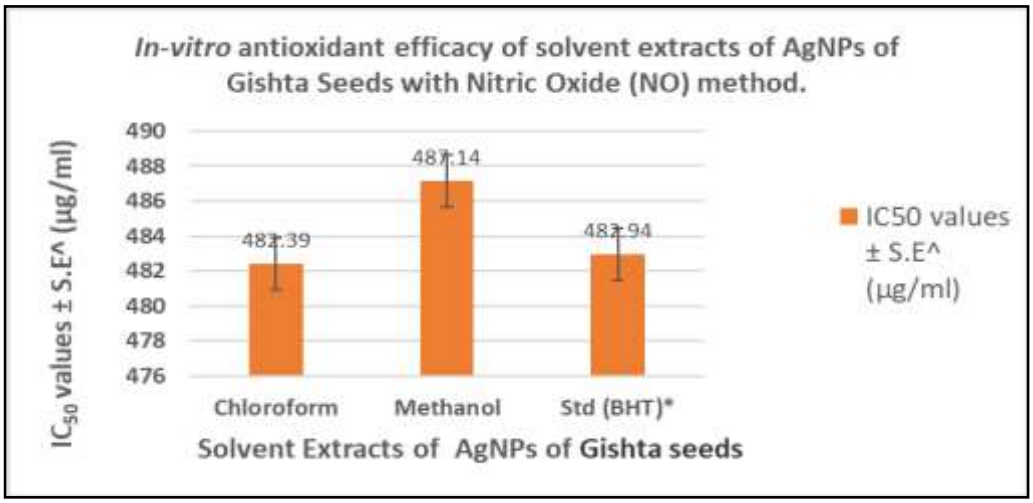
Among the solvent extracts of AgNPs from *Gishta* seeds, the methanol extract exhibited the strongest activity, followed by the chloroform extract. The IC_{50} value for the methanol extract was 487.14 ± 9.45 µg/ml, while the IC_{50} value for Rutin (the standard) was 482.94 ± 9.88

µg/ml, as shown in Table 3 and Graph 3. This indicates that the methanol extract demonstrated a comparable scavenging potency to the standard antioxidant, Rutin.

Table 3 *In-vitro* antioxidant efficacy of solvent extracts of AgNPs of Gishta Seeds

S.No.	Solvent extracts of seeds	IC_{50} values ± S.E^ (µg/ml)
1.	Chloroform	482.39 ± 7.36
2.	Methanol	487.14 ± 9.45
3.	Std*	482.94 ± 9.17

Std* Standard, Rutin. ^Results denotes mean of 10 determinants.



Graph 3 *In-vitro* antioxidant efficacy of solvent extracts of AgNPs of Gishta Seeds with Nitric Oxide (NO) method.

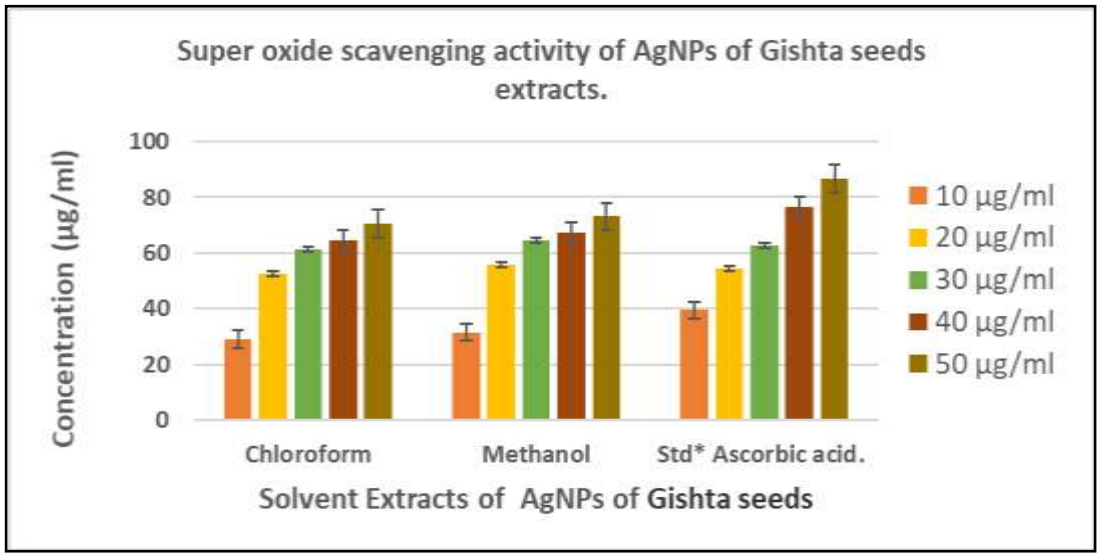
Super oxide anion (radical scavenging) activity of Gishta seeds extract

Superoxide radicals can cause significant damage to cellular components. The various solvent extracts of AgNPs from *Gishta* seeds effectively scavenged superoxide radicals and reduced the rate of NBT reduction. The superoxide radical scavenging activity exhibited a dose-dependent pattern, as shown in Table 4 and Graph 4. Among the extracts, the ethanol extract demonstrated the highest scavenging ability, indicating its potent antioxidant potential in neutralizing superoxide radicals.

Table 4 Super oxide scavenging activity of AgNPs of Gishta seeds extracts.

S. No.	Concentration	Absorbance at 560nm		
		Chloroform Extract	Methanolic Extract	Std*
1	10 µg/ml	29.07 ± 3.13	31.54 ± 3.62	39.41 ± 3.54
2	20 µg/ml	52.65 ± 2.52	55.67 ± 2.41	54.49 ± 2.18
3	30 µg/ml	61.29 ± 3.41	64.61± 1.45	62.64 ± 2.42
4	40 µg/ml	64.49 ± 2.46	67.48± 3.81	76.37 ± 3.15
5	50 µg/ml	70.58± 3.59	73.07± 2.46	86.63± 4.81

Std* Standard - Ascorbic acid.
Results denote the average ±S.D. of four experiments in duplicate(s).



Graph 4 Super oxide anion (radical scavenging) efficacy Gishta seeds extract

Among the solvent extracts of AgNPs from *Gishta* seeds, the methanol extract demonstrated the most remarkable activity, followed by the chloroform extract. As the concentration of the methanol extract increased, a corresponding increase in superoxide radical inhibition was observed. The inhibition values at various concentrations were as follows:

- 20 µg/ml: 31.54 ± 3.62
- 40 µg/ml: 55.67 ± 2.41
- 60 µg/ml: 64.61 ± 1.45
- 80 µg/ml: 67.48 ± 3.81
- 100 µg/ml: 73.07 ± 2.46

Ascorbic acid was used as a reference standard. Significant scavenging efficacy of superoxide anions was observed at all tested concentrations of the different solvent extracts, highlighting the potent antioxidant activity of the *Gishta* seed extracts.

4. Discussion

The aging process is often linked to chronic damage that leads to the production of harmful oxidants and oxygen-free radicals, which can cause significant toxicity to tissues, resulting in tissue necrosis and cellular damage. Various cellular mechanisms and external factors contribute to oxidative stress, including inflammation, the release of free radicals from mitochondria, auto-oxidation of catecholamines, activation of xanthine oxidase, prooxidant effects of toxins such as CCl_4, and exposure to ionizing radiation (Russo A. et al., 2005).

Aerobic cells have well-established antioxidant defense mechanisms, which include low molecular weight scavengers such as cysteine, reduced glutathione, and ascorbic acid, along with enzymatic systems like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Red), and glucose-6-phosphate dehydrogenase (G6PD). These systems work together to neutralize the harmful effects of reactive oxygen species (Halliwell B., 1994). However, when there is an imbalance between these reactive species and antioxidants, oxidative stress can occur, leading to irreversible cellular damage.

Our findings suggest that the methanol extracts of silver nanoparticles from *Gishta* exhibit remarkable antioxidant activity in cell-free systems. These extracts effectively quench synthetic DPPH radicals and display a superoxide dismutase (SOD)-like effect, inhibiting the

formation of superoxide anions ($O_2^{\bullet -}$) in a dose-dependent manner (Russo A. et al., 2005). This indicates the potential of *Gishta* seed-derived silver nanoparticles as an effective source of antioxidants, capable of mitigating oxidative stress and cellular damage.

DPPH Test:

In our study, we used the DPPH test as the primary screening method to evaluate the free radical scavenging activity of AgNPs derived from *Gishta* seed extracts. Among the various solvent extracts, the methanol extract exhibited the most significant antioxidant activity, followed by the chloroform extract, as illustrated in Graph 1. This indicates that the methanol extract of *Gishta* seeds, in the form of silver nanoparticles, has the highest potential for scavenging free radicals compared to other solvent extracts.

Reducing Power by FeCl3:

We assessed the reductive ability of AgNPs from *Gishta* seed extracts using the $FeCl_3$ method. The methanol extracts of AgNPs exhibited the highest antioxidant activity, surpassing the chloroform extracts, as shown in Graph 2. This highlights the superior reducing power of the methanol extract in neutralizing Fe^{3+} ions, further confirming its potent antioxidant potential.

Nitric Oxide Radical Inhibition Assay:

When evaluating the antioxidant activity of solvent extracts of AgNPs from *Gishta* seed using the nitric oxide radical inhibition method, the methanol extract demonstrated the strongest activity, followed by the chloroform extract. The methanol extract of AgNPs from *Gishta* exhibited an IC_{50} value of 487.14 ± 9.45 µg/ml, as shown in Graph 3. This indicates that the methanol extract has significant potential in inhibiting nitric oxide radicals, making it a promising candidate for further antioxidant applications.

Superoxide Anion Radical Scavenging Activity

Remarkable superoxide anion radical scavenging activity was observed in various solvent extracts of AgNPs from *Gishta* seeds. Among these, the methanol extract exhibited the highest superoxide anion radical scavenging activity, outperforming the chloroform extract, as shown in Graph 4. The promising results obtained in this study suggest that the bio-efficacy of the solvent extracts of AgNPs from *Gishta* seeds may be attributed to the presence of potent bioactive compounds, which contribute to their ability to neutralize superoxide radicals effectively.

5. Conclusion

Based on the results of these experiments, it can be confidently concluded that the methanolic and chloroform extracts of silver nanoparticles (AgNPs) synthesized from *Gishta* seeds exhibit strong primary and secondary antioxidant properties. These extracts effectively neutralize free radicals, thereby reducing lipid peroxidation. This suggests their potential to provide protective benefits against diseases associated with reactive oxygen species. The observed antioxidant activity is likely due to the presence of phenols and phenolic compounds in these extracts. However, to establish a definitive relationship between the chemical composition and antioxidant efficacy, further in vivo studies are required. These findings highlight the successful assessment of antioxidant activity in biologically derived AgNPs from *Gishta* seed extracts. Moreover, this study represents a pioneering exploration of the antioxidant potential of AgNPs from *Gishta* seeds, paving the way for future research in this promising area.

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