In-Vitro and In-Vivo Wound Healing Activity of Biogenically Synthesised S. Alternata Methanolic Extract Silver Nanoparticle Transdermal Patches

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Abstract

*StrobilanthesAlternata*is a well grown garden plant that is majorly grown for its attractive and energetic greenery. The present study silver nanoparticle synthesis by using *S. Alternata* methanolic extract and its *In-vitro* and *In-vivo* wound healing activity. Conventional and GC-MS phytochemical analysis confirm that *S. Alternata* methanolic extract bioactive secondary metabolites. By metal reduction method used to synthesised silver nanoparticles with help of *S. Alternata* methanolic extract. *S. Alternata* methanolic silver NPs studies cytotoxicity and *In-vitro* wound healing activity (scratch assay). Transdermal patches were prepared *S. Alternata* methanolic silver NPs by solvent casting method. *S. Alternata* methanolic silver NPs transdermal patches conducted *In-vivo* wound healing study in Wister albino rats. Phytochemical analysis of *S. Alternata* methanolic extract confirm that it having bioactive secondary metabolites (β-Sitosterol, Cyclobarbital, 4-Ethylbenzoic acid, Antra-9,10-quinone, 2-p-Nitrophenyl-oxadiazol-1,3,4-one-5 and butylparaben etc). *S. Alternata* methanolic silver NPs showing good migration properties (24 hrs) with L6 cell lines. F2 formulation *S. Alternata* methanolic silver NPs transdermal patch having good drug leasing properties (74.89%). *In-vivo* wound healing studies revolved that F2 formulation showing good wound healing properties as compared to standard drug. Animal wound size totally reduced at 14th day as equal to standard drug. Hence, we can conclude the *S. Alternata* methanolic silver NPs transdermal patches effectively can be used to treatment of wound healing. The effective wound healing activity due the presence of various bioactive phytoconstituents in developed formulation.

Keywords: *In-Vivo* wound healing activity, *S.Alternata*, Methanolic extract, silver nanoparticles, Transdermal patches.

1. INTRODUCTION

Nature is a shining example of the extraordinary symbiosis phenomenon. Nature has blessed us with a plethora of medicinal plants throughout human history, and folklore medicine has evolved as cultures and individuals sought novel methods of curing illness, which has been revived in the practise of medicine [1]. Traditional medicine has a long history in India. Medicinal plants are a globally significant local heritage. A wide variety of medicinal plants can be found all over the world. People all over the world strive to maintain their health in the face of chronic stress from today's work environment and various pollutions, as well as to treat disease with medicinal substances that work in tandem with the body's own defence mechanism. Herbs serve as a starting point for conventional drug isolation or synthesis. Medicinal plants have therapeutic value due to the presence of numerous complex active chemical constituents known as secondary plant metabolites [2, 3].

Inorganic hybrid materials show promise as drug delivery system materials. Nanoscience and nanotechnology have recently advanced, resulting in the continuous development of new nanomaterials with remarkable properties that make them appealing for use in pharmaceutical applications. Current research scientists are becoming more interested in

biocompatibility metallic nanoparticles in order to develop novel nano-based medicine [4, 5].

A wound is defined as any damage or disruption of living tissue caused by microbial, external, internal, physical, or chemical force [6]. Wound healing is a complicated process involving the regeneration or reconstruction of damaged tissue. The normal wound healing response is a sequence of events that begins with an injury. Inflammation is a highly complex process that can be thought of as the body's first protective response to the immune system. The immediate goal is to protect against bacterial invasions, antigen introduction, infections, and cell and tissue damage [7].

*S.Alternata*one of the most important ayurvedic plant for the treatment for various infectious and non-infectious disease [8, 9]. The present research study eco-friendly synthesis of metallic nanoparticles using the *S.Alternata*methanolic extract silver nanoparticles transdermal patches for the enhancement of wound healing activity by *In-vitro* and *In-vivo* study.

2. MATERIAL AND METHODS

2.1. *StrobilanthesAlternata*plant collection and authentication

*S. alternata*were gathered in the southern Indian state of Kerala's Ramamangalam Ernakulam District. Dr. K.R. Sasidharan, a plant taxonomist scientist, identified the specimen, which was then stored at the FishcherHebarium at the Institute of Forest Genetics and Tree Breeding and the Indian Council of Forestry Research and Education in Coimbatore, Tamil Nadu, India (Accession number: 24958).

2.2. Methanolic extraction of *S. alternata*leaf extraction by Soxhlet

S. alternata leaf was collected, sterile with 0.1% mercuric chloride, and rinsed with sterile water. A modified version of the Tzanova et al. (10) method was used to create a methanolic extract of *S. alternata* leaves. The extraction procedure is repeated until a drop of methanolic solvent emerges from the syphon tube without leaving any residue. A methanolic extract of *S. alternata* was used for additional phytochemical research.

2.3. Phytochemical evaluation

Mahire and Petel (11) improved the method for identifying phytochemicals in methanolic extracts of *S. alternata*.

2.4. Phytochemical evaluation by GC-MS

Phytochemical analysis of methanolic extract of *S. alternata*by using the GC-MS analysis followed by Konappa, Narasimhamurthy*et al* (12) method with slight changes by using the MS DSQ II GC-MS instrument - Thermo scientific Co. instrument with flow rate of mobile phase 0.1 ml/min, 40°C to 250°C at 5°C/min and 1µl volume injection was used. Mass values ware compared with the Willy spectral library.

2.5. Green synthesis of silver nanoparticles using methanolic extract of *S. alternata*

Functionally important sliver nanoparticles (AgNPs) are synthesised using the 1 mM silver nitrate solution in double distilled water was the source of silver. 1: 3 ratio of silver nitrate and methanolic extract reaction mixture stirred using the magnetic stiller with 500rpm beloving the boiling point. The experimental study conducted in dark condition to avoid the light reduction. Synthesised silver nanoparticles were centrifuged 10 min at 10,000 rpm. *S. alternata*methanolic extract silver nanoparticles pellet collected and washed with the distilled water and stored in 4°C for the further characterisation and biological activity analysis [13-15].

2.6. Characterization of nanoparticles by SEM, TEM, EDAX, FT-IR

Biogenically synthesised *S. alternata*silver nanoparticles were characterised by using the UV (UV-visible), XRD (X-ray diffraction), FT-IR (Fourier transform infrared)EDX (energy-dispersive X-ray spectroscopy), field emission scanning electron microscopy (FESEM) and high-resolution transmission electron microscopy (HRTEM) with EDAX (AgNPs). Particle size and zeta potential in liquid suspension were both assessed using the Zeta sizer Nano ZS (Malvern Instruments) [16-18].

2.7. MTT assay of *S. alternata*methanolic extract silver nanoparticles

*S. alternata*silver nanoparticles cytotoxicity on L6 cells was carried out as described by Karimi *et al*[19] and Satyavani*et al*[20] with modification. Fresh L6 cells concentration $1X10^4$ cells/mL cells cultivated in 96 well plate aseptically and cell concentration of measured by using the hemacytometer to maintain the constant volume of the cells for hole experiment. After 24hrs the cells were treated with the *S. alternata*silver nanoparticles

with various concentrations from 0 to 500 µg/mL and incubated for 24 hrs at 37°C, 95 % air and 5% CO2 conditions.After the 24hrs *S. alternata*silver nanoparticles treatment the cell culture wells were washed with culture media and added MTT (5 mg/mL in PBS) dye for the determination of live and dead cells. A cell viability determined using the multi-well plate reader at 540 nm. The cell viability results were expressed as a percentage of stable cells in contrast to the control. IC_{50} value calculated with optimal doses were investigated throughout time.

Proliferation inhibition (%) = (Ac-At/Ac) X 100

50% inhibition of the cells were calculated by using the *S. alternata*silver nanoparticles dose-response curve (n=3).

2.8. Acridine orange/ethidium bromide (AO/EB) staining technique was used to measure apoptotic induction.

Liu et al [21] used a fluorescence microscopic examination to study the apoptosis of S. alternata silver nanoparticles in comparison to L6 cell lines. PBS solution and treated S. alternata silver nanoparticles incubated L6 cell to create an AO/EB 1:1 ratio(100 µg/mL). The cells were examined under a fluorescence microscope at 40X after 5 minutes of incubation.

2.9. *In vitro* inflammatory activity-scratch assay of*S. alternata*silver nanoparticles

Fibroblast cells (L6 cell lines) were grown in sterile six-well plates to a confluent monolayer. Healthy monolayer cells were scraped straight line with sterile pipet tip. Un detached cells were removed by rinsing the sterile phosphate buffer solution. *S. alternata*silver nanoparticles(50 µg) and 0.2% FBS as control were added aseptically in the tested cells and incubated for 48hrs at 37°C cell culture incubator. After 48hr the 6 well plates were observed under the phase-contrast microscope and scratched cells growth pattern was observed and saved.

2.10. Formulation of transdermal patches preparation, evaluation, and drug release kinetics

Three batches of*S. alternata*silver nanoparticles transdermal patches were prepared using the drug with two different polymers in three different ratios (1:1,1:2&2:1). The composition of *S. alternata*silver nanoparticles incorporated Transdermal patch of *S. alternata* silver nanoparticles.PEG 400 was used as a plasticizer, and DMSO was a permeation enhancer. PVA and PVP were dissolved in the water-methanol mixture (1:1). DMSO and PEG 400 were added to the mixture. Different ratios of nanoparticles were added to the solution and mix well.The dispersion was transferred into a petri dish and kept for 24 hr. The dried patches are placed on a desiccator.

2.10.1. Physical Appearance

The Colour, clarity, smoothness, and flexibility of patches were visually inspected.

2.10.2. Thickness

At various spots along the patch, the thickness was measured with a screw gauge or vernier callipers. Three patches were chosen at random from each formulation. The thickness of a single patch was measured and the average value was found.

2.10.3. Weight variation

Individually weighing four randomly selected *S. alternata* silver nanoparticles transdermal patches was used to investigate weight variance. Average weight measured. The amount should not exceed the average weight.

2.10.4. Folding endurance

It was determined by folding the formed patch again and over until it broke. The folding endurance is measured by the number of times a patch can be folded before it breaks.

2.10.5. Drug content determination

A magnetic bead is used to swirl the produced patches in a beaker containing 100ml of distilled water for 5 hrs. Then, using correct dilution, filtered, and spectrophotometrically analysed at 258 nm.

2.10.6. Percent elongation

When a *S. alternata*silver nanoparticles patch sample is stressed, it stretches, which is referred to as a strain. Strain is calculated by dividing the patch's distortion by the sample's initial dimension. In general, as the amount of plasticizer in the patch grows, the patch lengthens. It is calculated using the formula below.

$$\text{Percent elongation} = \frac{\text{Increases in length of patch}}{\text{The initial length of patch}} \times 100$$

2.10.7. Percentage moisture content

The *S. alternata*silver nanoparticles patches measured the weight and incubated in desiccator with

$CaCl_2$ solution for 24 hrs and reweighed. Belove the formula was used to calculate the percentage moisture content.

$$\text{Moisture content} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

2.10.8. *In- vitro* drug dissolution study

*S. alternata*silver nanoparticle transdermal patch drug release study conducted using the Franz-diffusion cell.The dialysis membrane was divided into equal parts and soaked in distilled water for 12 hours. The *S. alternata*silver nanoparticle transdermal patch drug release tests were conducted phosphate buffer (10 ml) pH 7.4 at constant temperature and constant stirring. Using a phosphate buffer pH 7.4 as the blank, the amount of medication that diffused across the membrane was determined using a UV spectrophotometer at a wavelength of 258 nm.

2.10.9. Study of drug release kinetics

The *S. alternata*silver nanoparticles transdermal patches drug release kinetics were established by drawing the kinetic models below using data from *in vitro* release investigations. The release kinetics was followed by Cherukuri*et al*[22] method with slight changes and Korsmayer Peppa equations used to calculate the % of drug release from the developed formulation.

2.11. *In-Vivo* wound healing property of *S. alternata*silver nanoparticlespatch (F2)

In-Vivo wound healing activity of *S. alternata*silver nanoparticlespatch (F2) formulation was investigated in adult male albino rats with 200-250gm weight were employed. Selected animals were maintained sterile animal cages inside the animal house and provided commercial pellet rat chow and good water daily. As per the approval (IAEC: CBLRC/IAEC/08/01-2021) of the Institutional Animal Ethical Committee of Cape Bio Lab and Research Centre, Marthandam, KK Dist, Tamil Nadu, animal study was conducted which control and supervision of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPSCEA).

2.11.1. Four groups of animals containing each group four animals

The animal excision wound models were divided into three groups and each group with six animals.

Group G1= Treated with silver sulfadiazine ointment 10 mg/g applied topically.

Group G2= Negative control.

GroupG3=Transdermal patches formulation (F2 *S. alternata* silver nanoparticlessilver nanoparticle patch applied topically for 15 days).

Formulation F2, silver sulfadiazine ointment treated group, negative and untreated group animals were administrated the test medication and wound size was measured on the 2nd, 4th, 6th, 8th, 10th, 12th, and 14th post-wounding measurements. % of wound contraction was calculated using the formula.

$$\%\text{ of wound contraction} = \frac{\text{Initial wound size - Specific day wound size}}{\text{Initial wound size}} \times 100$$

2.11.2. Statistical analysis

The outcomes of the wound models were compared between the treated and control groups and given as mean SEM. Utilizing one-way ANOVA, the statistical differences between the treatment and control groups were evaluated.

3. RESULTS AND DISCUSSION

3.1. *S. alternata*collection, authentication, and extraction

The *S. alternata*authenticated plants was carefully collected without any adulterants (Figure 1). With our interest, healthy and good plant material was collected, and shade drying was used to dry and prevent the degradation of the bioactive phytoconstituents.*S. alternata*500 gm of dried leaf sample was weighted and subjected to successive hot continuous extraction process with the assistance of Soxhlet apparatus (Figure 2).

3.2. Phytochemical analysis *S. alternata*methanolic extract

Table 1 displays the findings of phytochemical screening of an methanolic extract of *S. alternata*. The presence of numerous phytochemical elements such as flavonoids, amino acids, carbohydrates, tannins, steroids, proteinsand phenolic compounds was discovered.

3.3. Phytochemical evaluation of methanolic extract of *S. alternata*methanolic extract by GC-MS

The tentative assignment of compounds detected from the *S. alternata*methanolic extractvia GC-MS analysis are presented. The compounds were detected and were identified by their fragmentation pattern and in conjunction with the NIST library.

Peak area and retention time were used for the identification of the compounds (Table 2).

3.4. Synthesis of silver nanoparticles using the *S. alternata* methanolic extract.

The visual colour change of the colloidal solution ensured the formation of the silver nanoparticles (Fig 3). The effective reduction of the Ag^{2+} was possible with *S. alternata*methanolic extract possibly due to the active secondary metabolites in *S. alternata* extract.

3.5. Characterization of nanoparticles *S. alternata*methanolic extract silver nanoparticles UV-Visible spectroscopy

The UV-Visible spectra of the *S. alternata*methanolic extract silver nanoparticles evidence SPR at 450 nm, ensuring the silver nanoparticle formation (Figure 3).

3.6. FT-IR spectroscopy of *S. alternata*methanolic extract silver nanoparticles

The functional groups present in the *S. alternata*methanolic extract silver nanoparticles weak peak at 455 cm-1 of ametal peak, which ensures the formation of silver nanoparticles. The common peaks seen in all the spectra are broad peaks around 3294 cm^{-1} corresponding to the –OH peak, and around 1635 cm^{-1} corresponding to the stretching vibration of the C=O group (Figure 4)

3.7. XRD analysis of *S. alternata*methanolic extract silver nanoparticles

The crystalline nature of nanoparticles was determined using XRD analysis. CBPPR1Au 2Ɵ= 38.83 and in *S. alternata*methanolic extract Ag 2Ɵ=32.40 and 38.78 show the crystalline nature of the nanoparticles (Figure 5)

3.8. Zeta potential and Zeta size of *S. alternata*methanolic extract silver nanoparticles

The stability and size of *S. alternata*methanolic extract silver nanoparticles were performed at 25 °c using the zeta sizer. The particle size of *S. alternata*methanolic extract AgNPswere found to be 635d. nm. The zeta potential is a measure of surface charge potential, which is a crucial metric in determining nanoparticle stability in aqueous solutions. The zeta potential value of *S. alternata*methanolic extract AgNPs were identified as -2.59 mV. It was confirmed that synthesized nanoparticles had a negative charge on the surface (Figure 6).

3.9. FE-SEM and HR-TEM observation of *S. alternata*silver nanoparticles

FESEM and HRTEM morphography of *S. alternata*silver nanoparticles showed the particles are spherical/oval and 18nm to 20 nm in size. The morphological features of prepared *S. alternata*silver nanoparticles, the results of which are presented in Fig 7.

3.10. EDAX spectroscopy of *S. alternata*methanolic extract silver nanoparticles

EDAX spectroscopy was used to determine the elemental composition of biosynthesised nanomaterials surfaces. Fig 8 is a typical TEM-EDX point-detection example for the composition, revealing the presence of silver elements simultaneously.

3.11. MTT assay of *S. alternata*silver nanoparticles on L6 cells

The antitumor activity of the *S. alternata*methanolic extract silver nanoparticles was examined by MTT assay in DMSO against L6 cells. The IC_{50} value was determined by plotting the cell viability against the complex concentration. The inhibitory concentration of the complex at 50% cell destruction, L6 IC_{50} was determined from the MTT assay at 24 hr as 70µM (Figure 9).

3.12. *S. alternata*methanolic extract silver nanoparticles treated L6 cell line apoptosis determination by acridine orange/ethidium bromide staining.

To investigate the morphological alterations of the L6 cell lines, the fluorescence staining investigation was carried out using acridine orange/ethidium bromide (AO/EB) staining (Fig 10). AO/EB staining was used to study the apoptotic features of L6 cells induced by *S. alternata*methanolic extract silver nanoparticles. The AO/EB staining fluorescence pattern predicts cell viability and membrane integrity. Dead cells are often permeable to EB, resulting in an orange-red fluorescence, whereas live cells are permeable to AO, resulting in green fluorescence.

3.13. *In vitro* inflammatory activity-scratch assay-*S. alternata*silver nanoparticles

In order to characterize the potential influence of *S. alternata*methanolic extract silver nanoparticles on skin fibroblasts cells (L6) were cultured. The extent of regrowth to close the scratch wound was measured after 0, 24 and 48 hr of incubation in medium containing *S. alternata*silver nanoparticles (50µg). Restoration of the full cellular density of the mesothelium in fibroblasts was faster in the *S. alternata*silver nanoparticles group than that in the control group. In other words, *S. alternata*silver nanoparticles promoted the migration rate of fibroblasts obviously at each time point (Figure 11).

3.14. Formulation of transdermal patches preparation, evaluation, and drug release kinetics and standard curve of *S. alternata* nanoparticles.

The neem nanoparticle was estimated using the UV-spectrometric method by measuring the absorbance at 218nm. It obeyed the Beer's lamberts law in the range of 2-10µg/ml. the correlation factor was found to be 0.9990 (Figure 12 and table 3).

3.15. Evaluation of *S. alternata*methanolic extract silver nanoparticles transdermal patches

3.15.1. Physical appearance

The color, clarity, smoothness, and flexibility of patches were visually inspected (Figure 13 and table 4).

The appearance of patches is evaluated and it was found that all patches were light green to colorless, clear, and flexible.

3.16. Thickness of patch, weight variation, folding endurance, drug content and % of moisture content of *S. alternata*methanolic extract silver nanoparticles patches.

The thickness of the patch was determined by a screw gauge. The mean thickness was measured at different points of the film.The thickness of the prepared patches was tabulated. It was found that F1(0.2mm) shows less thickness and F3(0.3mm) shows more thickness. The folding endurance values of all the patches were found satisfactory which indicates that the patches were prepared using *S.alternata* silver nanoparticles, HPMC and EC not showing any brittle. The drug content of F1 to F3 was measured spectrophotometrically at 218 nm. The drug content is calculated and it was found to be 80, 89 and 85 %. The moisture content of patches was determined and the patch F3(5.70%) shows more moisture content and F1(1.58%) shows less moisture content.

3.17. *In-Vitro*drug dissolution study

The *in-vitro* drug release studies were carried out for the formulations F_1 to F_3. It was found that amount of drug was released in phosphate buffer of pH 7.4 table 6shows that 74.89% of the drug was released in 8 hrs from the formulation.

F2It is clear from the figure that all the formulations demonstrate delayed release properties. Drug release was higher in the case of F2 formulation using pH7.4 phosphate buffer solution. The release data are given in table 6 and figure 14.

3.18. Study of drug release kinetics

The mathematical models were fitted to the obtained release data from the *in-vitro* dissolution research of several formulations. Zero order, first order, Higuchi equation, and Korsmeyer-Peppas models were among the kinetic models used. The release kinetics of all formulations are summarized in Table7-9. Table 10 provides the model fitting data for all four formulations evaluated, along with their R^2 values, K constants, and n exponential values. The drug release from the transdermal patches followed either a Zero-order or a Korsmeyer –Peppas model, according to the overall curve fitting. The values of the exponential factor 'n' were found to be between 0.45-0.85, indicating that the drug release was controlled by Non-Fickian diffusion.

The result of this study was a nanoparticle-loaded transdermal patch made with polyvinyl alcohol (PVA) and polyvinyl pyrrolidine (PVP) as a suitable carrier system for *S. alternata*silver nanoparticles integration.The transdermal patches were made with different materials and medication ratios. The content consistency, thickness, and weight variation of the formulated patches were tested, and the results were determined to be satisfactory.

Formulation F2 was determined to be the optimal formulation based on *in vitro* release experiments. In 8 h, approximately 74.89 % of the active medication was released. The drug release kinetics were assessed by replacing data from the Zero order, First order, Higuchi model, and Korsmeyer-Peppas models. The drug release from the patches appeared to follow a non-Fickian transport mechanism, according to kinetic analyses.

3.19. *In-vivo* anti-inflammatory activity of *StrobilanthesAlternata* methanolic extract Silver Nanoparticles transdermal patch (F2) on Wistar albino rats

The wound healing rate in F2 transdermal patch (*S.alternata* methanolic extract silver nanoparticlestransdermal patch) and other groups has been studied for 14 days, the percentage of wound healing increased in all rat groups. On days 10 and 14, the wound area decreased significantly in all treated groups compared to the control (P≤ 0.001). On the second day, drug treated had a significantly greater wound healing effect than the other groups (P≤ 0.001), whereas on the sixth day, there was no significant difference between the control and treated groups.On day 10, there was less significant difference in wound healing properties between cream base and standard. On the 14th day, there was a significant difference in wound healing activity between the F2 transdermal patch (*S.alternata* methanolic extract silver nanoparticlestransdermal patch) and the other groups (P≤ 0.001) (Figure 15 and Table 11).

3.19.1. Histopathological examination

S.alternata methanolic extract silver nanoparticlestransdermal patcheffect on wound induced and normal animals and its histopathological observation of *S.alternata* methanolic extract silver nanoparticlestransdermal patchtreated wound and control animals(Figure 16).In the F2 and Silver sulfadiazine-treated animals, there was complete re-epithelialization and well-formed granulation tissue of the epidermis, as well as remarkable neovascularization and mild inflammatory cell infiltration.

3.19.2. Body weight analysis in normal and experimental rats

All the animal group significantly regained the body weight by the completion of study period. The treatment with the extracts has improved the loss of body weight in rats brought on by wound healing conditions (Table 12 and Figure 17) (17).

4. CONCLUSION

Experimental observations suggest that an *S.Alternata*methanolic extract nanoparticles transdermal patches has wound protecting properties and requires correction of altered biological parameters. It also requires further research so that the understand the wound healing principles mechanism of this *S.Alternata*methanolic extract nanoparticles can be identified. From this study, the *S.Alternata*methanolic extract nanoparticles transdermal patches was found to have good wound healing activity. Thus, this plant can be used as an effective plant for the management of wound treatment.

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