International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
(A Peer Reviewed, Referred, Indexed and Open Access Journal)
DOI: 10.22192/ijarbs Coden: IJARQG (USA) Volume 10, Issue 5 -2023

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2023.10.05.005

Synthesis of silver nanoparticles from *Catharanthus roseus* and preparation of transdermal patches for wasp sting bite

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1. Abstract

Periwinkle is considered as one of the ancient medications. The production of the silver nanoparticles (AgNPs) was detected visually by a change in color of the reaction mixture from yellow to reddish brown by incubation for 8 hour at 37˚C. The biosynthesis of silver nitrate nanoparticles from the *Catharanthus roseus* flower was carried out using lyophilisation technique. Further, the AgNPs were analyzed by various standard characterization techniques such UV-Vis spectroscopy, Electron Microscopy (SEM), FTIR analysis. The antibacterial activities were carried out for the synthesized AgNPs and aqueous extract against superficial flora. The zone of inhibition was formed where the ZI was found to be larger for 200mg than 50mg for AgNPs and aqueous extract. The compounds isolated from *Catharanthus roseus* AgNPs had activity against Wasp stings. The phytochemical analysis was carried out to evaluate the possible medicinal utilities of a plant and also to determine the active principles responsible for the known biological activities exhibited by the plants. The green synthesized AgNPs were assessed for their angiogenic inhibition properties using zebrafish. The zebrafish were fed with the powdered extract and checked for their viability. At the same time the zebrafish were given intraperitoneal injection and checked for their viability. The study implies that *Catharanthus roseus* AgNP can be used for preparation of transdermal patches for wasp sting bite. The transdermal patches were prepared by following formulation and their characterization was carried out.

Keywords: AgNPs, *Catharanthus roseus*, phytochemical analysis, biological activities

2. Introduction

(1)The anticancer alkaloids Vinblastine and Vincristine are derived from stem and leaf of *Catharanthus roseus* These alkaloids have growth inhibition effect to some human tumors. Vinblastine is used experimentally for treatment of neoplasmas and is recommended for Hodgkins disease, chorio carcinoma. Vincristine another alkaloids is used for leukemia in children. Different percentage of the methanolic crude extracts of Catharanthus was found to show the significant anticancer activity against numerous cell types in the in vitro condition and especially greatest activity was found against the multidrug resistant tumor types. Vinblastine is sold as Velban or Vincristine as oncovin.

To synthesize AgNPs numerous physical and chemical techniques are adopted. However, it is essential to develop a biological cal method for silver nanoparticles synthesis. Nanoparticles have been used to stimulate plant metabolic activity. Biosynthesis of silver nanoparticles was carried out using periwinkle plant extracts. The production of silver nanoparticles (AgNPs) was detected visually by change in color of the reaction mixture from yellow to reddish brown. Various standard characterization techniques such UV-Visible spectrometry, GC-MS analysis. The UV-Visible spectrum showed the plasmon resonance peak of AgNPs, indicating the formation of silver nanoparticles. The synthesized silver NPs were in the range of 0-30 nm. Qualitative phytochemical analysis revealed the presence of the phytocompounds that were responsible for the capping, formation and stabilization of silver NPs. Phytochemical screening reveals the presence of alkaloids, phenol, saponins and protein. *Catharanthus roseus* contains significant amounts of volatile and phenolic compounds including caffeoylquinic acids and flavonoids glycosides which are known to antioxidant activity. It has a important role in the body defense system that is acts as a antioxidants against reactive oxygen species (ROS), which are harmful by forming such products through normal cell aerobic respiration. The antioxidant ability of the AgNPs and their respective leaf extracts were analysed using DPPH assay. The antimicrobial activity from ethanol leaf extract of *C.roseus* was investigated against some human pathogenic microorganisms. Biosynthesis of silver NPs from *C. roseus* leaf extract was carried out and their characterization, as well as antioxidant, antimicrobial activities were evaluated. The synthesized AgNPs showed strong antioxidant and antimicrobial activities against various pathogens. *C. roseus* demonstrate strong potential for synthesis of silver NPs by rapid reduction of silver ions. This study provides value added products for biomedical/nanotechnology based industries. Anti-oxidant property- The anti-oxidant potential of the ethanolic extract of the roots of the two varieties of *C. roseus* namely rosea (pink flower) and alba (white flower) was obtained by using different system of assay such as Hydroxyl radical-scavenging activity, uperoxide radical-scavenging activity, DPPH radical- scavenging activity and nitric oxide radical inhibition method. The result obtained proved that the ethanolic extract of the roots of Periwinkle varieties has exhibited the satisfactory scavenging effect in the entire assay in a concentration dependent manner but *C. roseus* was found to possess more antioxidant activity than that of *Catharanthus sp.* The *Catharanthus roseus* ethanol extract had high rate of wound contraction significantly decreased epithelization period, significant increase in dry weight and hydroxyproline content of the granulation tissue when compared with the controls. Wound contraction together with increased tensile strength and hydroxyproline content support the use of *C.roseus* in the management of wound healing.

3. Materials and Methods

3.1 Sample collection

The Madagascar periwinkle (pink) was collected from: Unigrow Gardens, Coimbatore - 11.038373665721194, 76.97914199508405.

3.2.1 Preparation of plant extract

20gm of the fresh flower were washed thoroughly with double distilled water and were then cut into small pieces. These finely cut pieces were then mixed with 200ml distilled water and the mixture was kept in a shaker overnight. The mixture was then filtered through Whatman Filter no. 1, after which the mixture was purified. The extract was then stored at 4˚C

3.2. Preparation of ethanol extract

20gm of the fresh leaves were washed thoroughly with double distilled water and were then cut into small pieces. These finely cut pieces were then mixed with 100ml distilled water and 100ml of 50% ethanol. The mixture was kept in the shaker overnight. The mixture was then filtered through Whatman Filter no. 1 after which the mixture was purified. The extract was then stored at 4˚C

3.3 Synthesis of Ag-NPs

3.3.1 Preparation of silver nitrate stock solution

1mM of the Silver Nitrate solution was prepared by adding 0.085mg of $AgNO_3$ in 100ml of distilled water. The solution was constantly stirred using magnetic stirrer for 5mins. The solution was then kept in dark for overnight at room temperature. [2]

50ml of (1mM) $AgNO_3$ solution was added to 50ml of aqueous extract in a conical flask. In another conical flask 50ml of (1mM) $AgNO_3$ solution was added to 50ml of ethanol extract. The $AgNO_3$ solution was treated with a fixed ratio to leaf extract (1:1).

3.3.2 Synthesis of silver nanoparticles

The extract was mixed with $AgNO_3$ stock solution in the ratio 1:1 and incubated overnight. The formation of the silver nanoparticles was confirmed with the occurrence of color change. The extract along with $AgNO_3$ solution was incubated overnight in the freezer. Then the solution was kept in lyophilizer for 4 days. After 4 days the solution was transformed into crystalline form.

3.4 Synthesis of AG-NPS by isolated compounds

Most plants usually produce two types of metabolites, namely primary and secondary metabolites. *Catharanthusroseus* contains more than 400 of useful alkaloids such as vincristine, vinblastine, catharanthine, tabersonine, yohimbine, vindosine, ajmalicine, lochnericine, vindolicine and vindoline [3]. The alkaloids that mainly present in aerial parts of the plant include actineoplastidemeric, vinblastine, vincristine, vindesine, vindeline and tabersonine. Meanwhile, ajmalicine, vinceine, vineamine, raubasin, reserpine and cathatanthine are usually found in roots and basal stem whereas anthocyanin pigment likes rosindin is present in the flower part of *C. roseus* (Monika and Vandana, 2013)[4]

3.5 Qualitative analysis of phytochemical activity

The aqueous extract was tested for phytochemicals [6]

3.6 Photocatalytic activity

Photocatalytic activity is the ability of AgNPs to catalyze a photoreaction under sunlight. When UV/visible light incidents on the surface of AgNPs that fulfills the bandgap energy, electrons from the CB get excited to the VB and form electron pairs. 1 mL of 5000 ppm PF AgNPs and 1 mL of 0.2 M $NaBH_4$ were added to 100 mL of 2 mM MO. The absorbance of the reaction mixture was measured from 300 to 560 nm using distilled water as a blank every 5 minutes for 20 minutes. The procedure was repeated for 333 ppm PF AgNPs. [5]

3.6.1 Methyl orange

The photocatalytic activity was carried out using methyl orange reduction [7]. 10mg of the synthesized nanoparticles was mixed with Methyl

Orange. A control was prepared without adding silver nanoparticles. The mixture was constantly mixed using magnetic stirrer. The color degradation was observed after every 10 minutes using UV spectrophotometer. The methyl orange absorption peak was observed at 470nm.

3.7 UV Spectrophotometer

Progression in the formation of Silver nanoparticles was examined with the help of UV-Vis spectroscopy. For mixtures having aqueous silver nitrate solution and *C.roseus* flower extract the UV-Vis spectra was carried out. UV-vis spectroscopic analysis was carried out at specific intervals i.e. after 10, 20, 45 and 60 mins.

3.8 Antioxidant activity

3.8.1 DPPH Assay

This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution. 0.1 ml, 0.2 ml and 0.3 ml of methanol leaf extracts were mixed with 1 ml of 0.1 mM DPPH. To all the tubes added 0.4 ml of 50 mMTris-HCl. Incubate the reaction mixture at room temperature for 30 minutes. The absorbance of the reaction mixture was read at 517 nm. [8, 9]

Percentage DPPH radical scavenging activity was calculated by the following equation

$$\% \text{ DPPH} = \frac{\text{Absorbance of control} - \text{Absorbance of Standard} \times 100}{\text{Absorbance of control}}$$

3.8.2 Total phenols

Total phenol content was determined by the Folin- ciocalteau reagent method. 1 ml of methanol and aqueous extract was mixed with 1 ml of folin's phenol reagent and 1 ml of 20% sodium carbonate. The mixture was allowed it for incubation at 45 C for 45 minutes and the absorbance was measured at 765 nm in spectrophotometer[10].

3.8.2 Total flavonoids

1 ml of methanol and aqueous extract was mixed with 0.1 ml of 10% AlCl3, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled H2O. The mixture was incubated in room temperature for 30 minutes. Then the absorbance was measured at 415 nm in spectrophotometer [11].

3.9 SEM Analysis

The silver nanoparticles synthesized by lyophilization was collected in an Eppendorf tube and SEM analysis was carried out for the following.

3.10 Feeding zebra fish

The zebra fish Danio variety was fed with Powdered extract each day twice. Their viability and activity was observed each day.

Intraperitoneal injection

The zebra fish Danio variety was given intraperitoneal injection. Their viability and activity was observed each day.

3.11 Anti microbial activity against superficial flora

The antimicrobial capacity of the extract and NPs was evaluated in terms of ZI measurement against the selected bacterial strains. Sterilized agar culture dishes were inoculated with respective strain of bacteria; 3 mm holes were made in the media at 5 cm distance from each other Leaf extract and the NPs were introduced into the holes through a micropipette. The culture dishes were incubated at 37°C for 24 h. ZI around each hole was measured. The whole procedure was carried out under laminar flow.

3.11.1 Identification of bacterial isolates

Bacterial isolates were cultured from normal skin flora using the method of Cheesbrough [12]. Pure cultures of the isolates were obtained by streaking representative isolates on freshly prepared nutrient agar and incubated at 37 for 24hours in the incubator. After incubation, identification was done using the gram staining technique and biochemical test such as Oxidase, Citrate, Indole, Catalase, sugar fermentation tests and Methyl Red Test

3.11.2 Antimicrobial sensitivity test

The agar well diffusion method was used in the sensitivity test. Bacteria suspension that was prepared to match 0.5% MC Farland standard was inoculated on the surface of prepared nutrient agar plates and distributed evenly by means of glass spreaders. Using a 3mm well puncher the wells were created on the agar plates 5 cm apart from each other. In each well different concentration of the samples were added and incubated for 48 hours at 37˚C.

3.12 Preparation of transdermal patch

The formulation for the transdermal patches are as follows:

S.no	Chemicals	Quantity
1.	Hexane extract of *C.roseus* flower (mg)	5
2.	Pectin (mg)	50
3.	DMSO(ml)	3
4.	Tween 80 (ml)	0.5
5.	Distilled water (ml)	6

The above compounds were mixed together and poured onto the sterile Petri plate and kept for drying. Finally the transdermal patches were obtained and those were stored in 4˚C.

3.13 Evaluation parameters of transdermal patch

3.13.1 Folding endurance

A strip of specific area (2 cm*2 cm) was cut evenly and repeatedly folded at the same place till it broke. The number of times the film was folded at the same place without breaking gave the value of the folding endurance [13].

3.13.2 Tensile strength

The tensile strength of the patch was calculated by using the tensiometer. It consisted of two loaded cell grips. The one at bottom was fixed and the upper one was mobile. Film strips had dimensions of 2*2 cm were fixed between the cell grips, and a gradual force was applied till the film broke. The tensile strength was taken directly from the dial reading in kg [14].

3.13.3 Percentage elongation break test

The percentage elongation break was determined by noting the length just before the break point, the percentage elongation was determined from the below mentioned formula [15].

$$\text{Elongation percentage} = \frac{(L_1 - L_2) \times 100}{L_2}$$

3.13.4 Thickness

Patch thickness was measured using digital micrometer screw gauge at three different places, and the mean value was calculated [13].

3.13.5 Drug content

A specified area of patch (2 cm*2 cm) was dissolved in 100 mL methanol and shaken continuously for 24 h. Then the whole solution was ultra-sonicated for 15 min. After filtration, the drug was estimated spectrophotometrically at wavelength of 281 nm and determined the drug content [16].

3.13.6 Percentage moisture content

The prepared films were weighed individually and kept in a desiccator containing fused calcium chloride at room temperature for 24 hour [13]. After 24 h, the films were reweighed and determined the percentage moisture content from the below mentioned formula
:

$$\% \text{ moisture content} = \frac{(\text{Initial weight} - \text{Final weight}) \times 100}{\text{Final weight}}$$

3.13.7 Percentage moisture uptake

The weighed films were kept in a desiccators at room temperature for 24 h containing saturated solution of potassium chloride in order to maintain 84% RH [13]. After 24 h, the films were reweighed and determine the percentage moisture uptake from the below mentioned formula:

$$\% \text{ moisture content} = \frac{(\text{Final weight} - \text{Initial weight}) \times 100}{\text{Initial weight}}$$

3.13.8 In vitro drug release studies

In vitro drug release studies were performed by using a Franz diffusion cell with a receptor compartment capacity of 60 mL. The cellulose acetate membrane was used for the determination of drug from the prepared transdermal matrix-type patches. The cellulose acetate membrane having a pore size 0.45 μ was mounted between the donor and receptor compartment of the diffusion cell. The prepared transdermal film was placed on the cellulose acetate membrane and covered with aluminum foil. The receptor compartment of the diffusion cell was filled with phosphate buffer pH 7.4. The whole assembly was fixed on a hot plate magnetic stirrer, and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads, and the temperature was maintained at 32 ± 0.5°C, because the normal skin temperature of human is 32°C. The samples were withdrawn at different time intervals and analyzed for drug content spectrophotometrically [16]. The receptor phase was replenished with an equal volume of phosphate buffer at each sample withdrawal.

4. Results

In the initial stage, as the temperature is one of the key influence factors in the nanoparticle synthesis, the AgNPs synthesis was done at different temperatures keeping other parameters constant. The optimum condition for all the varieties was at room temperature for 3 days. This may be due to the slow formation and growth at room temperature which usually takes more hours to complete the bioreduction [23]. However, the synthesis at room temperature is greener than using heat, which is a further advantage. It was also observed that the AgNPs were stable even for more than 6 months stored at 4°C. The formation of the AgNPs was initially observed by a color change to a reddish-brown solution. This is due to a phenomenon called the surface plasmon resonance (SPR) which occurs due to the excitation of the surface plasmons present on the outer surface of the AgNPs that get excited when light/UV is incident [24].

4.1 Synthesis of Ag-NPS

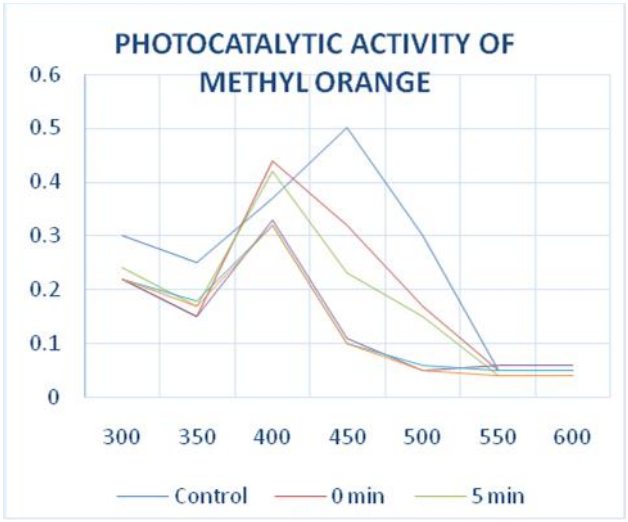
After lyophilization the liquid solution was transformed into a crystalline form. These crystalline form was crushed into powdered form aseptically and transferred into a clean Eppendorf tube and stored at room temperature.

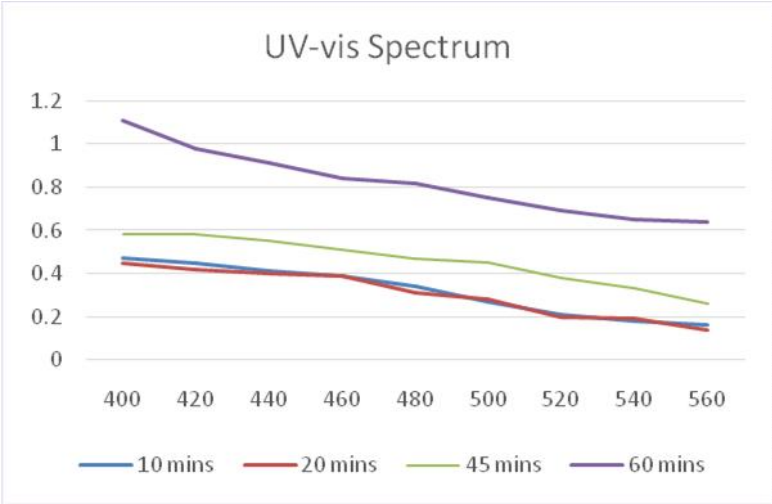
4.2 Phytochemical activity

S. NO.	Phytochemical analysis	Observation	Result
Alkaloids			
1	Iodine test	Blue color was seen which disappeared on boiling and reappears on cooling.	+ve
2.	Mayer's Reagent	A creamy white colored precipitate was obsereved.	+ve
Carbohydrates			
3.	Molish's test	A violet ring was observed	+ve
4.	Barfoed's test	A red precipitate was observed	+ve
Reducing agar			
5.	Benedict's test	Green color	+ve
6.	Fehling's Test	A red Precipitate	+ve
7.	Aqueous NaOH test	A yellow color	+ve
Cardiac Glycosides			
8.	Bromine water		-ve
Proteins and Amino acids			
9.	Ninhydrin test	A purple colored solution was formed	+ve
Flavanoids			
10.	Ferric Chloride test	A green precipitate was formed	+ve
Phenolic compounds			
11.	Iodine test	A red color was observed	+ve
Tannins			
12.	10% NaOH test	Emulsion formation	+ve
13.	Bromine water test	Bromine decolorization	+ve
Phytosterols			
14.	Salkowski test	Red color appearance	-ve
Cholesterol			
15.		No color change	-ve
Lignin			
16.			-ve
Fixed Oils and Fat			
17.	Spot test	No oil stain was observed	-ve
Terpenoides			
18.		A grayish color	+ve
Quinines			
19.		Blue green color appears	+ve
Saponins			
20.		1.3 cm of bubble formation	+ve
Protein			
21.		Formation of yellow color	+ve
Steroids			
22.		Formation of red color below the chloroform layer	+ve

4.4 Photocatalytic activity

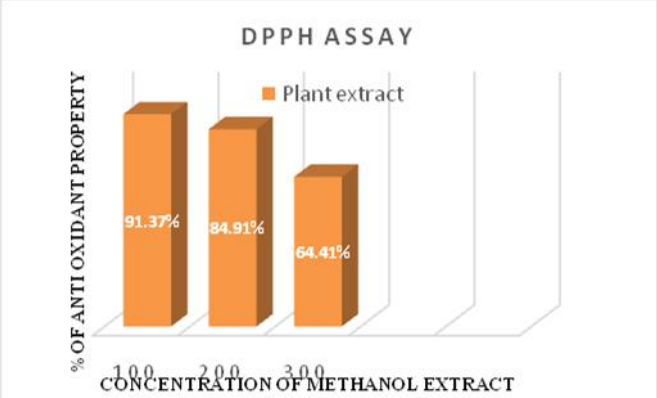
Methyl Orange UV Spectrophotometer



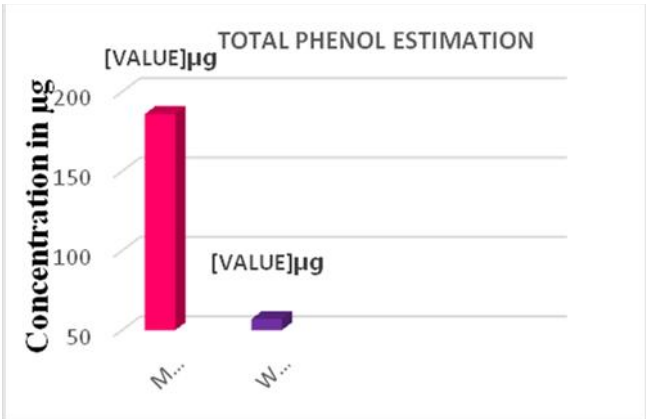


4.6Antioxidant activity

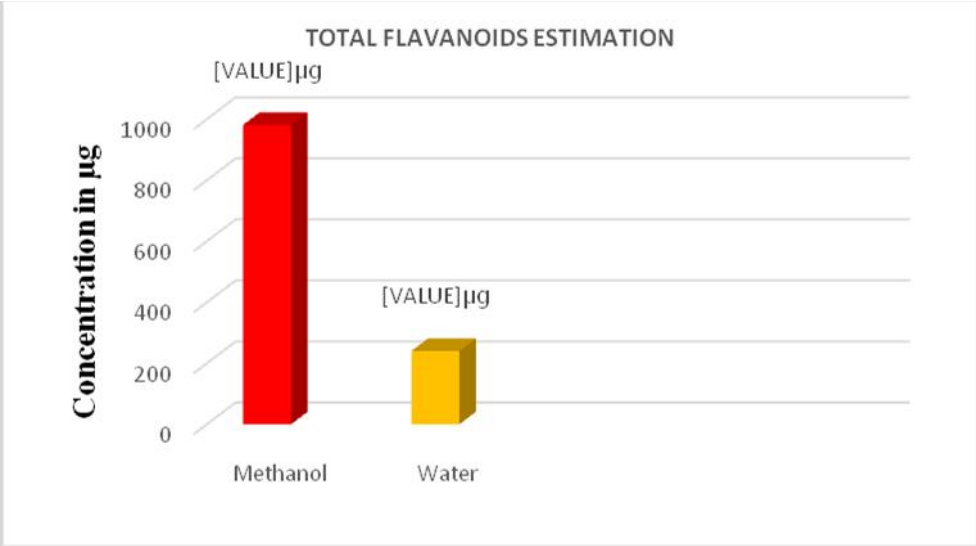
DPPH assay [15]



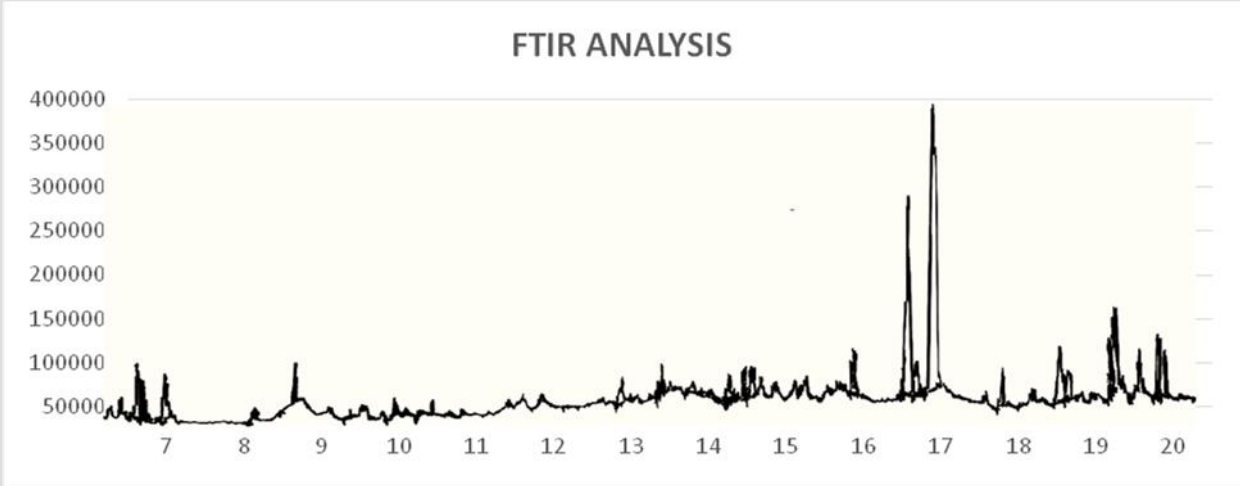
Total Phenols



Total flavanoids



FTIR analysis



4.10 Feeding zebra fish

4.10.1 Extract

The zebra fish were fed with 1gm of the powdered extract twice a day and their activity were observed where the fish were alive and active for more than 30 days. The color of the fish were slightly faded.

S.No.	Zebra Fish	Days Alive
1.	Control	32 days
2.	Feed	32 days
3.	Feed	32 days
4.	Feed	21 days

4.10.2 Intraperitoneal injection

The zebra fish were given intraperitoneal injection and again released into water again. These fish were fed with commercial fish food. Their activity were observed where the fish were alive and active for more than 30 days. The color of the fish were the same.

S.No.	Zebra Fish	Days Alive
1.	Control	32 days
2.	Feed	32 days
3.	Feed	26 days
4.	Feed	18 days

4.11 Anti microbial activity against superficial flora

After incubation period the zone were formed around the well diffused with respective concentration of the extract. These zone of inhibition against the superficial flora were measured in mm as follows:

Aqueous extract AgNPs

S. No.	Concentration	Zone of Inhibition
1.	50mg	1.2 cm
2.	100mg	1.0 cm
3.	150mg	0.7 cm
4.	200mg	0.4 cm

4.12 Preparation of transdermal patch

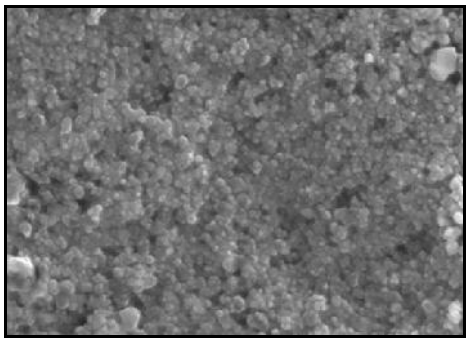
The physical appearance of the patch was analyzed by using naked eye for its appearance, colour, clarity, flexibility and smoothness.

S.no.	Parameters	Results
1.	Color	Light brown
2.	Appearance	Jellified preparation
3.	Flexibility	Yes
4.	Smoothness	Good
5.	pH	7±0.2
6.	Thickness(mm)	1
7.	Flexibility	Optimum and satisfactory
8.	Tensile strength	0.53 ± 0.017
9.	Folding endurance	> 200
10.	Drug content	78.3 %

4.13.1 In vitro drug release study

Artificial semipermeable membrane was used to study the *in vitro* conditions for the drug release characteristics. Due to low viscosity the formulation showed the release of about 82.74% in 7 hour.

SEM analysis



6.3 Anti microbial activity

Aqueous extract **AgNPs**

5.5 Transdermal patches

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DOI:10.22192/ijarbs.2023.10.05.005	

How to cite this article:
Arunkumar G, Sakena Joseph, Benedict Sruthika, Raveena Sherin, Raji. K, Yogeshwaran. (2023). Synthesis of silver nanoparticles from *Catharanthus roseus* and preparation of transdermal patches for wasp sting bite. Int. J. Adv. Res. Biol. Sci. 10(5): 27-39.
DOI: http://dx.doi.org/10.22192/ijarbs.2023.10.05.005