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TOPICAL ANTIMICROBIAL OINTMENT AND GEL PREPARATION FROM BIOSYNTHESISED SILVER NANOPARTICLE USING *TABERNAEMONTANA DIVARICATE*

Biological Science

Sruti S Nair PG & Research Department of Biotechnology, Women's Christian College, Chennai -6, Tamilnadu, India.

Anchana Devi C* PG & Research Department of Biotechnology, Women's Christian College, Chennai -6, Tamilnadu, India.*Corresponding Author

Pushpa, N PG & Research Department of Microbiology, Cauvery College for Women, Tiruchirappalli – 18, Tamilnadu, India.

ABSTRACT

Topical antimicrobials are mainly used to fight against infection caused by any micro-organism like fungi, virus and bacteria. The topical antimicrobial ointment and gel is less toxic compared to the chemical formulation. *Tabernaemontana divericata* have antimicrobial, antioxidant, antitumor and analgesic effect. It is known for its therapeutic effect in the healing of wound and treatment of various diseases like skin diseases, aches, ascaris, scabies, eye diseases and pain. In the present study, Silver nanoparticle was synthesised from the stem extract of *Tabarnaemontana divaricata*. 1mM of silver nitrate was used for the synthesis of silver nanoparticle. Ointment and gel preparations were made using biosynthesised nanoparticle and physical evaluation was carried out. Both ointment and gel forms are known to exhibit high antimicrobial activity as compared to the commercially available ointment and gel. They also have antioxidant, anticoagulation, anti-inflammatory, anticancer activity and are proved to exhibit less cytotoxic effects. This may be of therapeutic value and be a widely accepted treatment modality.

KEYWORDS

Tabarnaemontana divaricata, biosynthesis, nanoparticles, antimicrobials, antioxidant, anticoagulation, anti-inflammatory, anticancer, cytotoxicity.

Background information

Topical antimicrobials are substances that have the ability to destroy microbial cells and prevent its growth, when it's directly applied on a skin surface. They are mainly used to fight against infection caused by any micro-organism like fungi, virus and bacteria. An ideal topical antimicrobial should have low toxicity, low allergy, no irritation, high diffusion through tissues, fast and prolonged antimicrobial effect[1]. The topical antimicrobial ointment and gel is less toxic compared to the chemical formulation[2]. Its eco-friendly and require only less cost for manufacturing compared to other chemically formulated ointments and gels[3].

Nanoparticles are particles of any shape with size ranging from 10 to 100 nm. The IUPAC definition for nanoparticle is that the particle of any shape with dimensions in the 10^{-9} and 10^{7}. Nanoparticles have wide range of application in the field of physics, chemistry, biology, material science and medicine[4]. Nanoparticles have optical, catalytic, electronic and antimicrobial effect. Nanoparticles can be synthesised using chemical as well as physical method. Nanoparticles can be created using gas condensation, ion implantation, pyrolysis, hydrothermal synthesis[5].

Tabernaemontana divericata is shrub which is commonly seen in India. It usually grows in to a height of six feet. Almost all parts of the plant can be used for the treatment of various diseases. It's widely used in Ayurveda, Siddha and Unanai. *Tabernaemontana divericata* have antimicrobial, antioxidant, antitumor and analgesic effect. It's used for the healing of wound and treatment of various diseases like skin diseases, aches, ascaris, scabies eye diseases and pain[6]. The plant is used as traditional medicine of India, south East Asia, Indonesia, Malay Peninsula and Ambon Island[7].

MATERIALS AND METHODS

Sample Collection and Preparation of Plant extracts

Tabernaemontana divaricate stem samples free from diseases were collected from Chennai, Tamilnadu, India (Fig 1). The plant material was taxonomically identified and authenticated by Department of Botany, Women's Christian College, Chennai, India. It was then washed thoroughly 2-3 times with tap water and once with sterile water. 10 g of fresh stem were finely chopped and added to 100 mL of distilled water (1:10 ratio) and left undisturbed at room temperature for 48-72 hrs. Similarly ethanol extract was prepared in a capped bottle to prevent evaporation. The extracts were filtered with Whatman paper number 1. Filtrate was collected and stored for further analysis.

Green Synthesis of Nanoparticles:

Synthesis of Silver Nanoparticles[8]

0.1 M of aqueous solution of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. 5 mL of aqueous stem extract of *Tabernaemontana divaricate* was added to 45 mL of 0.1 M $AgNO_3$ solution for bio reduction process at room temperature overnight.

Characterization of Silver Nanoparticles

UV-Visible Absorbance Spectroscopy

UV-Visible spectroscopy analysis of synthesized was carried out using Shimadzu UV-Visible absorption spectrophotometer. 0.5 mL volume of sample (Silver nanoparticles) was taken and analyzed at room temperature.

The reaction between metal ions and the leaf extract was monitored by UV-Visible spectra.

For silver nanoparticles wavelength in nanometers from 340 to 800 nm was used. The reduction of silver ions and formation of silver nanoparticles was observed and the reading were noted[9].

Fourier-transform infrared (FTIR) Analysis:

Fourier transform infrared spectra (FT-IR) spectrometric analysis to specify the functional groups in the extract that may be responsible for reducing ions to NPs. For FTIR measurements, the synthesized nanoparticle solutions were centrifuged at 10000 rpm for 30 minutes. The pellets of silver, zinc and copper nanoparticles were washed thrice with 5 mL of deionized water to get rid of the free proteins or enzymes that are not capping the nanoparticles. The pellet was dried by using vacuum drier. It was analyzed by FTIR using potassium bromide (KBr) pellet method. Scan range was between 4000 and 400 cm−1 for silver nanoparticles where, possible biomolecules bonding interactions can be studied[10].

Scanning electron microscopy (SEM):

SEM is the type of electron microscope produces image of a nanoparticles using focused beam of electrons. The electrons interact with atoms in a sample, provides information on morphological and topographical characterization of the nanoparticles had been well established by the electron microscopic studies[11].

Preparation of ointment

50gm of bee wax was mixed with 100ml of coconut oil in a beaker and heated. It was mixed continuously until the bee was dissolved and 20 ml of biosynthesised silver nanoparticle was added to the ointment base and stirred for 5 minutes. The prepared ointment was poured on containers and kept for cooling[12].

Preparation of gel
50 ml of distilled water was taken in a beaker; 5 gm. of Carbopol 934 was added to the water and under continuous stirring until it forms a gel base. 10 ml of biosynthesised nanoparticle was added to the gel base and mixed well[12].

Physical evaluation of topical formulations[13]
Organoleptic characteristics: The physical characteristics like appearance, texture, colour, and homogeneity was evaluated by visual observation. The homogeneity and texture were tested by pressing the prepared ointment and gel between the thumb and index finger.

Spread ability: The spreadability was determined by placing excess of sample in between two slides which was uniformly compressed by placing a definite weight for definite time. The time required to separate the two slides was measured as spreadability. Better are the results in terms of spreadability with lesser time taken for the separation of two slides.

Spreadability was calculated by following formula

S=M×L/T

Where,
Spreadability, weight tide to the upper slide, length of glass slide and time taken to separate the slides are indicated by S, M, L and T respectively.

pH values: The pH of the prepared ointment and gel was checked using pH paper.

Antimicrobial assay of synthesised Ointment and gel
Antibacterial assay using well diffusion methodology
Antimicrobial assay of synthesised nanoparticles

Antibacterial analysis was done both Gram positive and negative bacteria (*Staphylococcus* spp, *Streptococcus* spp, *Bacillus* spp, *Escherichia coli*, Pseudomonas *aeruginosa*) by well-diffusion method. Overnight cultures were prepared in nutrient broth and inoculated with the organisms and incubated at 37°C for 24 h. 0.1 ml of bacterial culture was used for spread plate on Muller Hinton agar medium, Wells were punched using sterile well puncher. 200 µl of prepared gel and ointment were loaded on to the wells to each culture plate and incubated at 37°C for 24- 48 h. Commercially available gel and Ointment were used as standards. Antibacterial activity was measured based on the zone of inhibition obtained around the well.

Antifungal analysis was carried out using *Candida spp. Pencillium spp and Aspergillu niger* on SDA media using agar well diffusion method. The zone of inhibition was observed after 72 hours of incubation at 37°C[14].

Anti-oxidant assay – DPPH Assay
The free radical scavenging activity was assessed by using an ethanol solution of DPPH. The maximal absorbency exhibited by DPHH is at 517nm and in the presence of H-donor molecules this shows a decrease. The DPPH stable free radical was used for the determination of free radical scavenging activity of extract[15]. In 5 microliter of aqueous stem plant extract DPPH added to make the volume up to 100 μL in 96-well plates. The contents mixed and incubated at 37°C for 30 minutes and the optical density measured at 517 nm. Ascorbic acid was used as a standard, since it has a strong antioxidant property, that is why it was used as standard to evaluate the antioxidant activity of substances[16].

$$\%\text{DPPH scavenging activity} = 100 - \frac{\text{OD of test sample}}{\text{OD of control}} \times 100$$

Cytotoxicity activity (MTT Assay)
The cytotoxicity assay was performed on Vero cell lines by the MTT assay method. The cells were culture in MEM medium containing 10% FBS and 1% penicillin–streptomycin and seeded into 96 well plates and incubated at 37 °C for 24–48 h containing 5% CO_2. When the desired cell confluence was reached, fresh medium was added along with the 10µl of sample (ointment and gel) to the wells. At the end of the incubation time, the MTT dye was added and after re-incubation, formazan crystals were dissolved in 1ml DMSO. Absorbance of the wells was measured at 570 nm using a spectrometric ELISA microplate reader. Results were expressed as the percentage of inhibition compared with control cells in which cell survival was presumed 100%. All the assays assessing cell viability were reproduced in three-fold[17].

The percentage of cell viability can be calculated using the formula

% of cell viability = Absorbance at 570nm of treated cells /Absorbance of control cells X 100

RESULT AND DISCUSSION

Sample Collection and Preparation of Plant extracts
The sample was extracted using distilled water and ethanol. The stem of *Tabernaemontana divericata* and distilled water was taken in 1:10 ratio, grinded, filtered and stored in closed glass vessel. (Figure 1)

Stem was collected freshly from *Tabernaemontana divericata* to obtain the maximum amount of extract. Mechanical extraction using motor and pistil made the extraction easier. Aqueous and ethanol was used as solvents, various studies have reported the efficiency of aqueous and solvents containing alcohol are good for the extraction of plant materials[18].

Synthesis of Silver nanoparticles
Silver nanoparticle was synthesised from the aqueous stem extract of *Tabarnaemontana divaricata*. And 1mM of silver nitrate was used for the synthesis of silver nanoparticle. Colour change was observed from, mild yellow to brown indicated the formation of silver nanoparticle. (Figure 2)

The change in colours in synthesis, is due to the surface Plasmon excitation of metal ions, which is the primary evidence for the formation of nanoparticle[6,19].

FTIR- Fourier Transform Infrared Spectroscopy
The results of FTIR analysis of the AgNPs biosynthesised from *Tabarnaemontana divericata* stem extract shows different stretches of bonds shown at different peaks. The peaks were observed at 1639.70, 2110.54, 2363.02, 3322.66. The peak at 1639.70 indicate the presence of C=C – the stretching of alkenes, which is a weak bond and C=O – the presence of primary amides, which is a strong bond. The peak at 2110.54 indicates the presence C≡C in the biosynthesised AgNPs, which is a weak bond. The peak at 2363.02 shows the medium bond of C-H stretching. The peak at 3322.66 indicates the nanoparticles have amines (N-H) medium, and OH groups of alcohol, phenol and organic acids. (Figure 3; Table 1)

FTIR spectral measurements were performed to find out the potential Functional group in *Tabarnaemontana divericata* stem extract, which is responsible for the reducing and capping the bio reduced AgNPs showed the structure and respective bands of the biosynthesised AgNPs and the stretch of the bond. The stretch and bonds obtained were also similar to the results reported by Preetha *et al*., 2013[20].

Scanning Electron Microscopy (SEM) Analysis:
The SEM images obtained showed particle size of 2.5µm in Figure 4. The SEM image showed the presence of high-density and compact agglomerates of silver particles. The nature of AgNPs was found to be highly granulated. The images also showed the presence of various shapes such as spherical, ellipsoidal, and few irregular fused agglomerates. Similar kind of result was also obtained by Mehrdad F et al., 2010[21].

Preparation of ointment and gel
Ointment base was prepared using oil and wax. Biosynthesised AgNps was successfully incorporated to the base and topical antimicrobial ointment was prepared. The consistency of ointment was found to be same as that of commercially available antimicrobial ointment. (Figure 5)

Ointment base was prepared using bee wax and coconut oil; they are not harmful as compared to the paraffin wax, which is generally used for the preparation of ointment. The biosynthesised silver nanoparticles were successfully incorporated in to the base before it gets cooled. After cooling the ointment showed similar texture to that of the commercially available ointment.

Topical antimicrobial gel was prepared using Carbopol 934 and

biosynthesised AgNps. Gel base was prepared using double distilled water and Carbopol 934. Biosynthesised silver nanoparticle was successfully incorporated in to the gel base (Figure 4) .The prepared gel has the similar consistency as compared to the commercially available gel and it was transparent in nature. Both the Ointment and base were further subjected to Physical characterization studies. (Figure 6)

Physical chemical characterization of prepared ointment and gel

The colors of freshly prepared ointment and gel were white and transparent. There was no change in color of any samples at different storage conditions during study period. No change in color may be attributed to various factors that correlate to the stability of the preparation. The organoleptic characteristics, pH, colour, odour etc., were tabulated. (Table 2)

Anti-microbial assay of prepared ointment and gel using well diffusion methodology (Tables 3 & 4)

Anti-bacterial assay

The prepared ointment and gel exhibited high anti-bacterial activity against both the Gram positive and Gram-negative bacteria. The zone of inhibition was found to be more in *Bacillus spp, and Escherichia coli.* The prepared antimicrobial ointment and gel is having more antimicrobial activity when compared to the commercially available ointment and gel. (Table 3)

Antifungal assay

Both the samples showed antifungal activity. The zone of inhibition was higher for *Penicillium sp, Aspergillas sp.* The prepared ointment and gel exhibited more antifungal activity when compared with the commercially available antimicrobial ointment and gel. (Table 4)

Antimicrobial activity of ointment and gel was tested using well diffusion assay. The antimicrobial activity of prepared ointment and gel was compared with commercially available antimicrobial ointment and gel. The prepared ointment and gel exhibited more antimicrobial activity than that of the commercially available antimicrobial ointment and gel.

The formulated ointment and gel had better inhibitory effects on both bacteria and fungi than the commercially available formulations. The secondary metabolites of the stem extract act as reducing agent in silver nanoparticle synthesis aiding to be an excellent antimicrobial activity.

Anti-oxidant assay

The topical antimicrobial gel and ointment expressed high anti-oxidant activity. The scavenging activity of gel is more than ointment. Both samples expressed more than 80% percentage of activity. The anti-oxidant activity for gel was found to be 86% and for ointment it was 80.5 %. (Figure 7)

Antioxidants are compound which inhibit the oxidative activity or oxidation[22]. The total phenolic content was found to be high in stem extract of *Tabernaemontana divericata*. Phenolic have high antioxidant activity and they are effective in the prevention of oxidative stress. Manorenjitha *et al*., 2013 in their research using *Tabernaemontana divericata* leaf extract also reported that the plant has high antioxidant activity[23].

Cytotoxicity activity (MTT Assay)

Cytotoxicity of the prepared topical antimicrobial gel and ointment was tested using MTT Assay. The percentage of cell viability was calculated, both the samples have viable cells more than 100%. The ointment is having more viable cells than that of the prepared gel. (Figure 8)

The ointment has more than 192.73% of viable cells, were as the gel has more than 173.01% of viable cells. Hence the prepared ointment and gel is reported to be non-toxic in nature. The percentage of cell viability value was greater than 100%, reflecting that the samples are proliferating more than the control cells. Thus, the proliferating cells in the sample gives a higher value, which is directly proportional to cell viability.

CONCLUSION

Topical antimicrobial ointment and gel was prepared from biosynthesised silver nanoparticle using *Tabernaemontana divericata* stem extract. Stem extract of *Tabernaemontana divericata* was prepared using distilled water and ethanol. The phytochemical analysis of the plant indicated high amount of phenol and glycosides, than carbohydrates, protein, alkaloids and steroids. Silver, nanoparticle was biosynthesised from the stem extract, in which silver nanoparticle exhibited high antimicrobial activity. Characterization of the silver nanoparticle was done using UV spectrophotometry, FTIR, and SEM, the silver nanoparticle was spherical in shape with diameter of 25 μm.

Ointment and gel were prepared using biosynthesised nanoparticle and physical evaluation was carried out. The ointment and gel were found to have a high antimicrobial activity compared to the commercially available ointment and gel. The study also reported high antioxidant activity and less cytotoxic effects for the biosynthesized ointment and gel.

TABLES

Table 1: Functional groups present in the silver nanoparticle – FT IR

S. No	Wave number (cm)	Assignments	Bond strength
1	1639.70	C=C – the stretching of alkenes	weak bond
2	2110.54	C≡C stretching of alkynes	weak bond
3	2363.02	C-H stretching	Medium bond
4	3322.66	amines (N-H), and OH groups of alcohol, phenol and organic acids	Medium bond

Table 2: Physiochemical evaluation of the biosynthesized Ointment and gel

S. No	Physiochemical Parameters	Observation	
		Ointment	**Gel**
1.	Colour	White Creamy	Transparent
2.	Odour	Characteristic	Characteristic
3.	pH	7	7
4.	Consistency	Smooth	Smooth
5.	Spreadability	7	**6**

Table 3: Antimicrobial assay of prepared ointment

S. No	Organism	Ointment (mm)	Ointment base as control (mm)	Commercial ointment (mm)
1	***Streptococcus sp***	**20**	**0**	**16**
2	***Staphylococcus sp***	**18**	**0**	**16**
3	***Bacillus sp***	**25**	**0**	**16**
4	***Escherichia coli***	**24**	**0**	**17**
5	***Klebsiella sp***	**17**	**0**	**14**
6	***Pseudomonas sp***	**20**	**0**	**17**
7	***Penicillium sp***	**14**	**0**	**11**
8	***Aspergillus sp***	**23**	**0**	**15**
9	***Candida sp***	**18**	**0**	**14**

Table 4: Antimicrobial assay of prepared gel

S. No	Organism	Gel (mm)	Gel base as control (mm)	Commercial gel (mm)
1	***Streptococcus sp***	**14**	**0**	**12**
2	***Staphylococcus sp***	**16**	**0**	**12**
3	***Bacillus sp***	**12**	**0**	**13**
4	***Escherichia coli***	**21**	**0**	**12**
5	***Klebsiella sp***	**15**	**0**	**12**
6	***Pseudomonas sp***	**14**	**0**	**12**
7	***Penicillium sp***	**23**	**0**	**20**
8	***Aspergillus sp***	**30**	**0**	**16**
9	***Candida sp***	**15**	**0**	**12**

FIGURES

Figure 1: *Tabernaemontana divaricate* plant, Stem and extract preparation.

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$AgNo_3$ solution Ag Nps solution

Figure 2: Synthesis of Silver nanoparticles

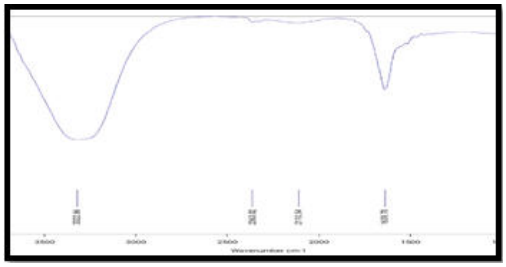

Figure 3: FTIR result of synthesised AgNPs

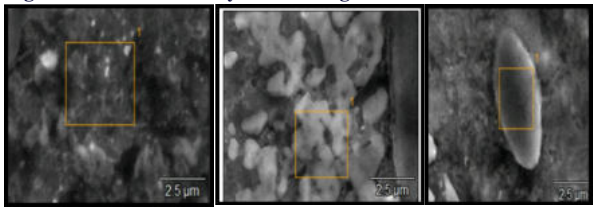

Figure 4: SEM image of synthesized Silver nanoparticles

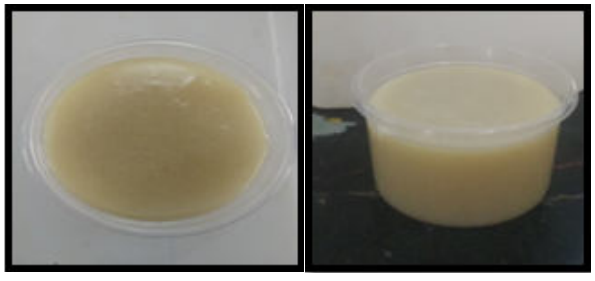

Figure 5: Biosynthesized Topical ointment

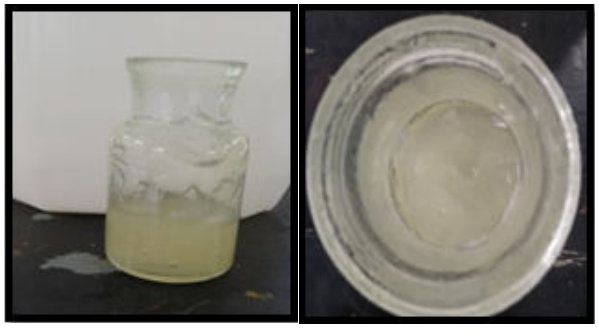

Figure 6: Biosynthesized Topical gel

Figure 7: Antioxidant activity of ointment and gel

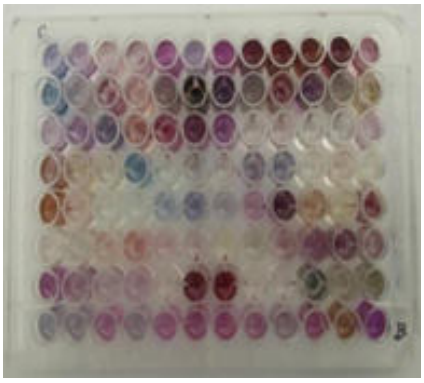

Figure 8: Anticytotoxicity activity of ointment and gel

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