Section A-Research paper

In-vitro and *In-vivo* Wound Healing Activity of Biogenically Synthesised *Azadirachta indica* Methanolic Extract Silver Nanoparticle Transdermal Patches

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ABSTRACT

Background: *Azadirachta indica* is a well-grown plant abundantly present in Asian countries in various climatic conditions.

Aim: The present study evaluation of woung healing activity (*In-vitro* and *In-vivo*) of the *A. indica* methanolic extract silver nanoparticle.

Material and Methods: Conventional and GC-MS phytochemical analysis confirms that *A. indica* methanolic extracts bioactive secondary metabolites. By metal reduction method was used to synthesise silver nanoparticles with the help of *A. indica* methanolic extract. *A. indica* methanolic silver NPs studies cytotoxicity and wound healing activity (*In-vitro* - scratch assay). Transdermal patches were prepared *for A. indica* methanolic silver NPs by solvent casting method. Wound healing activity (*In-vivo*) of *A. indica* methanolic silver NPs transdermal patches conducted in Wister albino rats.

Results: Phytochemical analysis of *A. indica* methanolic extract confirms that it has bioactive secondary metabolites (Copaene, Caryophyllene, Hexanedioic acid, bis(2-methyl propyl) ester, Triacontanoic acid, 2- n-Hexadecanoic acid, Octadecanoic acid, Tetradecanoic acid and 1,4-Eicosadiene etc.). *A. indica* methanolic silver NPs showing good migration properties (24

hr) with L6 cell lines. F2 formulation *A. indica* methanolic silver NPs transdermal patch having good drug leasing properties (%). *In-vivo* wound healing studies revealed that the F2 formulation outperformed the standard drug regarding wound healing properties. The size of the animals' wounds was completely reduced by the 14th day, just like with the standard drug.

Conclusion: As a result, *A. indica* methanolic silver NPs transdermal patches can effectively treat wound healing. Various bioactive phytoconstituents in the developed formulation result in effective wound-healing activity.

Keywords: *In-vivo* wound healing activity, *Azadirachta indica*, Methanolic extract, Silver nanoparticles, Transdermal patches.

INTRODUCTION

Nature is always a shining example of the exceptional symbiosis phenomenon. Throughout human history, nature has blessed us with many medicinal plants, and folklore medicine has evolved as cultures and individuals sought novel methods of curing illness, which has been revived in medicine. Traditional medicine has a long history in India. The globally significant local heritage is medicinal plants. Various medicinal plants can be found all over the world.[1-4] People worldwide strive to stay healthy in the face of chronic stress from today's work environment and various pollutions and treat diseases with medicinal substances that work in tandem with the body's defence mechanism. The herbs provide the starting material for conventional drug isolation or synthesis. Due to numerous complex active chemical constituents known as secondary plant metabolites, medicinal plants have therapeutic value.[5-8]

Inorganic hybrid materials are promising materials for drug delivery systems. The recent advancement of nanoscience and nanotechnology has resulted in the continuous development of new nanomaterials with remarkable properties that make them appealing for pharmaceutical applications. The biocompatibility metallic nanoparticles are showing more interest in current research scientists to words developing novel nano-based medicine.[9,10]

A wound is a damage or disruption of the living tissue caused by microbial, external, internal physical and chemical forces. Wound healing is a difficult process involving regenerating or reconstructing damaged cells. Inflammation is a highly complex process which could be viewed as the body's first protective response to the immune system. The immediate purpose is to defend against bacterial invasions, the introduction of antigens, and any infections or cell and tissue damage.[11]

A. indica is one of the most important ayurvedic plants for treating various infectious and non-infectious diseases. The present research study eco-friendly synthesis of metallic nanoparticles using the *A. indica* methanolic extract silver nanoparticles transdermal patches to enhance wound healing activity by *In-vitro* and *In-vivo* study.

MATERIALS AND METHODS

A. indica plant collection and authentication

A. indica were collected from Ramamangalam Ernakulam District Kerala, South India. The specimen was identified by plant taxonomist scientist Dr K.R SASIDHARAN and deposited at Fishcher Herbarium (Accession number: 24959) at IFGTB and ICFRE at Coimbatore, Tamil Nadu, India.

Methanolic extraction of *A. indica* leaf extraction by Soxhlet

A. indica leaf was collected, cleaned with sterile water, and sterilised using the sterilising solution (0.1% mercuric chloride). *A. indica* leaf methanolic extract was conducted using the Tzanova *et al*.[12] method with minor modification. The extraction processor is repeated continuously until drops of methanolic solvent form the syphon tube without leaving any residue. Methanolic extract of *A. indica* used further phytochemical analysis.

Phytochemical evaluation

Mahire and Petel (2020) modified the method used to identify phytochemicals from the methanolic extract of *A. indica*.[13]

Phytochemical evaluation by GC-MS

GC-MS used to identification of the phytochemical in *A. indica* methnolic extract followed by Narasimhamurthy *et al*.[14] method with the minor changes.

Green synthesis of silver nanoparticles using *A. indica* methanolic extract

A one millimolar solution of silver nitrate in double-distilled water is used to make functionally significant silver nanoparticles (AgNPs). 1: 3 ratio of silver nitrate and the methanolic extract reaction mixture stirred using the magnetic stiller with 500 rpm beloving the boiling point. The experimental study was conducted in dark conditions to avoid light reduction. Synthesised silver nanoparticles were centrifuged for 10 min at 10,000 rpm. *A. indica* methanolic extract silver nanoparticles pellet was collected and washed with the distilled water and stored at 4°C for further characterisation and biological activity analysis.[15-17]

Characterisation of nanoparticles by UV, SEM, TEM, EDAX, FT-IR

Biogenically synthesised *A. indica* silver nanoparticles were characterised followed by Urnukhsaikhan *et al*.[18], Chandrasekharan *et al*.[19] and Alby Babu *et al*. [20] methods with minor changes.

MTT assay of eco-friendly synthesised *A. indica* methanolic extract silver nanoparticles

A. indica silver nanoparticles cytotoxicity on L6 cells was carried out as described by Karimi *et al*. [21] and Satyavani *et al*.[22] with modification. Fresh L6 cells concentration of $1X10^4$ cells/mL cells cultivated in 96 well plates aseptically and cell concentration was measured using the hemacytometer to maintain the constant volume of the cells for the whole experiment. After 24hr the cells were treated with the *A. indica* silver nanoparticles with various concentrations from 0 to 500 µg/mL and incubated for 24 hr at 37°C, 95% air and 5% CO_2 conditions. After the 24hr *A. indica* silver nanoparticles treatment, the cell culture wells were washed with culture media. They added MTT (5 mg/mL in PBS) dye to determine live and dead cells. Cell viability was determined using the multi-well plate reader at 540 nm. The percentage of stable cells was compared to the control. Over time, optimal-dose IC50 values were examined.

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

50% inhibition of the cells was calculated by using the *A. indica* silver nanoparticles dose-response curve (n=3).

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

Liu *et al*.[23] method with slight changes study the apoptosis of the *A. indica* silver nanoparticles against L6 cell lines by examining the microscopic fluorescence. AO/EB 1:1 ratio was prepared using PBS solution and treated *A. indica* silver nanoparticles incubated L6 cell (100 µg/mL). After 5 min incubation, the cells were observed under the fluorescence microscope under 40X.

***In vitro* inflammatory activity-scratch assay- *A. indica* silver nanoparticles**

Fibroblast cells (L6 cell lines) were grown in sterile six-well plates to a confluent monolayer. Healthy monolayer cells were scraped in a straight line with a sterile pipet tip. Undetached cells were removed by rinsing the sterile phosphate buffer solution. *A. indica* silver nanoparticles (50 µg) and 0.2% FBS as control were added aseptically in the tested cells and incubated for 48hr at 37°C cell culture incubator. After 48hr the 6 well plates were observed

under the phase-contrast microscope, and a scratched cells growth pattern was observed and saved.

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

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

Physical Appearance

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

Thickness

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

Weight variation

The variation in weight was investigated by individually weighing four different randomly chosen A. indica silver nanoparticles transdermal patches. The weight taken as the average It is important that the total weight does not go above the average..

Folding endurance

Folding the patch repeatedly till it broke determined it. Folding endurance is the amount of times a patch can be folded before breaking.

Drug content determination

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

Percent elongation

When an *A. indica* silver nanoparticles patch sample is stressed, it stretches, referred to as a strain. Strain is calculated by dividing the patch's distortion by the sample's initial dimension.

As the amount of plasticiser in the patch grows, the patch lengthens. It is calculated using the formula below.

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

percentage of moisture content

The *A. indica* silver nanoparticles patches measured the weight, incubated in a desiccator with $CaCl_2$ solution for 24 hr, and reweighed. Beloved, the formula was used to calculate the percentage moisture content.

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

***In- vitro* drug dissolution study**

A. indica 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 hr. The *A. indica* silver nanoparticle transdermal patch drug release tests were conducted with phosphate buffer (10 ml) pH 7.4 at constant temperature and stirring. The amount of medication diffused across the membrane was determined using a UV spectrophotometer at 258 nm.

Study of drug release kinetics

The *A. indica* 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*.[24] method with slight changes and Korsmayer Peppa equations used to calculate the % of drug release from the developed formulation.

***In-vivo* wound healing property of *A. indica* silver nanoparticles patch (F2)**

In-vivo wound healing activity of *A. indica* silver nanoparticles patch (F2) formulation was investigated in adult male albino rats with 200-250gm weight were employed. Selected animals were maintained in 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, an animal study was conducted which control and supervision of the Committee for Control and Supervision of Experimental Animals (CPSCEA).

Four groups of animals containing each group of four animals

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

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

Group G2= Negative control.

Group G3=Transdermal patches formulation (F2 *A. indica* silver nanoparticles silver nanoparticle patch applied topically for 15 days).

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

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

Statistical analysis

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

RESULTS

***A. indica* collection, authentication, and extraction**

The *A. indica* authenticated plants were carefully collected without 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. *A. indica* 500 gm of dried leaf sample was weighted and subjected to successive hot continuous extraction process with the assistance of Soxhlet apparatus (Figure 2).

Phytochemical analysis *A. indica* methanolic extract

Table 1 displays the findings of the phytochemical screening of a methanolic extract of *A. indica*. Numerous phytochemical elements were discovered, such as alkaloids, flavonoids, amino acids, carbohydrates, saponins, tannins, and phenolic compounds.

Phytochemical evaluation of methanolic extract of *A. indica* methanolic extract by GC-MS

The tentative assignment of compounds detected from the *A. indica* methanolic extract via GC-MS analysis is presented. The compounds were detected and identified using their fragmentation pattern and the NIST library. Peak area and retention time were used to identify the compounds (Table 2).

Green synthesis of silver nanoparticles using methanolic extract of *A. indica*

The colloidal solution's visual colour change ensured the silver nanoparticles' formation (Figure 3). The effective reduction of the Au^{3+} and Ag^{2+} was possible with *A. indica*, possibly due to the active secondary metabolites in *A. indica* extract. Uniform heating in a microwave oven leads to the quick formation of the silver nanoparticles, ensured by the change in the Colour of the solution from Colour less to light green.

Characterisation of nanoparticles *A. indica* methanolic extract silver nanoparticles

UV-Visible spectroscopy

The UV-Visible spectra of the *A. indica* methanolic extract silver nanoparticles evidence SPR at 450 nm, ensuring the silver nanoparticle formation (Figure 4).

FT-IR spectroscopy of *A. indica* methanolic extract silver nanoparticles

The functional groups present in the *Azadirachta indica* methanolic extract silver nanoparticles have a weak peak at 455 cm-1 of a metal 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 5).

XRD analysis of *A. indica* methanolic extract silver nanoparticles

The crystalline nature of nanoparticles was determined using XRD analysis. *A. indica* methanolic extract Ag 2Ɵ=32.40 and 38.78 show the crystalline nature of the nanoparticles (Figure 6)

Zeta potential and Zeta size of *A. indica* methanolic extract silver nanoparticles

The stability and size of *A. indica* methanolic extract silver nanoparticles were performed at 25°C using the zeta sizer. The particle size of *A. indica* methanolic extract AgNPs was 265.4 d. nm. The zeta potential measures surface charge potential, a crucial metric in determining nanoparticle stability in aqueous solutions. The zeta potential value of *A. indica* methanolic extract AgNPs was identified as -24.1 mV. It was confirmed that synthesised nanoparticles had a negative charge on the surface (Figure 7).

FE-SEM and HR-TEM observation of *A. indica* silver nanoparticles

FESEM and HRTEM morphology of *A. indica* **s**ilver nanoparticles showed the particles are spherical/oval and 39 nm to 85 nm in size. The morphological features of prepared *A. indica* **s**ilver nanoparticles, the results of which are presented in Figure 8.

EDAX spectroscopy of

EDAX spectroscopy was used to determine the elemental composition of biosynthesised nanomaterials surfaces. Figure 9 is a typical TEM-EDX point-detection example for the composition, revealing the presence of silver elements simultaneously.

MTT assay of *A. indica* silver nanoparticles on L6 cells

The antitumor activity of the *A. indica* 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 68 µM (Figure 10).

***A. indica* 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 was carried out using acridine orange/ethidium bromide (AO/EB) staining (Figure 11). AO/EB staining was used to study the apoptotic features of L6 cells induced by *A. indica* 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.

***In vitro* inflammatory activity-scratch assay- *A. indica* silver nanoparticles**

A. indica methanolic extract silver nanoparticles used to evaluation of the migration activity of the skin fibroblast. After 0, 24, and 48 hours incubation in A. indica silver nanoparticle medium, scratch wound regeneration was measured (50 µg). The restoration of full mesothelium cellular density in fibroblasts was faster in the *A. indica* silver nanoparticles group than in the control group.(Figure 12).

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

Standard curve of neem nanoparticles

The neem nanoparticle was estimated using the UV-spectrometric method by measuring the absorbance at 258nm. It obeyed Beer's lamberts law in the 20-100µg/ml range. The correlation factor was 0.9990 (Figure 13 and Table 3).

Evaluation of *A. indica* methanolic extract silver nanoparticles transdermal patches

Physical appearance

Patches' colour, clarity, smoothness, and flexibility were visually inspected (Figure 14 and Table 4).

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

***A. indica* methanolic extract silver nanoparticle patches thickness, weight fluctuation, folding endurance, drug content, and moisture content.**

The *A. indica* silver nanoparticles thickness of the prepared patches was tabulated. It was found that F1 to F3 (0.1mm) show less thickness with uniform weight. Manually folding a 2×2 cm film strip at the same location until it broke measured folding endurance. F2 had the best flexibility and was not brittle. All patches were made with PVA:PVP. Drug content was noticed in the patches. The moisture content of patches was determined, and patch shows F1(1.82%) shows less moisture content (Table 5).

In-vitro Drug Dissolution Investigation

F_1 to F_3 the amount of drug was found to be released in a phosphate buffer of pH 7.4. Figure 14 shows that 92.6% of the drug was released in 8 hr from the formulation. F2 It is clear from the figure that all the formulations demonstrate delayed release properties. Drug release was higher in the case of F2 formulation using a pH 7.4 phosphate buffer solution. The release date is given in Table 6 and Figure 15.

Study of Drug Release Kinetics

The mathematical models were fitted using in-vitro dissolving data from multiple formulations. (Zero-order, first-order, Higuchi, and Korsmeyer-Peppas kinetic models). Table 7-9 summarises all formulation release kinetics. Table 10 shows model-relevant data for all four formulations, including r^2 values, K constants, and n exponential values. The total curve fitting showed that transdermal patch medication release followed a Zero-order or Korsmeyer –Peppas model. The exponential factor 'n' was 0.45-0.85, demonstrating Non-Fickian diffusion controlled drug release.

The result of this study was a nanoparticle-loaded transdermal patch made with polyvinyl alcohol PVA and PVP as a suitable carrier system for *A. indica* silver nanoparticles integration. The transdermal patches were made with different materials and medication

ratios. The formulated patches' content consistency, thickness, and weight variation were tested, and the results were determined to be satisfactory.

Formulation F2 was the optimal formulation based on *in vitro* release experiments. In 12 hr, approximately 92.6 per cent of the active medication was released. From the drug release kinetics study, the drug release from formulation appeared to follow a non-Fickian transport mechanism.

In-vivo anti-inflammatory activity of _A. indica_ nanoparticle transdermal patch (F2) on Wistar albino rats

The wound healing rate in F2 transdermal patches (*A. indica* nanoparticles treated animal) and other groups have 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 treated animals equal to the control ($P \leq 0.001$). Second day, the drug-treated had a significantly greater wound-healing effect compared with other groups ($P \leq 0.001$). From the sixt day on words no difference between the control and treated groups. On day 10, The cream base and the standard had somewhat different wound healing characteristics, but the difference was not statistically significant. The F2 transdermal patch (A. indica nanoparticles transdermal patches treated animal) had significantly higher wound healing activity on day 14 ($P < 0.001$). (Figure 16 and Table 11).

Histopathological examination

A. indica nanoparticles effect on wound-induced and normal animals and its histopathological observation of *A. indica* nanoparticles treated wound and control animals (Figure 17). In the F2 and Silver sulfadiazine-treated animals, there was complete re-epithelialisation and well-formed granulation tissue of the epidermis, as well as remarkable neovascularisation and mild inflammatory cell infiltration.

Body weight analysis in normal and experimental rats

All the animal groups significantly regained body weight by completing the study period. The treatment with the extracts has improved the body weight loss in rats brought on by wound healing conditions (Figure 18).

DISCUSSION

Traditional medicinal plants are being investigated for wound healing as potential sources of new therapeutic compounds. *A. indica* all the parts are used to treat various diseases, and it's also important in pharmaceutical applications.[25] In this present study, nanoparticles were

synthesised using the methanolic extract of *A. indica*. Phytochemical elements, such as alkaloids, flavonoids, amino acids, carbohydrates, saponins, tannins, and phenolic compounds, have been presented in *A. indica* methanolic extract confirmed by conventional phytochemical and GC-MS analysis. Sarkar *et al*.[26] reported the phytochemical present in the neem leaf and its functional importance in disease treatment. Using methanolic extract, silver nanoparticles formed because the colloidal fluid changed colour (Figure 3). Due to its active secondary metabolites, *A. indica* reduced Au3+ and Ag2+. The solution turns light green when uniformly heated, forming silver nanoparticles quickly. Chinnasamy *et al*.[25] Synthesised neem leaf silver nanoparticles (33nm size) and characterised using the UV, FT-IR, SEM and TEM. Methanolic extract synthesised nanoparticles average rang 43nm determined using the FE-SEM, and average Zetasizer (265nm) were confirmed. Experiment results indicate that *A. indica* methanolic extract nanoparticles transdermal patches have wound-protective properties and that altered biological parameters must be corrected. Maan *et al*.[27] studied wound healing activity, aqueous *A. indica* extracts significantly reduce the wound. Our study on methanolic extract nanoparticles transdermal patch formulation shows good wound healing activity. Incision and excision wound healing and tissue restoration are complicated biochemical and cellular reactions. Wound healing involves tissue inflammation, repair, and remodelling. The wound is a skin epithelial rupture caused by trauma. Epithelial rupture disrupts normal tissue shape and function.[28] Phytochemicals alkaloids, terpenoids, saponins and flavonoids enhance wound healing due to their antioxidant and antimicrobial activity. Further research is also required to determine the wound-healing principles mechanism of these *A. indica* methanolic extract nanoparticles. The wound-healing activity of *A. indica* methanolic extract nanoparticles transdermal patches were discovered in this study. As a result, this plant can be used as an effective wound treatment plant.

CONCLUSION

Experimental observations suggest that *A. indica* methanolic extract nanoparticles transdermal patches have wound-protecting properties and require correction of altered biological parameters. It also requires further research to identify the wound healing principles mechanism of this *A. indica* methanolic extract nanoparticles. This study found the *A. indica* methanolic extract nanoparticles transdermal patches to have good wound healing activity. Thus, this plant can be used as an effective plant for wound treatment management.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

IFGTB: Institute of forest genetics and tree breeding

ICFRE: Indian council of forestry research and education

GC-MS = Gass chromatography mass spectroscopy

AgNPs = Silver Nanoparticles

SEM = Scanning electron microscope

TEM = Transmission electron microscope

EDAX = Energy dispersive X-ray analysis

FT-IR = Fourier transform infrared spectroscopy

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

AO = Acridine orange

EB = Ethidium bromide

PVP = Polyvinyl pyrrolidine

PVA = Polyvinyl alcohol

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Table 1: Phytochemical screening of an methanolic extract of *A. indica.*

Sl. No	**Phytochemicals**	**Methanolic extract**
1	Alkaloids	-
2	Carbohydrate	+
3	Glycosides	-
4	Phenolic compounds	-
5	Protein and amino acid	-
6	Flavones and flavonoids	+
7	Saponins	-
8	Steroids and sterols	+
9	Tannins	+

Table 2: Phytochemical screening of an methanolic extract of *A. indica* by GC-MS analysis.

Sl. No	**Phytoconstituents**	**Mass**
1	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	330
2	Octadecanoic acid, 2,3-dihydroxypropyl ester	358
3	1,2,5-Oxadiazol-3-amine, 4-(4-methoxyphenoxy)-	207
4	Tetradecanoic acid	228

5	Octadecanoic acid	284
6	1,4-Eicosadiene	278
7	9,12,15-Octadecatrienoic acid, ethyl ester, (Z, Z, Z)	306
8	Ethyl 9,12,15-octadecatrienoate	306
9	1,5-Anhydro-d-mannitol	164
10	n-Hexadecanoic acid	256
11	Triacontanoic acid, methyl ester	466
12	Hexanedioic acid, bis(2-methylpropyl) ester	258
13	Caryophyllene	204
14	Copaene	204
15	Adipic acid, isobutyl 2-naphthyl ester	328

Table 3: Standard curve of *A. indica* methanolic extract silver nanoparticles.

Sl. No	**Concentration(µg/ml)**	**Absorbance at 258 nm**
1	0	0.00
2	20	0.2065
3	40	0.4133
4	60	0.620
5	80	0.8268
6	100	1.033

Table 4: Physical appearance.

Sl. No	**Patch code**	**Color**	**Clarity**	**Flexibility**	**Smoothness**
1	F1	Color less	clear	Flexible	Smooth
2	F2	Color less	clear	Flexible	Smooth
3	F3	Color less	clear	Flexible	Smooth

Table 5: Thickness of the patches.

Sl. No	**Patch code**	**Mean thickness**	**Weight**	**Folding endurance**	**Drug content**	**% Moisture content**
1	F1	0.1	0.900	120	78	1.82
2	F2	0.1	0.910	124	80	2.70
3	F3	0.1	0.920	110	79	5.60

Table 6: *In-vitro* drug release studies of *A. indica* silver nanoparticles.

Sl. No	F1	F2	F3
1	1.31	1.5	1.86
2	3.98	2.99	4.31
3	11.34	14.78	10.39
4	27.34	29.99	20.11
5	32.72	34.98	30.85
6	42.4	45.8	41
7	49.03	52.69	42.3
8	55.4	68.89	51.34
9	62.4	71.3	58.5
10	68.5	81.5	65.5
11	71.5	85.2	69.2
12	75.4	92.6	72.4

Table 7: Drug release kinetics of formulation 1.

Sl. No	Time(hr)	%CDR	Log % CDR	Log time	SQRT time
1	1	1.31	0.1172	0	1
2	2	3.98	0.5998	0.3010	1.4142
3	3	11.34	1.0546	0.4771	1.7320
4	4	27.34	1.4367	0.6020	2
5	5	32.72	1.5148	0.6989	2.2360
6	6	42.4	1.6273	0.7781	2.4494
7	7	49.03	1.6904	0.8450	2.6457
8	8	55.4	1.7435	0.9030	2.8284
9	9	62.4	1.7951	0.9542	3
10	10	68.5	1.8356	1	3.1622
11	11	71.8	1.8561	1.0413	3.3166
12	12	75.2	1.8762	1.0791	3.4641

Table 8: Drug release kinetics of formulation 2.

Sl. No	Time(hr)	%CDR	Log % CDR	Log time	SQRT time

1	1	1.5	0.1760	0	1
2	2	2.99	0.4756	0.3010	1.4142
3	3	14.78	1.1696	0.4771	1.7320
4	4	29.99	1.4769	0.6020	2
5	5	34.98	1.5438	0.6989	2.2360
6	6	45.8	1.6608	0.7781	2.4494
7	7	52.69	1.7217	0.8450	2.6457
8	8	68.89	1.8381	0.9030	2.8284
9	9	71.3	1.8530	0.9542	3
10	10	81.5	1.9111	1	3.1622
11	11	85.2	1.9304	1.0413	3.3166
12	12	92.6	1.9666	1.0791	3.4641

Table 9: Drug release kinetics of formulation 3.

Sl. No	**Time(hr)**	**%CDR**	**Log % CDR**	**Log time**	**SQRT time**
1	1	1.86	0.2552	0	1
2	2	4.31	0.6344	0.3010	1.4142
3	3	10.39	1.0166	0.4771	1.7320
4	4	20.11	1.3034	0.6020	2
5	5	30.85	1.4892	0.6989	2.2360
6	6	41	1.6127	0.7781	2.4494
7	7	42.3	1.6263	0.8450	2.6457
8	8	51.34	1.7104	0.9030	2.8284
9	9	58.5	1.7671	0.9542	3
10	10	65.5	1.8162	1	3.1622
11	11	69.2	1.8401	1.0413	3.3166
12	12	72.4	1.8597	1.0791	3.4641

Table 10: *Azadirachta indica* silver nanoparticles patches *in-vitro* drug release kinetics.

Formulation code	**Zero-order**		**First-order**		**Higuchi**		**Korsmeyer - Peppas**		**n values**
	r^2	**K_0**	**r^2**	**K_0**	**r^2**	**K_0**	**r^2**	**K_0**	
F1	0.9688	6.609	0.5355	0.002	0.4966	29.00	0.9722	1.86	0.722

F2	0.9689	7.764	0.5657	0.002	0.4567	33.79	0.9538	1.938	0.772
F3	0.9695	6.216	0.5335	0.002	0.4609	27.11	0.9638	1.829	0.744

Table 11: Effect of *A. indica* methanolic extract nanoparticles transdermal patches patch on wound.

Wounding days	**Negative control**	**F2**	**Silver sulfadiazine**
2	4.15 ± 1.92	11.60 ± 2.61	14.80 ± 6.40
4	23.5 ± 1.77	26.51 ± 06.10	30.50 ± 08.20
6	32.22 ± 3.96	40.83 ± 8.60	47.25 ± 8.81
8	44.5 ± 7.33	53.61 ± 09.90	57.77 ± 5.74*
10	65.37 ± 6.50	71.26 ± 8.24*	77.30 ± 5.90**
12	83.77 ± 5.19	88.10 ± 2.10*	94.61 ± 5.23**
14	87.01 ± 4.21	94.70 ± 2.15**	99.21 ± 0.42**

Figure 1: *A. indica* leaf.

Figure 2: Methanolic extract of *A. indica* leaf – Soxhlet apparatus.

Figure 3: synthesis of silver nanoparticles using methanolic extract of *A. indica.*

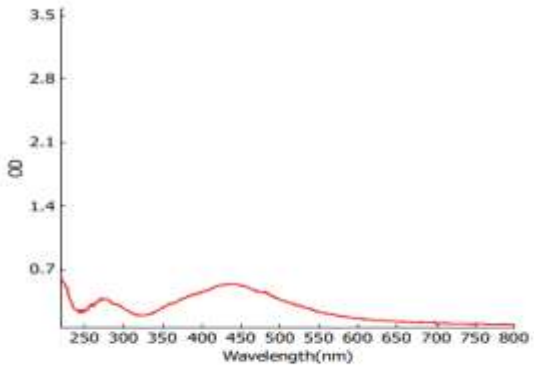

Figure 4: UV-Visible spectra of *Azadirachta indica* methanolic extract silver nanoparticles.

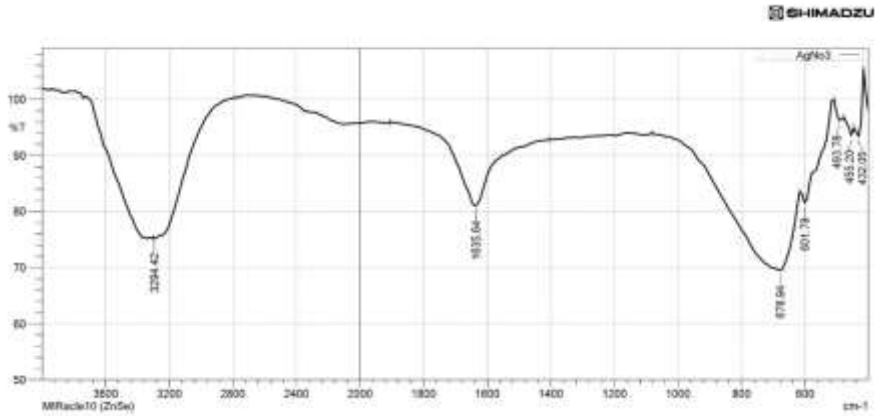

Figure 5: FT-IR spectra of *A. indica* methanolic extract silver nanoparticles.

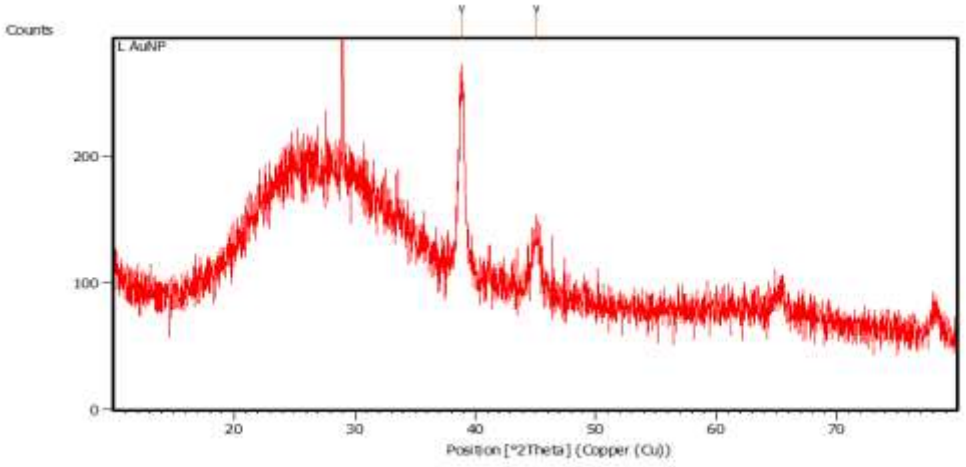

Figure 6: XRD spectra of *A. indica* methanolic extract silver nanoparticles.

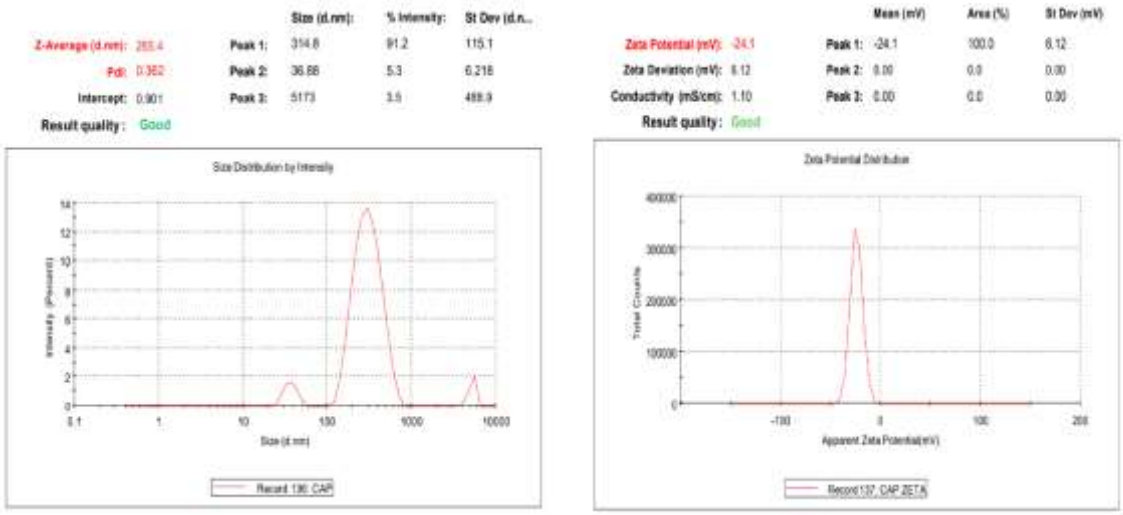

Figure 7: Zeta size and Zeta potential of *A. indica* silver nanoparticles. (A): *A. indica* methanolic extract silver nanoparticles Zeta size (265.4 d. nm). (B) *A. indica* methanolic extract silver nanoparticles Zeta potential -24.1 mV.

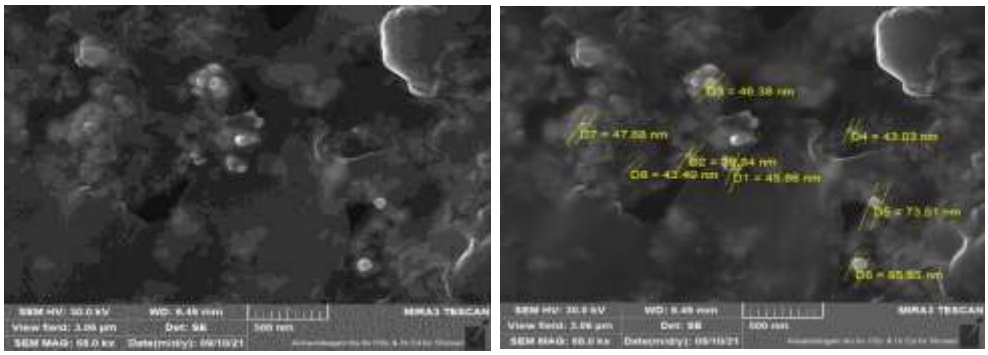

Figure 8A: FE-SEM image of *A. indica* methanolic extract silver nanoparticles.

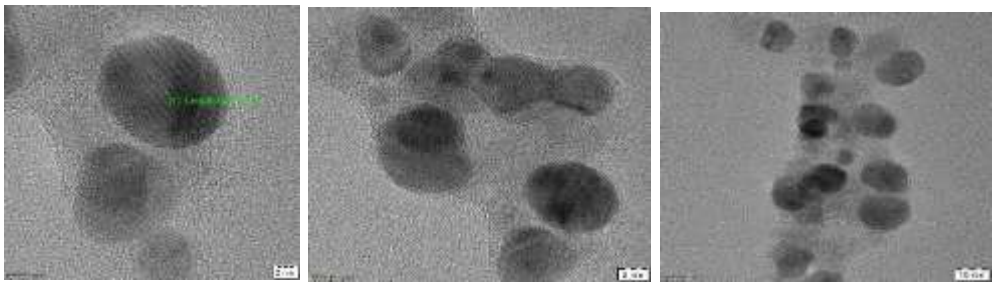

Figure 8B: HR-TEM observation *A. indica* methanolic extract silver nanoparticles at the scale of 2, 5 and 10 nm.

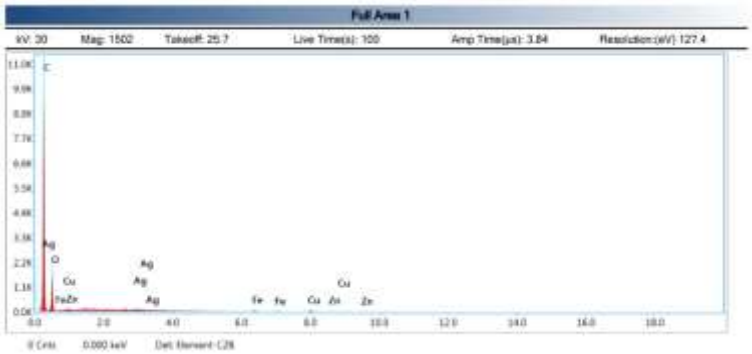

Figure 9: EDAX spectra and elemental composition of *A. indica* methanolic extract silver nanoparticles.

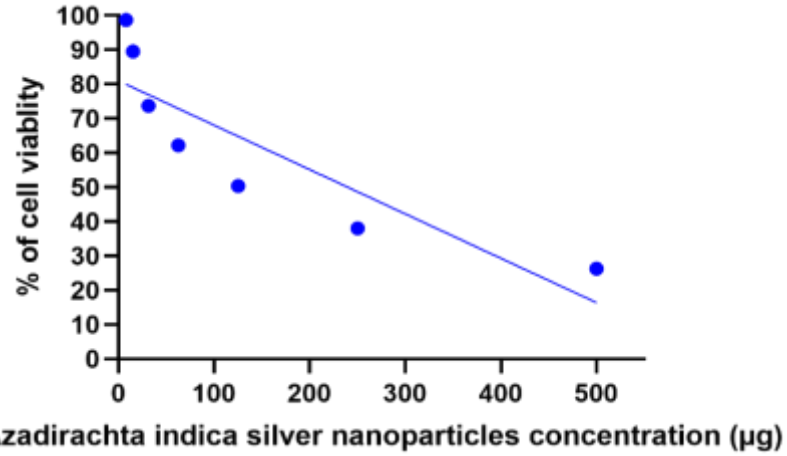

Figure 10: *A. indica* methanolic extract silver nanoparticles cytotoxicity on L6 cells.

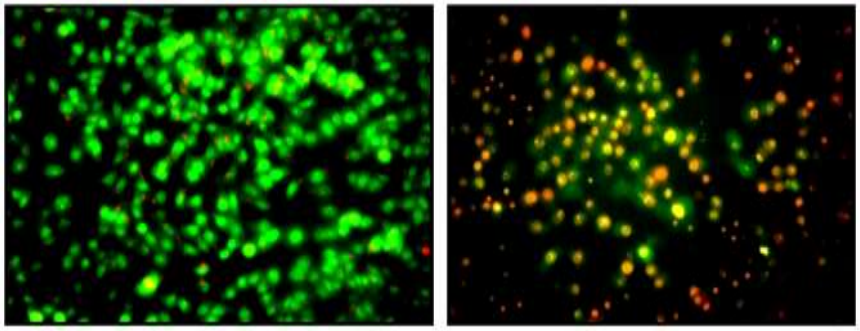

Figure 11: A: L6 control cells, B: *A. indica* methanolic extract silver nanoparticles treated L6 cells (24 hr).

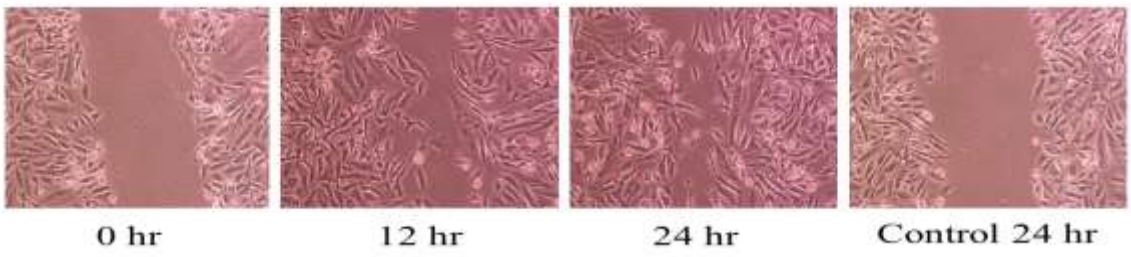

Figure 12: *A. indica* methanolic extract silver nanoparticles accelerated migration fibroblast in scratch assay. (A) Scratch assay of fibroblast treated with *A. indica* methanolic extract silver nanoparticles for 0, 12, and 24hr.

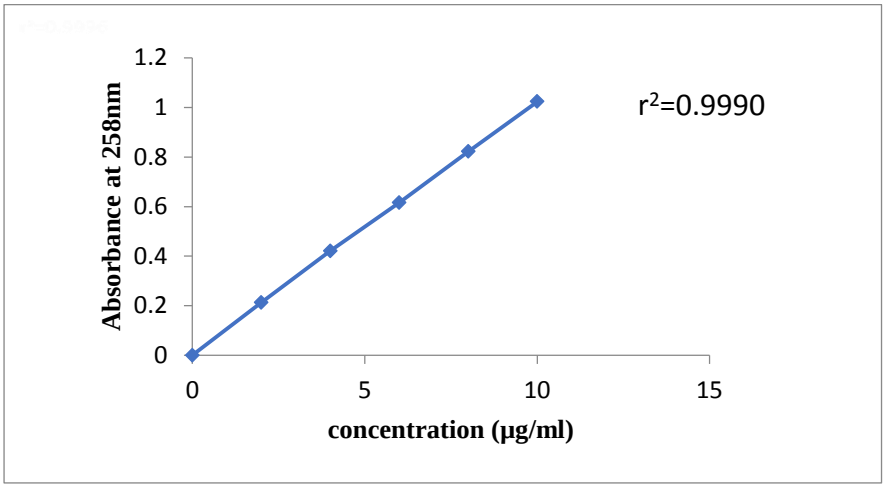

Figure 13: Standard curve of *A. indica* methanolic extract silver nanoparticles.

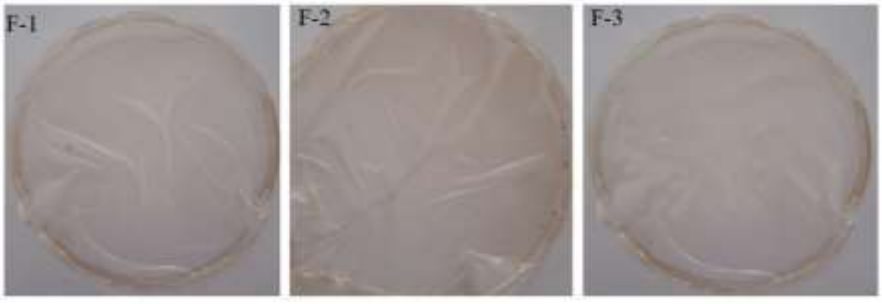

Figure 14. *A. indica* methanolic extract silver nanoparticles transdermal patches.

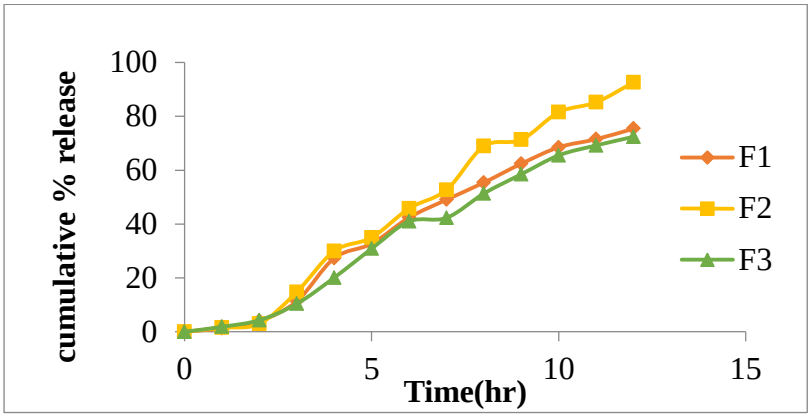

Figure 15: *In-vitro* drug release studies of *A. indica* silver nanoparticles.

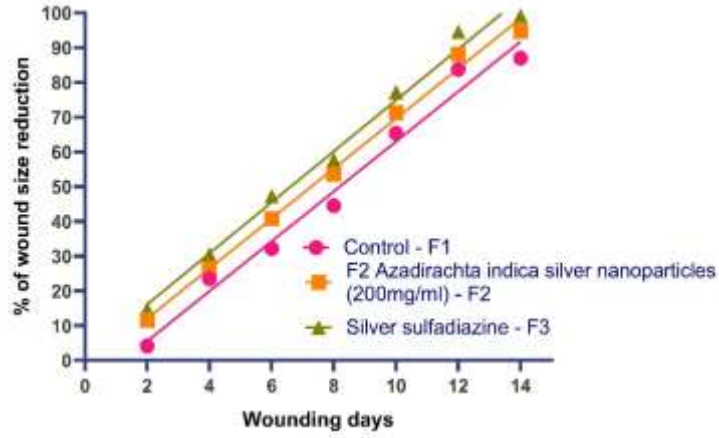

Figure 16: F1: Control, F2: Formulation F2 patch applied topically for 15 days. F3: Treated with silver sulfadiazine ointment 10 mg/g applied topically.

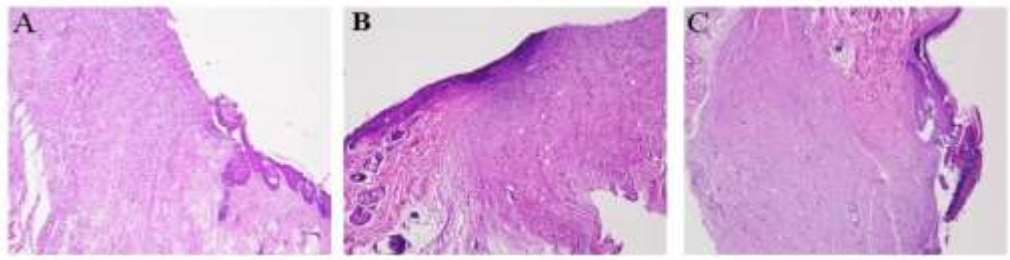

Figure 17: Microscopic observation (40X) of experimental rats wound histology that were given *A. indica nanoparticles*. A: Control, B Silver sulfadiazine and C: F2 patch treated.

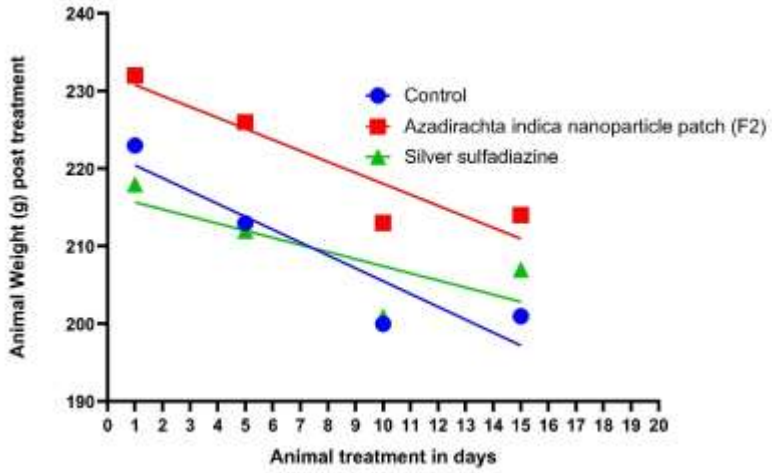

Figure 18: Effect of *A. indica* methanolic extract nanoparticles transdermal patches body weight of experimental animals.