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ASSESSMENT OF ANTI-BACTERIAL & ANTI-FUNGAL ACTIVITIES OF GREEN SYNTHESIZED SILVER NANOPARTICLES (AGNPS) FROM THE AQUEOUS LEAF EXTRACT OF CASSIA ALATA

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ABSTRACT

The ability of plants to accumulate and detoxify heavymetals is well proved. In nanotechnology nanoparticles research is an important aspect due to its numerable applications. The main challenge in the development of catalytic NPs is to prepare nanomaterial's that are highly active, selective, stable, robust, and inexpensive. Among the metal nanoparticles, SNPs are considered to be of great importance because of their properties such as antiviral, antibacterial, antifungal, etc. Silver nanoparticles are nanoparticles of silver, i.e. silver particles of between 1 nm and 100 nm in size. *Cassia alata* (also known as Senna alata) is a shrub belonging to the fabaceae family, found in tropical areas. AgNPs synthesized from the aqueous leaf extract of *Cassia alata*, show significant anti-bacterial, anti-fungal activities.

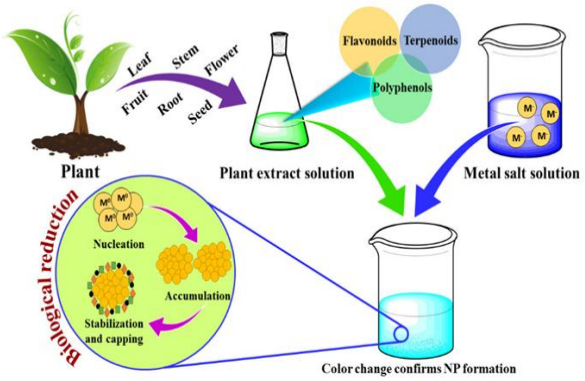
KEYWORDS: Green synthesis, *Cassia alata*, Silver nanoparticles (AgNPs), Anti-microbial activities.

1. INTRODUCTION

Metal based nanoparticles are synthesized for numerous applications from the extracts of different plant parts such as leaves, roots, flower, seeds, etc. The ability of plants to accumulate and detoxify heavymetals is well proved. Generally it is used as bioreductant in the process of synthesizing Ag nanoparticles. A plant extract contains a number of metabolites and reductive biomolecules responsible for the reduction of metal ions. This includes (Water soluble plant metabolites) alkaloids, terpenoids, flavonoids, ketones, aldehydes, amides, carboxylic acids, carbohydrates, proteins, and vitamins. AgNPs nanoparticles of silver, i.e. silver particles of between 1 nm and 100 nm in size. The presence of some toxic chemicals adsorbed on the surface of the nanoparticles that are synthesized by chemical methods may have adverse effect on biomedical applications. Thus Green synthesis is an apt method, safer than chemical and physical method.

Green synthesis is effective, eco-friendly and without difficulty scaled up on a large scale. There is no need of high pressure, energy, temperature, and toxic chemicals. The inhibitory effect on silver on microbes, it was detected in the past, is generally used in medical and industrial processes (Jose *et al*., 2005; Lok *et al*., 2007). *Cassia alata* (also known as *Senna alata*) is a shrub belonging to the fabaceae family, found in tropical areas. It is commonly known as candle bush, with reference to the shape of inflorescence. It is annual or bi annual shrub with an offensive smell, 1-4 m tall, preferring sunny and moist areas.

Synthesis of these nanoparticles use as an alternate antibiotic therapy straightly depend on some physical and chemical properties such as their size, shape, concentration and zeta potential. (Z. Lu *et al*., 2013), (S. Pal *et al*., 2007) Therefore, the properties of nanoparticles should be considered and given more attention during the green synthesis. Reduction of Ag+ ions is proposed to come from the combinations of bio molecules (enzymes/proteins, amino acids, polysaccharides, and vitamins etc.) found in these extracts. But the exact mechanism is not till understood. Most accepted mechanism is based on the presence of enzyme "nitrate reductase". (S. Ahmed *et al*., 2016, P. Mohanpuria *et al*., 2008) The enzyme converts nitrate (NO3) to nitrite (NO2). During this reduction process where NO3is converted into NO2, the electron is transferred to the silver ion (Ag+) which then reduced to metallic silver (Ag0) (A. Roy *et al*., 2013), (S. Anil Kumar *et al*., 2007).



2. MATERIALS AND METHODS

2.1 Collection of leaf samples from *C. alata*

Leaves that appeared healthy were collected from different branches of C. alata from ottapalam, Palakkad, Kerala. The plant authentication were done in Botanical Survey of India, Southern Regional Centre, Coimbatore with reference no: BSI/SRC/01/18/2021/Tech/93.

2.2 Preparation of plant extract

The plant leaf sample were washed thoroughly with running tap water followed by double distilled water twice, to remove the adhering dust particles and the leaves were dried under the shades for two weeks. It is then grinded into fine powder form and stored in an airlift container. The plant extract of C. alata was prepared by, boiled the 20g of leaf powder with 250ml distilled water. After 15 minutes the aqueous extract was cooled and filtered through What man No.1 filter paper and get clear plant extract.. The aqueous leaf extract was used as reducing agent for the synthesis of silver nanoparticles.

2.3 Phytochemical analysis (Qualitative)

The qualitative phytochemical study was performed on the extracts by using below standard tests.

2.3.1 Test for Alkaloids (Drangendroff test)

About 2ml of the extract was treated with 4-5 drops of drangendroff reagent. The formation of orange red precipitate confirms the presence of alkaloids.

2.3.2 Test for Flavonoids (Lead acetate test)

To about 2ml of plant extract, few drops of lead acetate solution was added. The formation of yellow colour indicates the presence of flavonoids.

2.3.4 Test for Phenol (Ferric chloride test)

About 2ml of the extract was treated with 10% ferric chloride solution and observed for the formation of deep blue / black colour.

2.3.5 Test for reducing sugars (Benedict's Test)

To 1 ml of the extract added few drops of benedict's reagent and the mixture was boiled in a boiling water bath for 5minutes and observed for the appearance of orange red precipitate.

2.3.6 Test for carbohydrates (Molisch's test)

To 1 ml of the extract add 2 drops of alpha napthol solution then shaken well. Add concentrated H2SO4, development of a violet ring at the junction of a two liquids confirmed the presence of carbohydrates.

2.3.7 Test for Phytosterols (Salkowski's Test)

One ml of the plant extract was treated with 2 ml of chloroform and few drops of acetic anhydride were added. To that mixture added equal amount of concentrated sulphuric acid was added. The formation of bluish green colour indicates the presence of phytosterols.

2.3.8 Test for Aminoacids and Proteins (Ninhydrin test)

To a few ml of plant extract added small amount of Ninhydrin reagent. A purple or violet colour formed indicates the presence of amino acids and proteins.

2.4 Preparation of 1mM silver nitrate solution:

Dissolve 0.169 AgNO3 (silver nitrate) in 1000ml of distilled water and used for the green synthesis of silver nanoparticles (AgNPs).

2.5 Green synthesis of silver nanoparticles

10 ml of filtered aqueous extract of cassia alata leaves was added to 90 ml of 1mM AgNO3in a 250 ml Erlenmeyer flask. Then kept in room temperature for 48 hours at dark. The process was continued till the change of colour occurred from yellow to dark brown indicating the completion of silver nanoparticle synthesis. After 24 hour centrifuge the reaction mixture and discard the supernatant. added 1 ml of distilled water to the pellet and washed by centrifugation. The pellet was collected and the nanoparticles were stored for further characterization.

2.6 Characterization of silver nanoparticles

2.6.1 UV-Visible spectral analysis

UV-Visible spectroscopy is an important technique for analyzing the formation of SNPs in aqueous solution. The bio reduction of pure silver ions to silver nanoparticles was observed by UV-Visible spectroscopy. Taking 4ml of the sampe, compared with 4ml of 1mM silver nitrate used as blank. The absorption maxima were measured by using UV spectrophotometer between 300-800nm wavelength.

2.6.2 Fourier transform infra-red spectroscopy (FTIR)

The fourier transform infrared spectroscopy (FTIR) analysis was carried out to know the different functional groups that act as bioreductors to reduce Ag+ ions to Ag0. 2mg of the dried sample (AgNPs) were mixed with 200mg of KBr and pressed into a pellet. it was placed into the sample holder for FTIR analysis, in which the samples were irradiated by a broad spectrum of infra-red light and the level of absorbance at a particular frequency was plotted after fourier transformation of the data.

Compounds contained in the dried sample were identified according to standard infra-red chart.

2.6.3 Scanning Electron Microscopy (SEM Analysis)
SEM analysis was carried out to determine the particle morphology. The biologically synthesized silver nanoparticle sample was centrifuged. The pellet was collected and dried. The fine sample was used for SEM analysis.

2.6.4 Dynamic light scattering analysis (DLS)
Dynamic light scattering (DLS) sometimes referred to as Quasi Elastic Light Scattering(QELS) or Photon Correlation Spectroscopy(PCS)is a non-invasive, well established technique for measuring size distribution and zeta potential of molecules and particles (related to the magnitude of the electrical charge at the particle surface) or stability of the SNPs dispersed in water. In this technique the sample is illuminated by a laser beam and the fluctuations of the scattered light are detected at a known scattering angle Θ by a fast photon detector.

2.7 Antimicrobial Activity (Agar Well Diffusion Method)
2.7.1 Anti-bacterial activity
Preparation of inoculum
Stock cultures were maintained at 4℃ on slant of nutrient agar. Active cultures for experiments were prepared by transferring a loop full of cells from the stock cultures to test tubes of nutrient broth for bacteria that were incubated at 24hrs at 37℃. The assay was performed by agar diffusion method.

Antibacterial activity of sample was determined by well diffusion method on Muller Hinton Agar (MHA) medium.

The Muller Hinton Agar medium was weighed as 3.8 gms and dissolved in 100 ml of distilled water and add 1 gm of agar. Then the medium is kept for sterilization. After sterilization the media was poured in to sterile petriplates and were allowed to solidify. After the medium was solidified, the inoculums were swabbed on the MHA plates with sterile swab moistened with the bacterial suspension. Wells were made by using cork borer. Different concentration of samples (20µl, 40µl and 60µl) and positive control (streptomycin 1mg/ml- 10µl) were loaded in respective wells. These plates were incubated for 24 hours at 37℃ . Then the microbial growth was determined by measuring the diameter of zone of inhibition.

2.7.2 Antifungal activity
Preparation of inoculum
Stock cultures were maintained at 4℃ on slant of potato dextrose agar. Active cultures for experiments were prepared by transferring a loop full of cells from the stock cultures to test tubes of potato dextrose broth for fungi that were incubated at 4 days at room temperature. The assay was performed by agar diffusion method.

Antifungal activity of sample was determined by well diffusion method on Potato Dextrose Agar (PDA) medium.

The Potato Dextrose Agar medium was weighed as 4.4gms and dissolved in 100 ml of distilled water and add 1 gm of agar. Then the medium is kept for sterilization. After sterilization the media was poured in to sterile petri plates and were allowed to solidify for 1 hr. After the medium was solidified, the inoculums were swabbed on the PDA plates with sterile swab moistened with the fungal suspension. Wells were made by using cork borer. Different concentration of samples (20µl, 40µl and 60µl) and positive control (Griseofulvin 1mg/ml- 10µl) were loaded in respective wells. These plates were incubated for 48-96 hours at 28℃ . Then the microbial growth was determined by measuring the diameter of zone of inhibition.

3. RESULT

3.1 Phytochemical analysis
Phytochemicals are naturally found in plants, they are biologically active and function to protect plants against invasion, disease and infection. The present study is based on the synthesis of silver nanoparticles using aqueous extract of *Cassia alata* leaves. Table.1.

3.2 Green Synthesis of Silver Nanoparticles
The plant extract were pale brown in colour before the addition of silver nitrate solution. As the aqueous extract was mixed in the aqueous solution of silver, it started to change the colour from pale brown to dark brown due to reduction of silver ion which may be the indication of formation of silver nanoparticles.

3.3 Characterization of Green Synthesized AgNPs
3.3.1 UV-Visible spectral analysis
The absorption spectrum showed maximum peak at 430nm for the nanoparticle synthesized using aqueous extract of Cassia alata leaves. (Fig.)

3.3.2 Fourier Transform Infrared Spectroscopy (FTIR)
The FTIR analysis of the nanoparticle sample is given in the Fig. 12. The FTIR analysis of nanoparticle sample (aqueous leaf extract of *Cassia alata*) shows peaks at 3564.45, 3525.88 indicate the presence of OH group. The peak at 2927.94 indicates the presence of OH stretch. The peaks at 2364.73 indicates NH stretch. The peak value of 1516.05 indicates the presence of C=C groups (preferably belonging to benzene ring). The peak in between 1000-1500 indicates strong C-F stretching (fluro compound).

3.3.3 SEM Analysis
SEM images shows similar appearance for the presence of silver nanoparticles synthesized from C. alata. The SEM image shows cluster of nanoparticles in different size. The particles are spherical in shape. The SEM analysis confirmed the presence of nanoparticle. (Fig.).

3.3.4 Particle Size Determination

The particle size of AgNPs obtained was detected by Dynamic Light Scattering Analysis (DLS). The size of synthesized AgNPs ranges from 35 to 50 nm and the average diameter of the AgNPs were found to be >42nm. (Fig.)

3.3.5 Zeta Potential Measurement

The higher negative value of zeta potential confirms the repulsion among the particles and there by increases the stability of the formulation. The electrostatic repulsive forces between the nanoparticles when they are negatively charged possibly protect them from forming an association. This prevents the particles from agglomeration in the medium leading to long term stability. The AgNPs in this study were negatively charged with a zeta potential -0.383mV (Fig.), which proves that the particles were dispersed in the medium, that they are stable.

3.4 Antibacterial Activity

The inhibitory effect of silver nanoparticles synthesized from the aqueous leaf extract of *C.alata* were tested by using well diffusion method on MHA (Muller Hinton Agar) plates against human pathogens such as *Bacillus subtilis*, *Escherichia coli*. And the results are obtained are given in Table.2.

On the basis of antimicrobial activities of silver nanoparticles synthesized from the leafs of *C.alata*, it was evident that they shows significant antibacterial activity against Bacillus sp., and *E.coli*. Fig.

3.5 Antifungal Activity

The inhibitory effect of silver nanoparticles synthesized from the aqueous leaf extract of *C.alata* were tested by using well diffusion method on PDA (Potato Dextrose Agar) plates against 2 fungus such as *Candida albicans* and *Candida trophicalis* and the results obtained are given in the Table.3.

On the basis of antimicrobial activities of silver nanoparticles synthesized from the leafs of *C.alata*, it was evident that they shows significant antifungal activity against *Candida albicans* and *Candida trophicalis*. Fig.8.

Table 1: Phytochemical Evaluation of *Cassia alata* Leaf Extract.

TEST	RESULT
1. Alkaloids (Dragendroffs test)	++
2. Flavonoids (lead acetate test)	++
3. Phenolic compounds(ferric chloride test)	++
4. Phytosterols (salkowskis test)	++
5. Carbohydrates (molischs test)	++
6. Reducing sugars (benedicts test)	++
8. Proteins and amino acids (ninhydrin test)	++

Table 2: Zone Of Inhibition Produced by Silver Nanoparticles Prepared from the Leaves of *Cassia alata* against Bacterial Pathogens.

Bacterial Pathogens	50 µl	100 µl	Standard (Chloramphenicol)
E.coli	1.3 cm	2.0 cm	2.2 cm
Bacillus subtilis	1.5 cm	2.2 cm	2.5cm

Table 9: Zone Of Inhibition Produced by Silver Nanoparticles Prepared from the Leaves of *Cassia alata* against Fungal Pathogens.

Fungal pathogen	50 µl	100 µl	Standard (Flucanazole)
C. albicans	1.5 cm	1.8 cm	2.0 cm
C.trophicalis	1.5 cm	2.2 cm	1.8cm

Figure 1: *Cassia alata* Plant Showing Leaves.

Figure 2: Aqueous Leaf extract of *Cassia alata* Before and After the Formation of AgNPs.

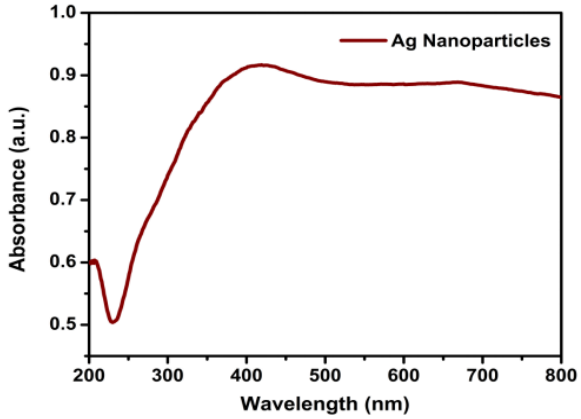


Figure 3: UV-Visible spectrum of silver nanoparticles synthesized from the aqueous leaf extract of *C.alata.*

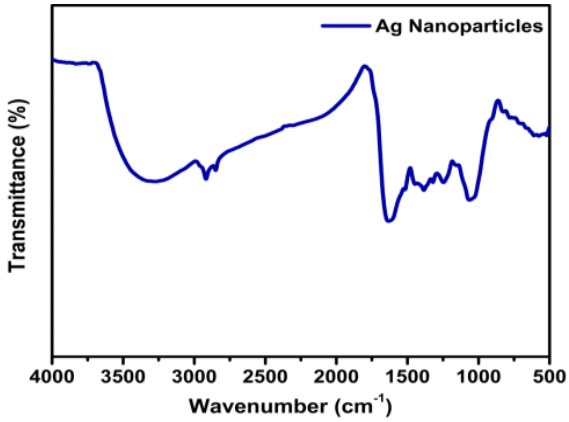


Figure 4: FTIR spectra of AgNPs synthesized from the aqueous leaf extract of *C.alata*

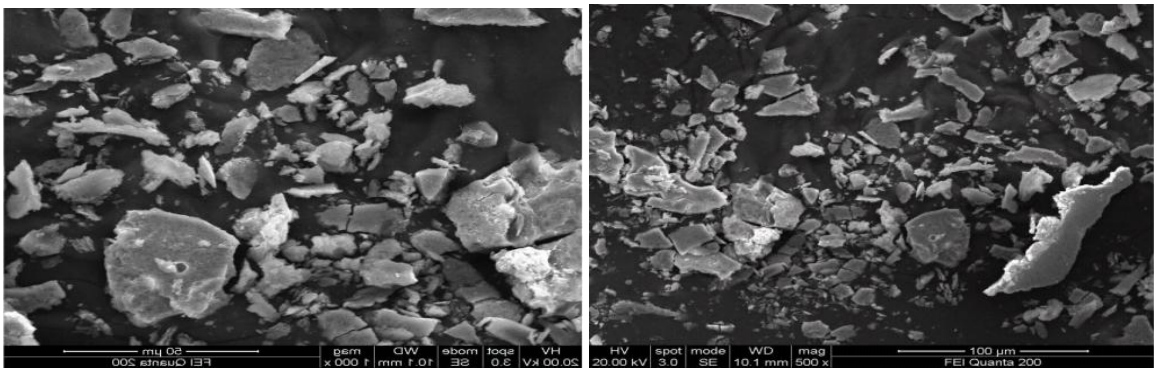


Figure 5: SEM images of AgNPs synthesized from aqueous leaf extract of *C.alata.*

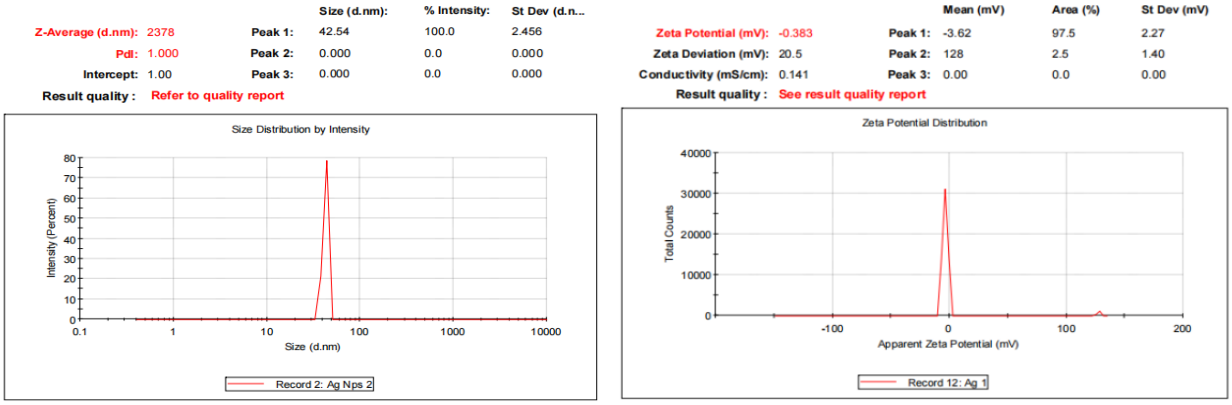


Figure 6 &7: DLS Analysis of AgNPs synthesized from aqueous leaf extract of *C.alata.*

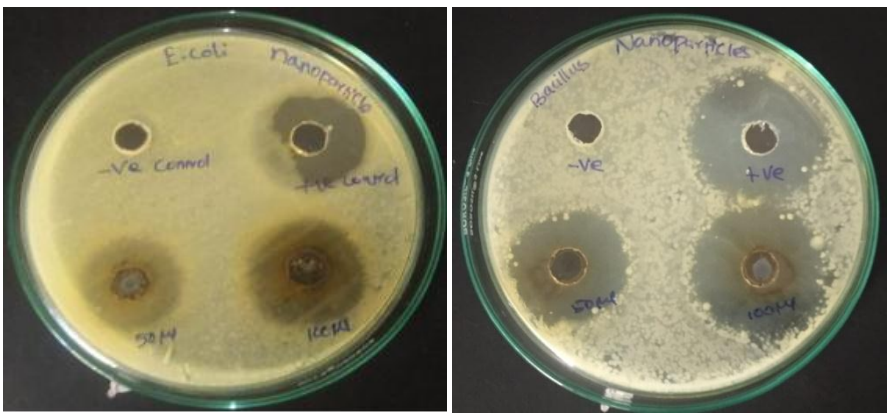


Figure 8: Antibacterial activity.

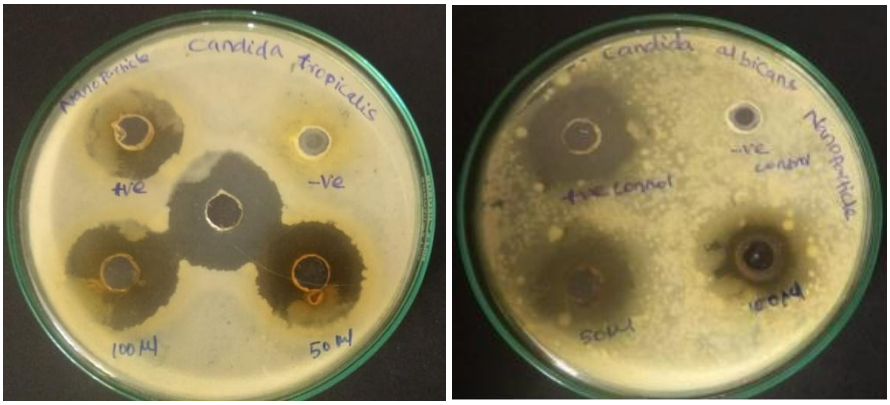


Figure 9: Antifungal activity.

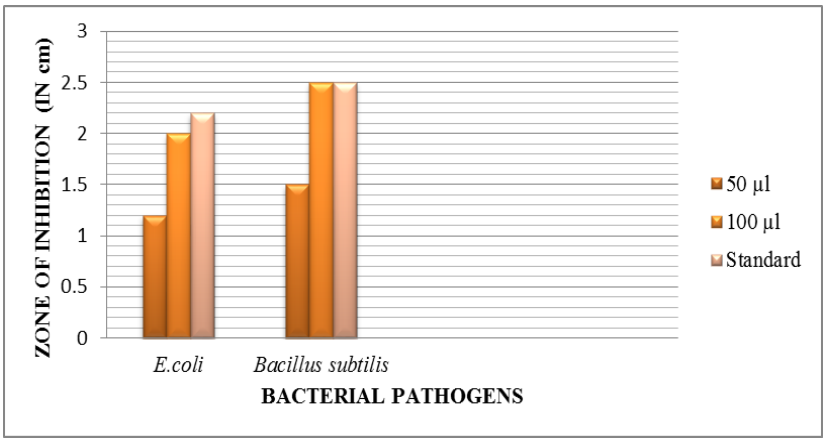


Figure 10. Zone of Inhibition Produced by Silver Nanoparticles Prepared from the Leaves of *Cassia alata* against Bacterial Pathogens.

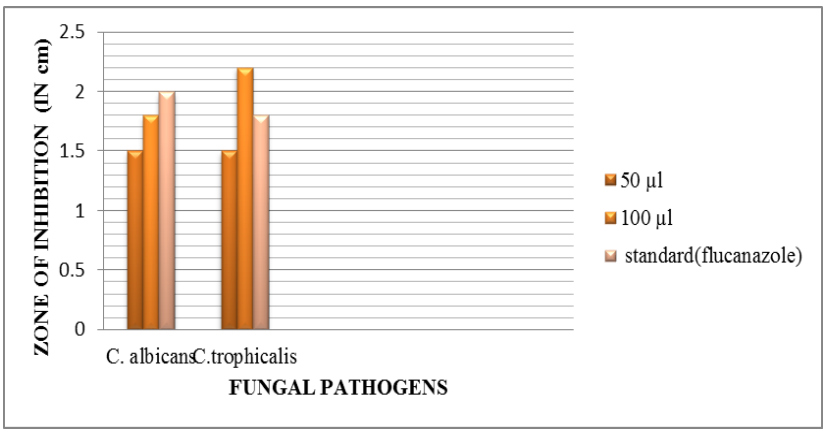


Figure 8: Zone of Inhibition Produced by Silver Nanoparticles Prepared from the Leaves of *Cassia alata* against fungal Pathogens.

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