Journal of Chemical Health Risks
www.jchr.org
JCHR (2024) 14(5), 297-304 | ISSN:2251-6727

Antimicrobial Efficacy of Ag-NPs of Gishta seed Extracts

Harikrishna Ramaprasad Saripalli[1*], Prasanna Kumar Dixit[2], Rajasekhar Dega[3], Uma Devarapalli[4]
[1]Research Scholar (D.Sc in Biotechnology), Berhampur University, Bhanja Bihar, Berhampur 750007, India. And Technical Consultant, IANZ Technical Expert, Twinlands NewZealand Limited, 10 Salisbury Place, Hamilton East, Hamiton 3216, Waikato, New Zealand. And
Manager-Operations, Technical & Quality, AgriTesting Laboratories (Accredited IANZ, New Zealand), A Division of King Honey Limited, Me|Today Limited, 1/6 Mahoe Street, Tauhara, RD2, Taupo 3378, New Zealand.
[2]Reader, P.G. Department of Zoology, Berhampur University, Bhanja Bihar, Berhampur 750007 (Odisha), India.
[3]Lecturer in Botany, Katta Rama Koteswara Rao Government Degree College, Addanki, Andhra Pradesh, India.
[4]Lecturer in Botany, Government College for Women (Autonomous), Guntur, Andhra Pradesh 522002, India.

(Received: 11 June 2024 Revised: 16 July 2024 Accepted: 10 August 2024)

KEYWORDS
Gishta, Ag-NPs, Antibacterial, Antifungal.

ABSTRACT:
Integration of plant-based silver nanoparticles as therapeutic agents opens up new horizons for combatting microbial infections, Nanotechnology is a congenial and proved technology for nanoparticle synthesis over conventional wet chemical techniques as it was proved ecofriendly and feasible over the latter. The study aims at evaluating the antibacterial, antifungal activity of Gishta seed nano particles. Ag-NPs prepared by green synthesis method and evaluated in vitro exhibited antibacterial, antifungal activity towards ten clinically significant microorganisms both bacteria and fungi. All the tested fractions exhibited good antibacterial and antifungal activity.

1. INTRODUCTION:

Graviola, a short, erect and evergreen tree species grows about 5–6 m long, leaves are shiny and dark green. It produces large, green, heart shaped fruits having white fleshy edible pulp. The fruit measures about 15–20 cm in diameter. The different parts of the plant have been utilized conventionally for treating various ailments (Edwards, S., *et al.*, 2000). *Graviola* is usually present in the rain forests of different regions of the Earth. The other names of the plant are thorny custard apple, cherimoya and Brazilian pawpaw. The fruit is also called differently in various languages like soursop (regions of America), and cachiman (épineux), shul-ram-fal, hanuman fal, and mullaatha (India), Harar and yebere lib (Ethiopia) means heart of cow (Blackherbals, 2019).
As traditional folkloric medicine seeds and leaves of Gishta are very effective in treating diseases, various parasitic infections etc. Seeds are known for their emetic function, seed oil kills lice, floral parts are antispasmodic in nature, fruit pulp has been used as diuretic and to treat some other ailments. The raw fruit has astringent properties, cures intestinal problems, whereas ripened fruits are used as antiscorbutic and anthelmintic. The beverage is used as medical supplement to treat gaustric problems. The bark is effective in curing diarrhoea and dysentery. The medicinal properties of the species are attributred to bio-active compounds acetogenins (Ruppercht, J.K., *et al*., 1982). To our knowledge investigation of Gishta for antibacterial, antifungal, has not been done especially on this plant species (Saripalli, Harikrishna & Dixit, Prasanna., 2016). This study explored on synthesis of AgNPs of Gishta seeds and studying their antibacterial and antifungal activity.

2. MATERIALS AND METHODOLOGY:

2.1 Ghista fruits collection:

Fruits of Ghista (Fig. 1a), collected from the Jimma (7°40'26.01"N, 36°50'8.85"E) Oromo region, Ethiopia, in January 2016 after confirmation and authentification deposited voucher specimen number:

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JCHR (2024) 14(5), 297-304 | ISSN:2251-6727

AAU/CBS/G/2014-01 in College of Biological Sciences, Addis Ababa University, and supplied by Ato Behailu Etana Disasa of Natural Resource Management, College of Agriculture and Veterinary Medicine, Jimma University, Jimma, Ethiopia.

2.2 Extract Preparation:

Seeds of Gishta were dried in the shade at 28 $\pm 3^0$C temperature under aseptic conditions. Then the dried seed materials were powered and subjected to standardized Soxhlet procedure using chloroform and methanol to extract the sample (Harikrishna Ramaprasad Saripalli., 2004). Thus prepared sample was preserved at 4^0C for further research.

2.3 Making of Silver Nitrate solution:

A quantity of 0.1699g of $AgNO_3$ particles dissolved in 1liter purified water to prepare 1mM solution and it was preserved in dark coloured glass jar for controlling the silver auto-oxidation.

2.4 Synthesis of Ag-NPs:

1mM $AgNO_3$ was taken in the flasks containing chloroform and methanol plant extract(s) separately and brought the volume of the solution to 200ml. The mixtures were subjected to the centrifugation at 18,000 rpm for 25min and later exposed to the sand bath for 10 min at 60^oc. Each solvent alone without seed extract served as a control. The process of incubation was continued till the solution turned to dark colour. Dried plant extract was subjected to flash evaporator for half-an-hour to get the powered Ag-NPs. The leftover material was treated as the absolute extract powder and different concentrations of the extract powder were prepared as shown in the table by dissolving it in the corresponding organic solvent (Harikrishna Ramaprasad Saripalli., 2007).

2.5 UV-VIS Spectral analysis of Ag-NPs:

The synthesis of Gishta mediated Ag-NPs were further confrmed by ultraviolet–visible spectroscopy (UV/VIS). A small quantity of the sample dissolved in the distilled water, suspended for 5 hours and subjected to spectral analysis (Shah M *et al.,* 2015).

2.6 Scanning Electron Microscope (SEM) analysis of Ag-NPs:

Studied physical characters of AgNPs using standard procedure and Scanning Electron Microscope (SEM). The re-dispersed nanoparticles were dried in oven to obtain a powdered form and 10mg of the sample was re-dispersed in ethanol. The sample was made into thin layers on a carbon supported copper grid and dried using mercury-vapor lamp.

2.7 Test organisms (Bacteria and Fungi):

As listed in Table-1 and 2 total ten different test organisms were selected for the study, of them seven were bacteria and the three were fungi, all are pathogenic. The test bacteria were cultured, inoculated in the broth medium and incubated following standard protocols. The bacterial suspensions were subjected to centrifugation and the pellets were suspended in the distilled water and brought to required concentration. OD value of each solution was noted at 0.45Å (610nm) and used for further studies. 9-10 days old fungal colonies grown on potato dextrose agar medium were harvested and the spore concentration was brought to required level.

2.8 Antibacterial activity of Ag-NPs:

Germ-destroying activity of biologically synthesized plant AgNPs was tested at various concentrations such as 250, 500, 750 and 1000µg using Kirby Baur test (Wang, Q.F., *et al.*, 2006; Lu, Y., *et al.*, 2007; Barry, A.C., 1976; Benson, H.J., 1990 and Cowan, M.M., 1999). The uniform mixture of medium and microbial suspension was poured into petri plates and allowed to be solidified. After proper solidification, wells of 6 mm were prepared in the medium and loaded with 50µl of silver nanoparticles of plant extract. The same process was adopted for three replicates. The plates were suspended at room temperature for 1hour for proper diffusion of extract into the medium and the bacterial test plates were incubated at 37°C for 1 - 2 days. The inhibition zone around the each well (Cheesbrough, M., 2000) was measured and recorded the mean value. Minimum inhibitory concentration was also measured. The solvents were taken as control and antibiotic Streptomycin (10µg/ml) was tested against the all test bacteria to compare the degree of inhibition of biologically synthesized silver nanoparticles. The total process was done under complete sterile conditions.

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Table 1. Bacterial Test Organisms:

S. No.	Organisms	Microscopic Traits	Ailments Caused
1.	*Escherichia coli*	–ve and rod	Gastroenteritis
2.	*Bacillus subtilis*	+ve and rod	Food poisoning
3.	*Pseudomonas aeruginosa*	–ve and rod	Lesions and Urinary tract diseases
4.	*Corynebacterium diphtheriae*	+ve and rod	Diphtheria
5.	*Xanthomonas citrovorum*	–ve and rod	Urinary tract diseases
6.	*Proteus vulgaris*	–ve and rod	Urinary tract and wound infections
7.	*Staphylococcus aureus*	+ve and round	Boils, respiratory tract infections, Meningitis, bone and joint infections, Endocarditis, Toxic shock syndrome

ATCC Codes: 1. 25922, 2. 6633, 3. 27853, 4. 75415, 5. 8082, 6. 638, 7. 25923

2.9 Antifungal activity of Ag-NPs:

Germ-destroying activity of biologically synthesized plant AgNPs was tested at various concentrations such as 250, 500, 750 and 1000µg using Kirby Baur test (Wang, Q.F., *et al*., 2006; Lu, Y., *et al*., 2007; Barry, A.C., 1976; Benson, H.J., 1990 and Cowan, M.M., 1999). The uniform mixture of medium and microbial suspension was poured into petri plates and allowed to be solidified. After proper solidification, wells of 6 mm were prepared in the medium and loaded with 50µl of silver nanoparticles of plant extract. The same process was adopted for three replicates. The plates were suspended at room temperature for 1hour for proper diffusion of extract into the medium and the fungal plates were incubated at 25°C for 4 – 5 days. The inhibition zone around the each well (Cheesbrough, M., 2000) was measured and recorded the mean value. Minimum inhibitory concentration was also measured. The solvents were taken as control and antibiotic Nystatin (10µg/ml) were tested against the all fungi to compare the degree of inhibition of biologically synthesized silver nanoparticles. The total process was done under complete sterile conditions.

Table 2. Final Test Organisms

S.No.	Organisms	roscopic Traits	Ailments Caused
1	*Aspergillus niger*	Branched and filament	Allergy, Asthma
2	*Aspergillus fumigatus*	Branched and filament	Lung disorders
3	*Candida albicans*	Dimorphic	Oral candidiasis, Intestinal disorders, skin infections

NCIM Codes: 8. 596, 9. 291, 10. 670

3. RESULTS:

3.1 Antibacterial activity:

Antibacterial potency of AgNPs was studied against the following pathogenic bacteria, using well diffusion method and zone of inhibition formed around was measured comparing with positive controls (Table 7). The antibacterial efficacy of the test material was found more (30 mm) against *Corynebacterium diphtheria* and less (15 mm) against *E. coli*. The results were perfectly correlated with the results of Gurunathan S *et al.*, 2009. Chloroform extracts of the test material showed activity at 500µg against *Corynebacterium diphtheria* and at 750 µg for the remaining bacteria under study. Methanol extract started working against *Bacillus subtilis*, *Corynebacterium diphtheria*, *Escherichia coli* and *Staphylococcus aureus* at 500 µg concentration and growth of other test organisms was controlled at 750 µg.

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Table 3. Zone of inhibition (mm) of Gishta mediated Ag-NPs against bacterial pathogens **Standard Antibiotics**:

Solvent extracts of Silver nanoparticles	**Product (µg)**	**Inhibition effect of Test material (mm)**						
		1	2	3	4	5	6	7
Chloroform	**control**	11.3	11.3	11.3	11.3	11.3	11.3	11.3
	250	11.3	11.3	11.3	11.3	11.3	11.3	11.3
	500	11.3	11.3	11.3	12.5	11.3	11.3	11.3
	750	16.3	16.3	22.5	20.0	20.0	18.8	18.8
	1000	22.5	22.5	28.8	28.8	27.5	28.2	22.1
Methanol	**control**	10.8	10.8	10.8	10.8	10.8	10.8	10.8
	250	10.8	10.8	10.8	10.8	10.8	10.8	10.8
	500	11.3	11.3	10.8	16.3	10.8	10.8	15.3
	750	15.0	15.0	11.3	21.3	11.3	15.8	26.9
	1000	20.0	20.0	16.3	30.0	17.5	15.8	39.1
Standard	**10**	26.0	28.6	13.7	25.8	26.0	17.0	19.1

Streptomycin (10µg/ml)

Test organisms:

1. *Escherichia coli*
2. *Bacillus subtilis*
3. *Pseudomonas aeruginosa*
4. *Corynebacterium diphtheria*
5. *Xanthomonas citrovorum*
6. *Proteus vulgaris*
7. *Staphylococcus aureus*

3.2 Antifungal activity:

The below are the details (Table 8) of antifungal naure of the plant under study. The studies were done invitro using agar plate method against three fungi viz. *Aspergillus niger*, *A. fumigatus* and *Candida albicans* at different concentration ranging from 250µg/ml to 1000µg/ml. Moderate activity was found against *Aspergillus niger* at 1000 µg/ml concentration, followed by *Aspergillus fumigatus* and *Candida albicans.*

Table 4. Zone of inhibition (mm) of Gishta mediated Ag-NPs against fungal pathogens

Solvent extracts of Silver nanoparticles	**Product (µg)**	**Zone of Inhibition (mm)**		
		8	9	10
Chloroform	**control**	19.5	19.5	19.5
	250	19.5	19.5	19.5
	500	19.5	19.5	25.0
	750	27.0	24.5	30.0
	1000	31.3	29.1	29.4
Methanol	**control**	19.5	19.5	19.5
	250	23.3	23.3	23.3
	500	23.3	23.3	23.3
	750	27.7	25.2	27.7
	1000	40.5	37.2	32.7
Standard	**10**	12.7	12.7	17.0

Standard Antibiotic: Nystatin (10µg/ml) – Fungi

Test organisms:

8. *Aspergillus niger*
9. *Aspergillus fumigatus*
10. *Candida albicans*

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The AgNPs synthesised by green technology of both chloroform and methanol were found very effective against the test organisms and the silver nanoparticles of (50 µL) showed higher efficacy. Chloroform extracts of the test material showed its activity against *Candida albicans* at 500 µg and *Aspergillus niger* and *A. fumigatus* at 750 µg. Methanol extract started working against *Aspergillus niger, A. fumigatus* and *Candida albicans* at 250 µg concentration. Growth of other test organisms was controlled at 750 µg.

Table 9. Details of the various factor variables for ANOVA model

Factor	**Type**	**Number of Levels**	**Values**
Test Samples	fixed	1	Gishta seeds
Solvents	fixed	2	Chloroform, Methanol
Concentration	fixed	5	Control, 250µg/ml, 500µg/ml, 750µg/ml, 1000µg/ml
Bacteria	Fixed/Block	7	1) *E. coli* 2) *B. subtilis* 3) *P. aeruginosa* 4) *C. diphtheria* 5) *X. citrovorum* 6) *P. vulgaris* 7) *S. aureus*
Fungi	Fixed/Block	3	8) *A. niger* 9) *A. fumigatus* 10) *C. albicans*

3.3 Analyzed data as follows:

Factorial design of Analysis of Variance for Response, using Adjusted SS for Tests					
Source	**D.F**	**Seq.SS**	**Adj.SS**	**Adj. MS**	**Fac**
Test Materials (Seeds)	1	565.22	565.22	282.6	21.93
Solvent Extract	2	95.46	95.46	31.82	7.47
Concentration	4	6130.3	6130.3	1532.58	118.92
Test materials*Solvent Extract	2	900.605	900.605	150.1	11.65
Test material*Concentration	4	5093.28	5093.28	636.66	49.4
Solvent Extract*Concentration	8	2534.42	2534.42	211.95	16.44
Test material*Solvent Extract*Concentration	8	2530.92	2530.92	105.45	8.18
Microorganisms	9	1265.46	1265.46	801.96	62.23
Error		865.51	11153.6	11153.6	6.44
Total		**899.5**	**31899.91**		

The results of Table 9 demonstrate that there is a significant effect of test sample and the solvents extracts in controlling the pathogens. The factor level effect was studied by graphical evaluation.

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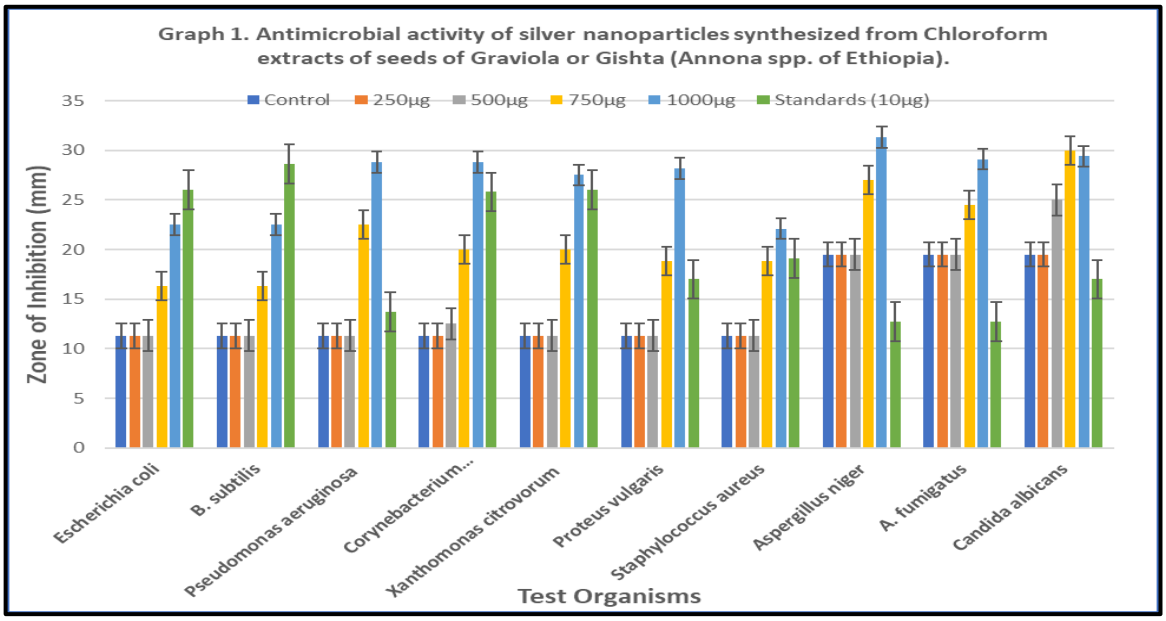
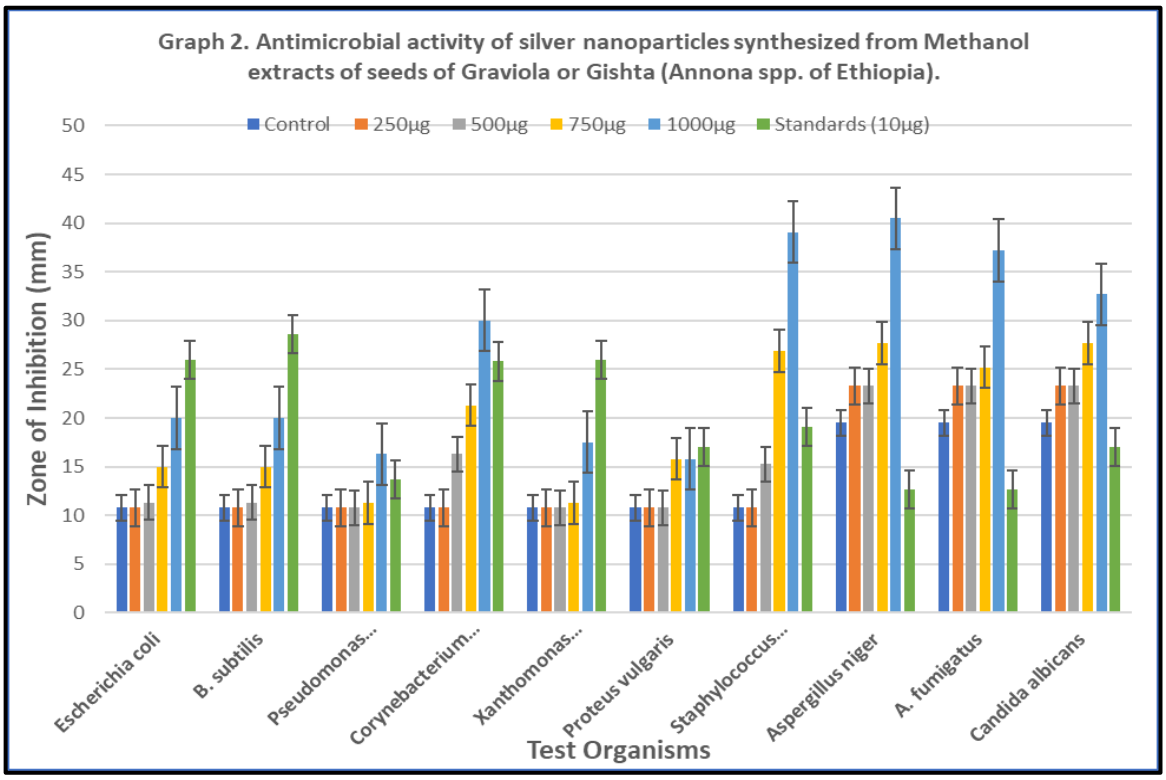
JCHR (2024) 14(5), 297-304 | ISSN:2251-6727



Antimicrobial activity of Gishta mediated Ag-NPs of chloroform extract. Standard- Streptomycin (10µg/ml) - Bacteria, Nystatin (10µg/ml) against fungi



Antimicrobial activity of Gishta mediated Ag-NPs of methanol extract Streptomycin (10µg/ml) - Bacteria, Nystatin (10µg/ml) - Fungi) are the standard antibiotics

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4. CONCLUSION:

Of all the solvent extracts of Gishta mediated Ag-NPs, methanolic extracts were proved the most effective in controlling all the test organisms. Inhibitory activity of the plant under study was in directly proportional with the concentration of the extract (Graph 2). The silver nanoparticles of chloroform and methanol extracts of Graviola seeds are very effective on microorganisms tested (Graph 1 and Graph 2). The silver nanoparticles of solvent seeds extract exhibited around 50% high efficacy in comparison with conventional solvent extracts (Saripalli, Harikrishna & Dixit, Prasanna., 2016).

Authors Contribution Statement:

Dr Harikrishna Ramaprasad Saripalli conducted research work, result analysis and prepared the whole manuscript including graphs, figures, statistical analysis and bibliography.

Dr Prasanna Kumar Dixit revised the draft and managed the project.

D. Raja Sekhar reviewed and corrected different versions of the manuscript.

Funding and Acknowledgements:

The research work conducted in this study received financial support from Biosquare (TwinLands NZ Limited) (NZBN: 9429051249311) located in Hamilton, New Zealand, 3216. The authors whole heartedly express their sincere thanks to A to B E Disasa of Natural Resource Management for their valuable assistance, which greatly contributed to the completion of this research project. Furthermore, the authors acknowledge the support of Aksum University for giving the essential instrumentation requirements for conducting the study.

The corresponding author would like to express deep gratitude to Prof. Sambasiva Rao, K.R.S, Prof. Madan Prasad, Chris Bowman (CEO of Cosana New Zealand), the Department of Chemistry, Waikato University, Hamilton, New Zealand, and Dr. Ammani. K for their invaluable motivation throughout the study.

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