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Research Article

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Formulation and Evaluation of Silver Nanoparticles for Effective Treatment of Topical Fungal Disease

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Abstract Silver nanoparticle gels are a novel form of plant-based biopesticide that can be used as an alternative to chemical-based pesticides for the treatment of fungal diseases on tomato plants. In this investigation, our attention will be focused on the preparation and evaluation of colloidal silver nanoparticles as antibacterial and antifungal agents. The colloidal silver nanoparticles have been prepared employing standard chemical reduction methods. The colloidal silver nanoparticles were characterized using transmission electron microscopy TEM, zeta potential, photo correlation spectroscopy PCS, and in vitro release kinetics. The particles thus obtained were spherical in shape and having an average particles size of 5-20 nm, zeta potentials of -25.5 to -38.3 mV, and the release kinetics was following zero order kinetics with r 2 >0.96. The dissolution data indicates that the release of the silver nanoparticles is inversely correlated with the size of the nanoparticles i.e. the release increased with smaller particles. The results suggest that the Ag NPs would be stable in the pharmaceutical preparations and will be easily to the infection site. The colloidal silver nanoparticles were found to be very efficient antibacterial agents for different types of bacteria. The bacteria studied were namely: E. coli, S. coccus, Salmonellae, and P. aeruginosa. The associated antifungal effects were also investigated for Aspergillus and Pencillium. Cytotoxicity of the nanoparticle was studied using human fibroblast cell line. It was concluded that cytotoxicity is concentrations dependant. The results provided strong evidence that could warrant the consideration of silver nanoparticles as antibacterial and antifungal agent that could circumvent the side and passive effects of the conventional antibiotics.

Keywords Formulation, Evaluation, Silver Nanoparticles, Topical Fungal Disease

Introduction

The emergence of fungi causing human infection is a growing serious public health problem. Fungi of medical importance may be characterized as primary or opportunistic. Primary which causes infection in healthy population who are not exposed to endemic fungi, whereas opportunistic infect immunosuppresed individuals. With the increase in numbers of individuals with low immunity and people with more susceptibility for fungal infections generate pressure on cost of human life and health care. Fungal Infections have been on the ascending since the past three decades, due to the emergence of HIV, influenza virus, various other immunodeficient conditions including solid organ transplantations, cancers, genetic defects and autoimmune conditions.

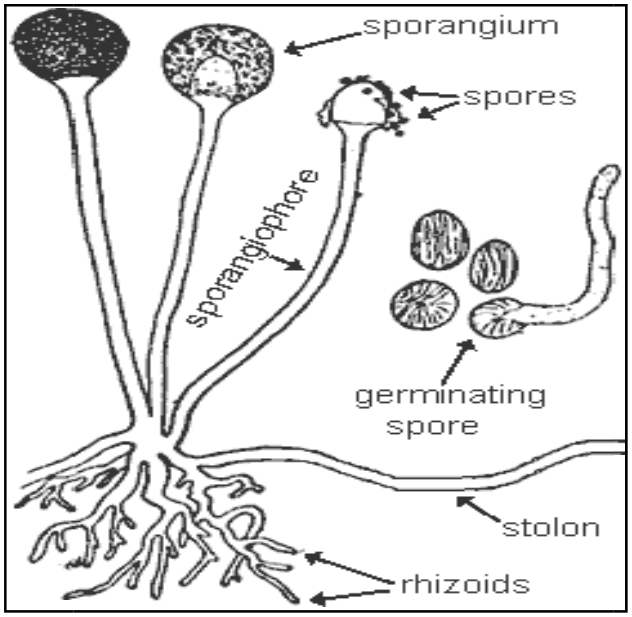

Figure 1: Structure of fungi

Silver nanoparticles

Nanoparticles exhibit novel properties which depend on their size, shape and morphology which enable them to interact with plants, animals and microbes [1-7]. Silver nanoparticles (Ag NPs) have shown excellent bactericidal properties against a wide range of microorganisms [8-11]. They are prepared from different perspectives, often to study their morphology or physical characteristics. Some authors have used chemical method [12] and mistaken it with green synthesis, although they have done it inadvertently. Silver has been used extensively from last5000 years for its antibacterial nature. Ag is preferred as nanoparticle for the reason that it has antibacterial property and non- toxic to human beings. Either killing or reducing the growth of bacteria without affecting surrounding cells is known as antibacterial activity.

Material and Method

Collection of plant material

The plants have been selected on the basis of its availability and folk use of the plant. Leaves of *Lagerstroemia parviflora* were collected from Bhojpur in the month of August, 2022. Drying of fresh plant parts was carried out in sun but underthe shade. Dried leaves of *Lagerstroemia parviflora* were preserved in plastic bags, closed tightly and powdered as per the requirements.

Extraction procedure

Following procedure was adopted for the preparation of extract from the shade driedand powdered herbs:

Defatting of plant material

Leaves of *Lagerstroemia parviflora* were shade dried at room temperature. 56.4 gram dried powdered plant material was coarsely powdered and subjected to extraction with petroleum ether by maceration. The extraction was continued till thedefatting of the material had taken place.

Extraction by maceration process

Defatted dried powdered leaves of *Lagerstroemia parviflora* has been extracted with hydroalcoholic solvent (ethanol: water: 70:30) using maceration process for 48 hrs, filtered and dried using vacuum evaporator at 40 ºC [45-46].

Phytochemical Screening

Detection of alkaloids: Extract were dissolved individually in diluteHydrochloric acid and filtered.

Mayer's Test: Filtrates was treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence ofalkaloids.

Wagner's Test: Filtrates was treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test: Filtrates was treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

Hager's Test: Filtrates was treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

Detection of carbohydrates: Extract was dissolved individually in 5 ml distilledwater and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch's Test: Filtrates was treated with 2 drops of alcoholic α-naphthol solutionin a test tube. Formation of the violet ring at the junction indicates the presence of Carbohydrates.

Benedict's Test: Filtrates was treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Fehling's Test: Filtrates was hydrolysed with dil. HCl, neutralized with alkali andheated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

Detection of glycosides: Extract was hydrolysed with dil. HCl, and then subjected to test for glycosides.

Detection of saponins

Froth Test: Extract was diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of phenols

Ferric Chloride Test: Extract was treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Detection of flavonoids

Alkaline Reagent Test: Extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extract was treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Detection of proteins

Xanthoproteic Test: The extract was treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Detection of diterpenes

Copper acetate Test: Extract was dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence ofditerpenes.

Total flavonoids content estimation

Determination of total flavonoids content was based on aluminium chloride method

Preparation of standard: 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5-25µg/ml were prepared in methanol.

Preparation of extract: 10mg of dried extracts of were dissolved in 10 ml methanol and filtered. 3 ml (1mg/ml) of this solution was used for the estimation of flavonoid.

Procedure: 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

Total Phenolic content estimation

The total phenolic content of the extract was determined by the modified Folin- Ciocalteu method

Preparation of Standard: 10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 10-50µg/ml was prepared in methanol

Preparation of Extract: 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Two ml (1mg/ml) of this solution was used for the estimation ofphenol.

Procedure: 2 ml of each extract or standard was mixed with 1 ml of Folin-Ciocalteureagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15 min for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Biosynthesis of Silver nanoparticles

$AgNO_3$ powder was dissolved in distilled water to prepare 10 mM $AgNO_3$ stock solution from which a series of 1 mM, 2 mM and 3 Mm $AgNO_3$ solutions were prepared [49]. The $AgNO_3$ solutions were mixed with the extract of leaves of *Lagerstroemia parviflora* at a ratio of 1:1, and 1:2 (v/v) to a volume of 50 mL in a flask. The flask was wrapped with an aluminum foil and was then heated in a water bath at 60°C for 5 hours. Furthermore, the mixture was stored in the refrigerator forthe further use.

Table 1: Different formulation of Silver nanoparticles

Formulation Code	Extract (mg)	$AgNO_3$ (mM)	Ratio
F1	500	1	1:1
F2	500	2	1:1
F3	500	3	1:1
F4	500	1	1:2
F5	500	2	1:2
F6	500	3	1:2

Formulation development of gel

Measured amounts of methyl paraben, glycerin, polyethylene glycol and hydroalcoholic extract of leaves of *Lagerstroemia parviflora* were dissolved in about 100 ml of water in a beaker and stirred at high speed using mechanical stirrer(or sonicator) [53]. Then Carbopol 940 was slowly added to the beaker which contained above liquid while stirring. Neutralized the solution by adding a slow, constantly stirring triethanolamine solution until the gel formed.

Table 2: Formula

Ingredients (mg)	F1	F 2	F3
Lagerstroemia parviflora extract	500	500	500
Carbopol 940	250	500	750
Polyethylene Glycol 600	0.2	0.2	0.2
Methyl Paraben	0.08	0.08	0.08
Triethanolamine	1.0	1.0	1.0
Distilled Water	100 ml	100ml	100ml

Evaluation of gel

Appearance and consistency:

The physical appearance was visually checked for the texture of gel formulations and observations reported in table 3.

Washability

Prepared formulations were added to the skin and then manually tested for ease anddegree of washing with water, and findings were recorded in table 7.9.

Extrudability determination of formulations

The gel formulations were filled into aluminium collapsible tubes and sealed. Thetubes were pressed to extrude the material and the extrudability of the formulation was noted.

Determination of Spreadability

For gels an significant requirement is that it must have strong spreadability. Spreadability is a concept defined to denote the degree to which the gel applies readily to the skin upon application. A formulations medicinal potency also dependson its spread-value.

Method:

Two normal dimensional glass slides (6x2) were chosen. The gel formulation the spreadability of which had to be determined was placed over one of the diapers. Thesecond slide was mounted over the slide in such a way as to sandwich the formulation over the slide over a length of 6 cm between them. The upper slide had 20 grams of weight, so that the gel formulation between the two was placed uniformly to form a thin layer [54].

Determination of pH

Digital pH meter had calculated the pH of the gels. One gram of gel was dissolved in 25 ml of purified water and the electrode was then dipped into gel solution until steady reading was achieved. Measurements of pH were repeated twice for each formulation.

Drug content

The composition of the medication was measured by taking 1gm of gel mixed with methanol in 10 ml volumetric flask. 3 ml of stock solution has been mixed with 1 ml $AlCl_3$ solution of 2 percent. The mixture was vortexed for 15s and allowed for the color production to stand at 40°C for 30min, using a spectrophotometer the absorbance was measured at 420 nm [55].

***In vitro* diffusion profile (*In vitro* permeation in rat skin)**

In vitro diffusion experiments were performed using Franz diffusion cell for all formulations. Locally assembled as an open-ended cylindrical tube with an area of 3.7cm^2 and a height of 100 mm with a diffusion area of 3.8 cm^2. Phosphate buffer (pH 7.4) was used as substrate for receptors. Rat abdominal skin used as membranefor dialysis. The skin was tied to the diffusion cell (donor cell) such that the stratumcorneum side of the skin was in intimate contact with the release surface of the formulation in the donor cell. Isotonic phosphate buffer solution, pH 7.4 (100 ml) was added to a donor compartment prior to be mounted on the diffusion cell. A weighed quantity of formulation equivalent to 1g of gel was taken on to the rat skinand was immersed slightly in 100 ml of receptor medium, which was continuously stirred. The whole network had been held at 37±1ºC. At different time intervals of up to 4 hours, an aliquot of 5 ml was extracted, and spectrophotometrically measured at 295 nm. The diffusion media was replaced with an equal volume of fresh diffusion medium after each withdrawal. For each time period the total percentrelease was measured.

Mathematical treatment of *in-vitro* release data:

Table 3: Interpretation of diffusional release mechanisms

Release exponent (*n*)	Drug transport mechanism	Rate as a function of time
0.5	Fickian diffusion	$t^{-0.5}$
0.5<*n*<1.0	Anomalous transport	$t^{n - 1}$
1.0	Case-II transport	Zero-order release
Higher than 1.0	Super Case-II transport	$t^{n - 1}$

Antifungal activity of silver nanoparticle gelMedia preparation (broth and agar media) Composition of potato dextrose agar media;

Potato infusion- 20 gms.
Dextrose- 2 gms.
Agar - 1.5 gms.
Distilled water- to make 100 ml.pH – 7

This agar medium was dissolved in distilled water and boiled in conical flask of sufficient capacity. Dry ingredients are transferred to flask containing required quantity of distilled water and heat to dissolve the medium completely. The flask containing medium was cotton plugged and was placed in autoclave for sterilizationat 15 lbs /inch^2 (121^{o}C) for 15 minutes. After sterilization, the media in flask was immediately poured (20 ml/ plate) into sterile petri dishes on plane surface. The poured plates were left at room temperature to solidify and incubate at 37^{o}C overnight to check the sterility of plates. The plates were dried at 50^{o}C for 30 minutes before use.

The fungal cultures used in the study were obtained in lyophilized form. With the help aseptic techniques the lyophilized cultures are inoculated in sterile potato dextrose broth than incubated for 48 hours at 25^{o}C. After incubation the growth is observed in the form of turbidity.

These broth cultures were further inoculated on to the potato dextrose agar plates with loop full of microbes and further incubated for next 24 hours at 37^{o}C to obtainthe pure culture and stored as stocks that are to be used in further research work.

The well diffusion method was used to determine the antifungal activity of silver nanoparticle gel prepared from the leaves of *Lagerstroemia parviflora* using standard procedure [56]. There were 3 concentration used which are 25, 50 and 100 mg/ml for each extracted phytochemicals in studies. It's essential feature is the placing of wells with the antibiotics on the surfaces of agar immediately after inoculation with the organism tested. Undiluted over night broth cultures should never be used as an inoculums. The plates were incubated at 25^{o}C for 48 hr. and then examined for clear zones of inhibition around the wells impregnated with particular concentration of drug.

Result and Discussion

Table 4: Determination of percentage yield

S. No.	Solvents	% Yield (w/w)
1.	Pet ether	1.53%
2.	Hydroalcoholic	6.74%

Phytochemical screening of extract

Table 5: Phytochemical screening of extract of leaves of *Lagerstroemia parviflora*

S. No.	Constituents	Hydroalcoholic extract
1.	**Alkaloids**	
	Mayer's Test	-ve
	Wagner's Test	-ve
	Dragendroff's Test	-ve
	Hager's Test	-ve
2.	**Glycosides**	
	Legal's Test	+ve
3.	**Flavonoids**	
	Lead acetateAlkaline	+ve
	test	+ve
4.	**Phenol**	
	Ferric chloride test	+ve

5.	**Proteins** Xanthoproteic test	 +ve
6.	**Carbohydrates** Molisch's Test Benedict's Test Fehling's Test	+ve +ve +ve
7	**Saponins** Froth Test Foam Test	 +ve +ve
8	**Diterpenes** Copper acetate test	 -ve
9	**Tannins** Gelatin Test	 +ve

Results of estimation of total flavonoids and phenol content

Table 6: Preparation of Calibration curve of Quercetin

S. No.	Concentration (µg/ml)	Absorbance
1	5	0.204
2	10	0.412
3	15	0.604
4	20	0.798
5	25	0.971

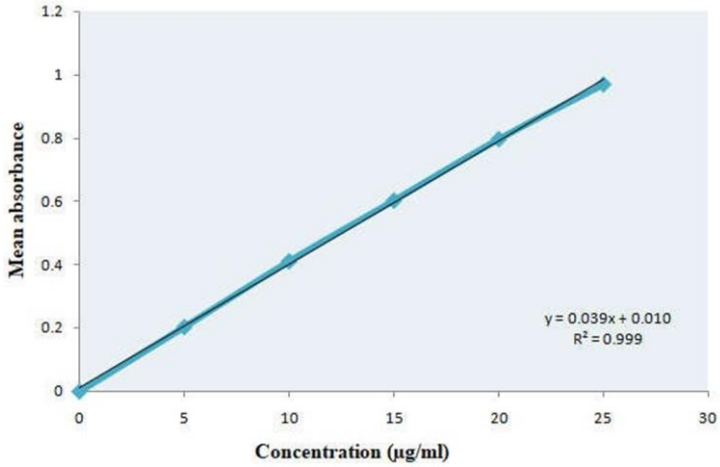

Figure 2: Graph of calibration curve of Quercetin

Total Phenolic content estimation (TPC)

Table 7: Preparation of calibration curve of Gallic acid

S. No.	Concentration (µg/ml)	Absorbance
1	10	0.185
2	20	0.347
3	30	0.514
4	40	0.676
5	50	0.857

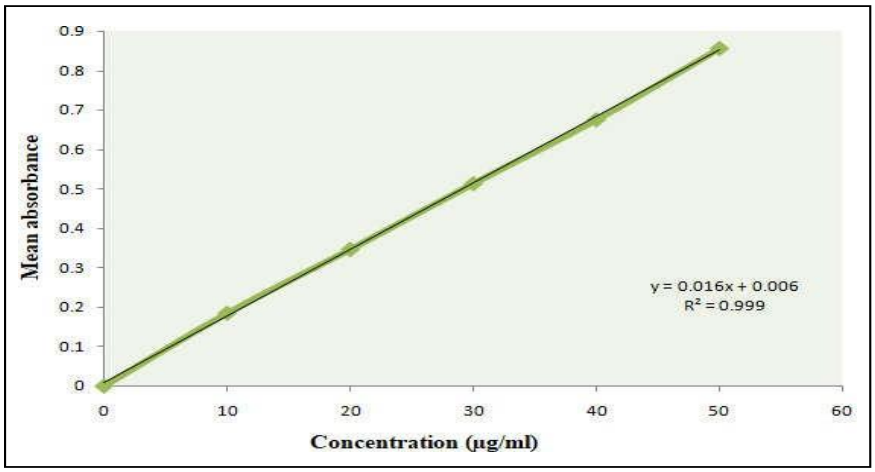

Figure 3: Graph of estimation of total Phenolic content

Table 8: Estimation of total flavonoids and phenol content of leaves of *Lagerstroemia parviflora*

S. No.	Extract	Total flavonoids content (mg/ 100 mg of dried extract)	Total phenol content (mg/ 100 mg of dried extract)
1.	Hydroalcoholic	0.674	0.287

Characterization of optimized formulation of silver nanoparticles

Percentage Yield

The yield of silver nanoparticles is an important factor to consider when producing nanoparticles because it directly impacts the cost of production and the amount of nanoparticles that can be produced. It is also important to consider the quality of the nanoparticles produced as this can affect their performance. The yield of silver nanoparticles can be determined by measuring the amount of silver nanoparticles produced in relation to the amount of silver precursor used. This can be done by analyzing the silver nanoparticles using various UV-visible spectroscopy.

Table 9: Determination of % yield of prepared formulations

Formulation code	% Yield
F1	65.58±0.25
F2	69.98±0.32
F3	78.85±0.15
F4	70.23±0.23
F5	68.87±0.18
F6	67.45±0.21

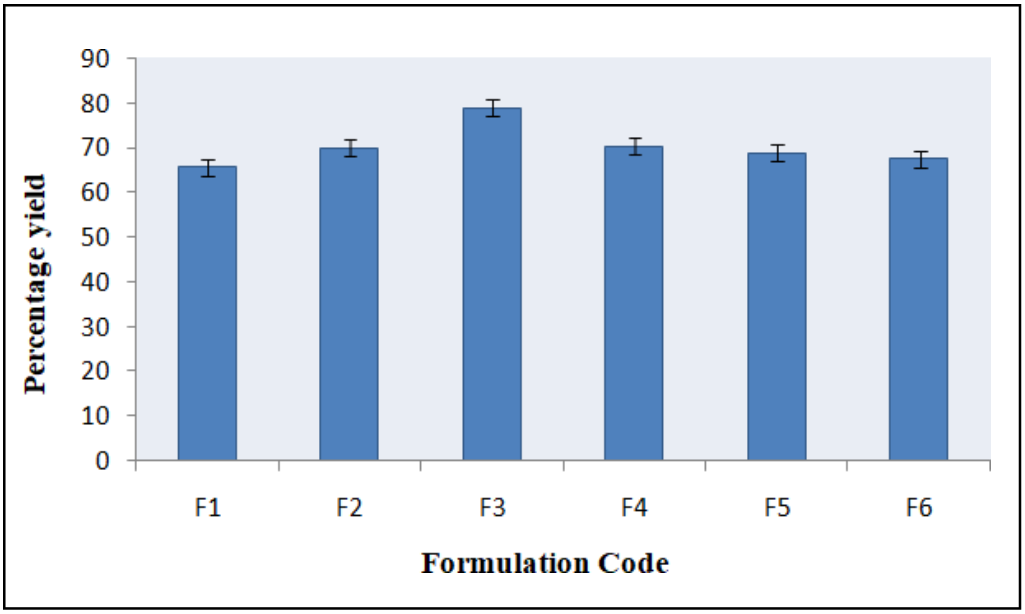

Figure 4: Graph of determination of % yield of prepared formulation

Results of % entrapment efficiency

Table 10: Determination of entrapment efficiency of prepared formulations

Formulation code	Percentage entrapment efficiency (Flavonoid mg/100mg quercetin equivalent)
F1	0.612±0.015
F2	0.605±0.025
F3	0.652±0.014
F4	0.587±0.016
F5	0.605±0.012
F6	0.582±0.017

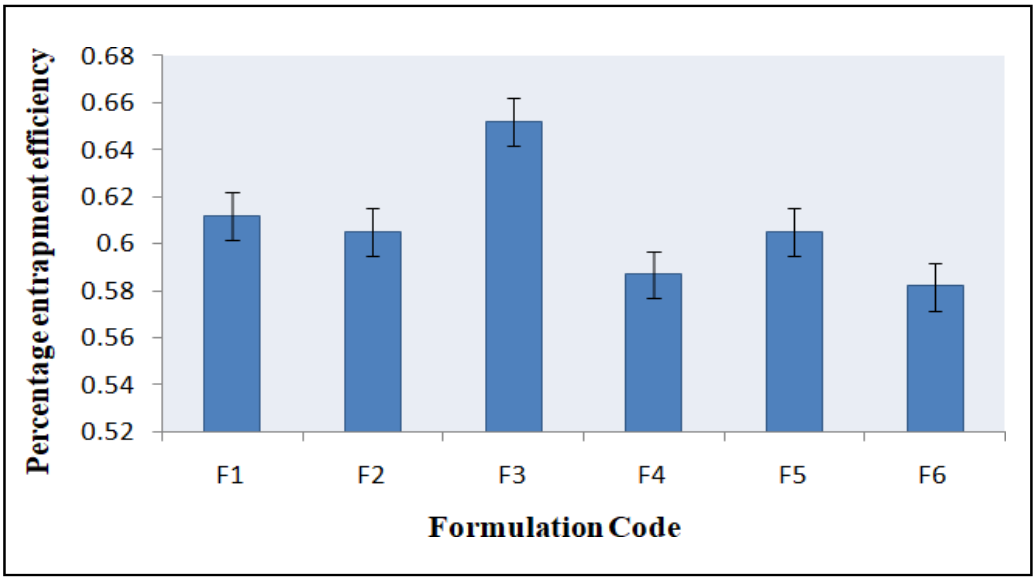

Figure 5: Graph of % entrapment efficiency

Average particle size and zeta potential

Table 11: Characterization of average particle size and zeta potential

Formulation code	Average Particle size (nm)	Zeta Potential (mV)
F3	220.5	- 38.5 mV

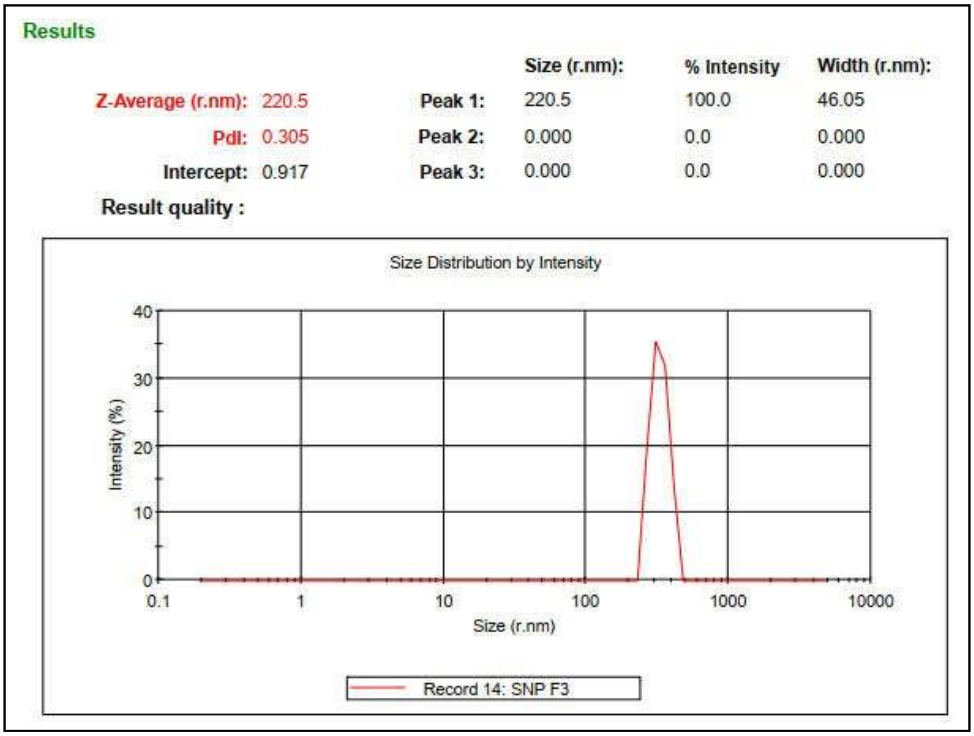

Figure 6: Graph of Average particle size of formulation F3

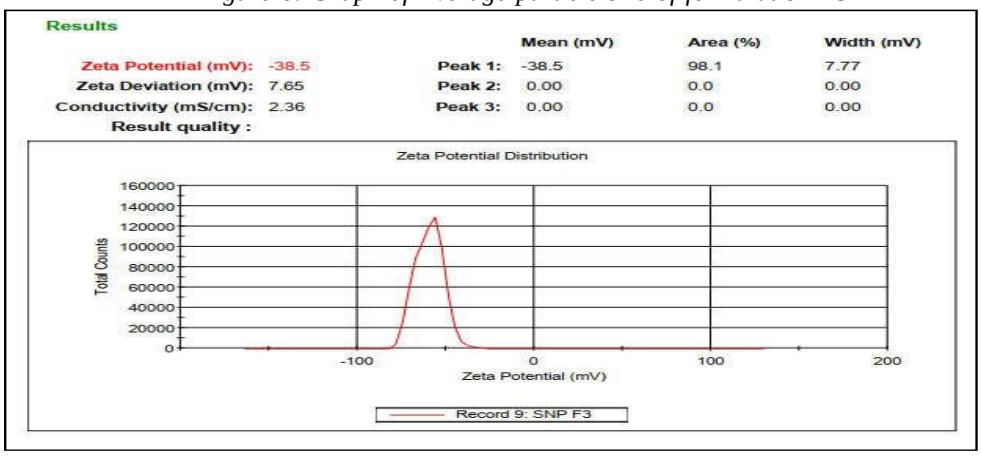

Figure 7: Graph of Average vesicle size formulation F3

Results of gel Formulation

Evaluation of gel formulation of gel

Table 12: Results of physical characteristics

Formulation code	Colour	Clogging	Homogeneity	Texture	Washability	Extrudability
F1	Brown	Absent	Good	Smooth	Good	Good
F2	Brown	Absent	Good	Smooth	Good	Good
F3	Brown	Absent	Good	Smooth	Good	Good

Results of Spreadability

The results of the spreadability of the silver nanoparticles gel show that the spreadability of the gel is good. The results show that the gel has a good spreadability and can easily spread on a surface. This can be attributed to the small size of the silver nanoparticles, which increases the surface area and enhances the spreadability of the gel. The Spreadability of Formulation F1, F2, F3 was found 9.12±0.25, 8.45±0.15 and 7.36±0.10 respectively.

Table 13: Results of spreadability of gel

Formulation code	Spreadability* (gcm/sec)
F1	9.12±0.25
F2	8.45±0.15
F3	7.36±0.10

Results of Viscosity: - The Viscosity of Prepared formulation was found to be 3245, 3165 and 3047 cp for formulation F1, F2, F3 respectively.

Table 14: Results of Viscosity of gel

Formulation code	Viscosity* (cp)
F1	3245
F2	3165
F3	3047

*Average of three determinations (n=3 ±SD)

Results of flavonoid Content

Table 15: Results of flavonoid content in gel using $AlCl_3$ method

Formulation code	Flavonoid Content (mg/100mg)
F1	0.589±0.054
F2	0.854±0.032
F3	0.789±0.042

*Average of three determinations (n=3 ±SD)

Results of pH

The results of the pH of the gel of prepared silver nanoparticles gel show that the gel has a pH of 7.25±0.02, 7.05±0.01 and 7.25±0.02 for formulation F1, F2, F3 respectively. This is slightly higher than the neutral pH of 7.0.

Table 16: Results of pH of gel

Formulation code	pH
F1	7.25±0.02
F2	7.05±0.01
F3	7.25±0.02

*Average of three determinations (n=3 ±SD)

Results of *in vitro* drug release study

Table 17: *In vitro* drug release study of prepared gel formulation

S. No.	Time (hr)	% Cumulative Drug Release		
		F1	F2	F3
1	0.25	28.85	22.36	18.85
2	0.5	35.65	33.36	23.36
3	1	48.85	45.58	39.98
4	1.5	63.32	59.98	42.25
5	2	79.98	67.74	56.65
6	2.5	88.85	79.98	67.74
7	3	98.98	86.65	75.65
8	4	99.12	98.78	86.65

***In-vitro* drug release study of F1, F2 and F3**

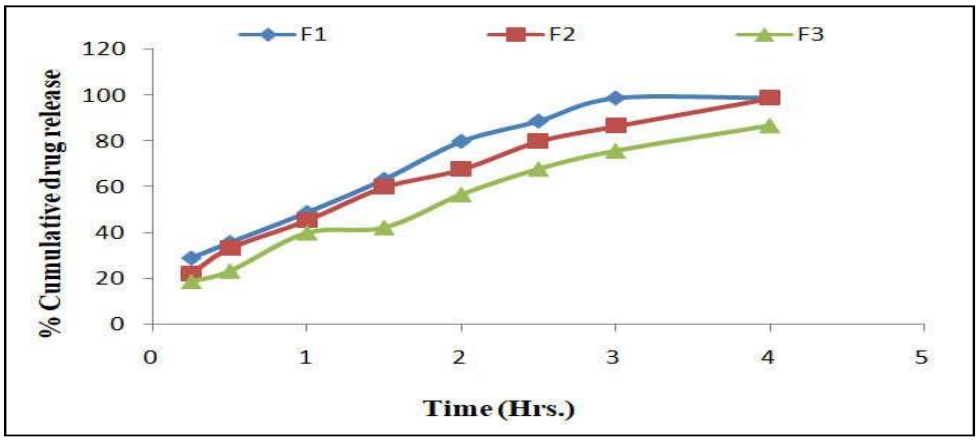

Figure 8: Graph of gel formulation F1, F2 and F3

Release Kinetics of gel F2

Table 18: *In-vitro* drug release data for gel F2

Time (h)	Square Root of Time(h)$^{1/2}$	Log Time	Cumulative*% Drug Release	Log Cumulative % Drug Release	Cumulative % Drug Remaining	Log Cumulative % Drug Remaining
0.25	0.5	-0.602	22.36	1.349	77.64	1.890
0.5	0.707	-0.301	33.36	1.523	66.64	1.824
1	1	0	45.58	1.659	54.42	1.736
1.5	1.225	0.176	59.98	1.778	40.02	1.602
2	1.414	0.301	67.74	1.831	32.26	1.509
2.5	1.581	0.398	79.98	1.903	20.02	1.301
3	1.732	0.477	86.65	1.938	13.35	1.125
4	2	0.602	98.78	1.995	1.22	0.086

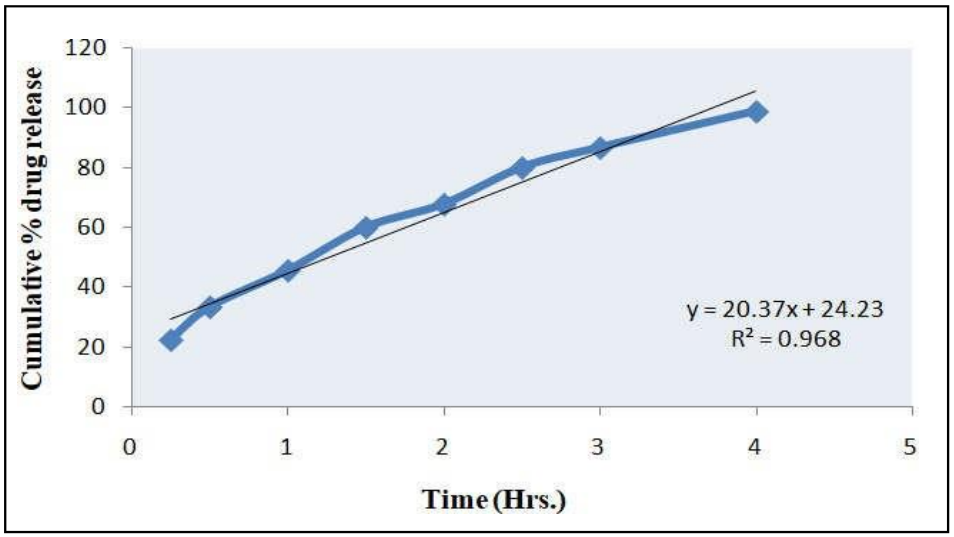

Figure 9: Graph of Zero order Release Kinetics of gel F2(Cumulative % drug released Vs Time)

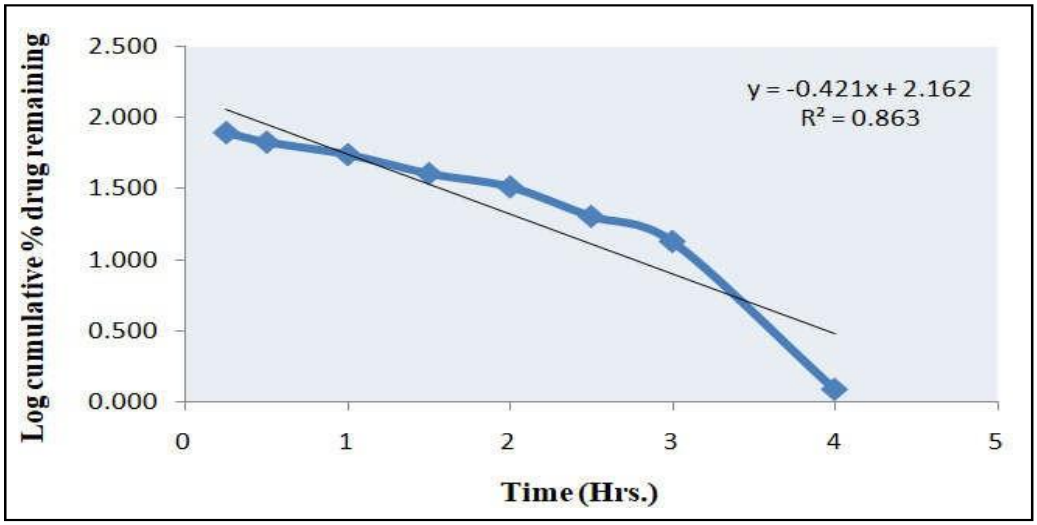

Figure 10: Graph of first order Release Kinetics of gel F2 (Log cumulative % drug remaining Vs Time)

Table 19: Release kinetics regression values of formulation F1-F3

Formulation code	Zero order	First order
F2	0.968	0.863

***In vitro* antifungal activity of extract and prepared silver nanoparticles gel**

Table 20: Antifungal activity against *Candida albicans*

S. No.	Name of drug	Microbes	Zone of inhibition		
			25 mg/ml	50 mg/ml	100 mg/ml
1.	Extract	*Candida albicans*	7±0.5	8±0.74	10±0.47
2.	Silver nanoparticles gel		10±0.86	11±0.94	14±0.57

Conclusion

Silver nanoparticle gels are a novel form of plant-based biopesticide that can be used as an alternative to chemical-based pesticides for the treatment of fungal diseases on tomato plants. Silver nanoparticle gels are made from the leaves of Lagerstroemia parviflora, a tropical tree native to India and Southeast Asia. These gels are formulated to contain silver nanoparticles, which are known for their antimicrobial and antifungal properties. When applied to tomato leaves, the silver nanoparticle gel forms a protective layer that prevents the growth of fungal pathogens. The gel also provides nutrients to the plant, helping to improve its health and vigor. Silver nanoparticle gels are highly effective and have been found to be safe for both the environment and humans. They are an ideal solution for organic farmers who want to protect their crops from disease without the use of chemical-based pesticides. Based on the results obtained, it can be concluded that the prepared gel formulation of silver nanoparticles exhibits antifungal activity against *Candida albicans*. The results showed that the prepared gel formulation of silver nanoparticles has significant antifungal activity on *Candida albicans* and could be used as a potential alternative for antifungal agent in the treatment of fungal infections.

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