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Evaluation of wound healing potential of chemical and phytogenic nanosilver

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Abstract
Green plants possess a variety of bioactive constituents like alkaloids, flavonoids, tannins, terpenoids and saponins which possess wound healing properties. The reducing properties of the bioactive constituents have been exploited in the synthesis of silver nanoparticles. The aim of present study was to evaluate the wound healing properties of *B. ovalifoliolata* ethanolic extract mediated silver nanoparticles (BENS) in comparison with 1% povidone iodine, citrate coated silver nanoparticles (AgNPs) and *B. ovalifoliolata* ethanolic extract (BE). The wound healing activity was evaluated in three wound models i.e., excision, incision and dead space wound model. The topical application of BENS showed better wound healing properties as evidenced by higher wound breaking strength, complete re-epithelialization with regrowth of hair. BENS promoted wound healing by improving angiogenesis, collagen production and fibroblast proliferation and by reducing oxidative stress. Further a significant increase in biochemical parameters viz, hydroxyproline, hexosamine, protein content and antioxidant parameters viz, catalase and vitamin C confirmed its wound healing potential. The present study showed that *B. ovalifoliolata* can be successfully used for the synthesis of nanoparticles and this phytogenic nanosilver could be a better option in wound management.

Keywords: Silver nanoparticles, wound healing, phytogenic nano silver, wound breaking strength

1. Introduction
Wound is defined as disruption of cellular, anatomical and functional continuity of a living tissue. Wounds are very common in veterinary practice which include incisions, abrasions, burns, bite wounds, avulsions, punctures, contusions, lacerations and shot wounds. At present, wound care and management involves a number of measures including dressing and administration of pain killers, use of anti-inflammatory agents, topical and systemic antimicrobial agents and healing promoting drugs [1]. In spite of tremendous advances in pharmaceutical industry, the availability of drugs capable of stimulating the process of wound repair is still limited [2]. Moreover, the management of chronic wounds is another major problem due to high cost of therapy and presence of unwanted side effects [3]. The rentless use of antibiotics has led to the emergence of antibiotic resistance strains of pathogens. For the last two decades, extensive work has been done to develop new drugs from natural products because of the resistance of the microorganisms to existing drugs [4].
The recent emergence of nanotechnology has provided a new therapeutic modality in silver nanoparticles for its use in wounds [5]. Silver nanoparticles possess many beneficial properties for wound healing like antibacterial [6, 7], antifungal [8], antiviral [9] and anti-inflammatory properties [10]. It was reported that silver ions and silver based compounds are toxic to microorganisms, possessing strong biocidal effects on at least 12 species of bacteria including multi-resistant bacteria like Methicillin resistant *Staphylococcus aureus* (MRSA), as well as multidrug resistant *Pseudomonas aeruginosa*, ampicillin resistant *E.coli* O157:H7 and erythromycin resistant *S.pyogenes* [11]. Traditional method of nanoparticle synthesis requires the use of harmful reductants such as sodium borohydride or hydrazine which produces larger amounts of heat and harmful byproducts during synthesis. Ecofriendly methods using bacteria, yeast, fungi, plants and plant extracts are becoming popular these days as they are nontoxic to environment [12]. In recent years, green synthesis of silver nanoparticles using plant extracts was exploited to a vast extent because the plants are widely distributed, easily available, safe to handle and with a range of metabolites [13].
Boswellia ovalifoliolata is medium sized deciduous tree, belongs to the family Burseraceae.

The bioactive constituents present in the stem bark of the plant are flavanoids, indoles, anthocyanins, steroids, phenols, saponins, tannins and lignans [14]. Further, antibacterial properties of *B. ovalifoliolata* [15], anti-inflammatory [16] and wound healing properties of *B. serrata* [17] were reported. Therefore, ethanolic extract of *B. ovalifoliolata* was used for the synthesis of silver nanoparticles i.e., phytogenic nanosilver. The present study is therefore an attempt to assess the efficacy of phytogenic nanosilver using different parameters of wound healing in rats.

2. Material and Methods

2.1 Materials

Silver nitrate (99.5%), sodium tricitrate (99%) and sodium borohydrate were from Sigma Aldrich. The bark of *Boswellia ovalifoliolata* was obtained from local market and identified and authenticated by Department of Botany, Sri Venkateswara University, Tirupati. Nutrient agar, nutrient broth, Roswell Park Memorial Institute (RPMI)-1640 medium and 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were from Himedia, India. All chemicals used were of analytical grade.

2.2 Preparation of ethanolic extract of *Boswellia ovalifoliolata*

About 100 g of stem-bark powder was soaked in 500 ml of 95% ethanol (v/v) with intermittent mixing using a glass rod for 72 hrs and then filtered using muslin cloth followed by whatman No. 1 filter paper [16]. The filtrate was evaporated at 55ºC in hot air oven. The dried extract obtained was used for synthesis of phytogenic nanosilver.

2.3 Synthesis of citrate coated nanosilver

Citrate mediated AgNPs were synthesized as per the method described [18]. Briefly 100 ml each of equimolar (1mM) concentrations of sodium citrate and silver nitrate were mixed in a conical flask and aged for about 2 hr. The aqueous solution was heated to 95˚C and 2-3 drops of 0.01 M sodium borohydrate was added to solution during heating. Finally the solution was cooled to room temperature. Gradual development of yellow colour indicates the formation of AgNPs. The prepared AgNPs were stored in a polyvinyl bottle for further studies.

2.4 Synthesis of *B. ovalifoliolata* ethanolic extract mediated nanosilver

About 100ml each of 10% ethanolic extract of *B. ovalifoliolata* and 1mM of silver ion solution were mixed and centrifuged at 18,000 rpm for 10 minutes. The supernatant was separated, heated to 95ºC until the colour of solution changes from pale to dark. The change in colour of the solution indicated the synthesis of *B. ovalifoliolata* ethanolic extract mediated AgNPs.

2.5 Characterization of nanoparticles

The reduction of pure Ag^+ ions was monitored by UV-Visible spectral analysis using UV-VIS spectrophotometer UV-2450 (Shimadzu). FT-IR (Fourier Transform Infrared Spectroscopy) analysis was carried out using TENSOR-27 (BRUCKER) in the diffuse reflectance mode operated at a resolution of 4 cm^{-1} in the range of 400 to 4000 cm^{-1} to evaluate the functional groups that might be involved in nanoparticle formation. SEM (Scanning Electron Microscope) analysis was done using Hitachi S-4500 SEM machine to study the morphology and size of synthesized nanoparticles. DLS (Dynamic Light Scattering) analysis was carried out using the instrument Nanopartica SZ-100 (HORIBA) to measure hydrodynamic radius or diameter, which is calculated using the Stokes-Einstein equation.

2.6 Agar Well Diffusion Assay

Agar well diffusion method [19] was suitably modified to test the anti-bacterial susceptibility of the compounds. Pure isolate of *S.aureus* was obtained and grown in nutrient broth at 37°c overnight. The resulting culture was subcultured in fresh nutrient broth for 3-6 hrs to obtain log phase culture. The concentration of log phase culture was adjusted to 0.5 Mc Farland standards (10^8 CFU/ml). Add 1ml of this culture to 100 ml of molten nutrient agar which was cooled to 45 ºC to obtain approximately 10^6 CFU/ml. This was poured onto sterile petri dishes. After solidification of agar, wells of 4 mm diameter were punched. The bottom of the well was sealed with 1 % agar. About 100 µl each of *B. ovalifoliolata* ethanolic extract, *B. ovalifoliolata* extract mediated nanosilver and citrate coated nanosilver were delivered in to wells separately. The nutrient agar plates were incubated at 37 ºC for 24 hrs. The diameters of zones of inhibition were measured in millimeters with vernier callipers. The test was repeated thrice to obtain average zone of inhibition.

2.7 Minimum inhibitory concentration

Tube dilution method was adopted to determine the MIC (Minimum Inhibitory Concentration) of the test compounds [20]. 10 sterile test tubes were taken**.** Briefly, one mL of the compound was serially diluted and five mL of sterile nutrient broth was added to all the test tubes. About 50 µL of 0.5 Mc Farland units log phase culture of *S. aureus* was added to all the tubes. The tubes were incubated for 18 hrs at 37 ºC and the lowest concentration of the test compound at which there was no visible growth was considered as MIC of the respective compound.

2.8 Cytotoxicity study (MTT Assay)

Cytotoxicity study was carried out in mouse spleenocytes [21]. Mouse spleenocytes (100µL of 1x 10^7cells /mL) were incubated with serially diluted test compound in RPMI-1640 medium in a microtiter plate for 16 h at 37 ºC in a 5 % CO_2 incubator.10 µl MTT was added to the wells 4 hours before the completion of incubation time. The plate was centrifuged at 1200 g for 10 min and the supernatant was discarded. 100 µl of dimethyl sulfoxide was added to dissolve the formazan formed. The absorbance was read at 530 nm after 10 min in an ELISA reader.

2.9 Experimental design

About 90 albino rats of both sexes (45 male and 45 female) were randomly divided into five groups. Group I served as control (0.9% normal saline), Group II as standard (1% povidone iodine solution), Group III as Citrate coated silver nanoparticles (AgNPs) (170 µg per dressing), Group IV as *B. ovalifoliolata* ethanolic extract (BE) (50 mg per dressing) and Group V as *B. ovalifoliolata* ethanolic extract mediated silver nanoparticles (BENS) (85 µg per dressing) (Phytogenic nanosilver). In each group the wound healing activity was evaluated in three different models viz., incision, excision and dead space wound model(n=6).The animals were housed in polypropylene cages and fed with standard pellet feed. Permission was obtained from the Institutional Animal Ethics

and Biosafety Committee before the start of the experiment.

2.10 Excision wound model

Rats were anaesthetized using ketamine (90 mg/kg.bwt, i/m) and xylazine (10 mg/kg.bwt, i/m). Excision wounds are inflicted on dorsal thoracic region 1-1.5 cm away from the vertebral column on either side and 5 cm away from the ear. After wound preparation with 70% alcohol, a circular skin piece was excised to its full thickness to obtain a wound area of about 500 mm^2 and 2 mm depth [22]. The respective treatments were topically applied once in a day till complete epithelialization of wound. The progressive reduction in wound area was monitored by tracing the raw wound on a sterile transparency sheet without causing any damage to the wound. The wound area recorded was then measured using a mm^2 graph paper on day 4, 8, 12, 16 and 20. The degree of wound healing was calculated as percentage wound contraction using the formula. The period of epithelialization was also calculated i.e., the number of days required for the falling of eschar without any residual raw wound [23].

Percentage wound contraction = 1-AD/AO x 100

Where AO= wound area on zero day; AD= wound area on corresponding days

2.11 Incision wound model

After wound preparation with 70%alcohol, two longitudinal paravertebral incisions of 6 cm length are made through the skin and cutaneous muscle at a distance of about 1.5 cm from the midline on either side of the vertebral column [24]. The parted skin was sutured with interrupted sutures using black braided silk thread (No. 000) and curved needle (No. 11). The respective test compound was taken on sterile surgical bandage and applied topically till 7th day. The sutures were removed on eighth post wounding day and skin breaking strength was measured on Day 10 as per the method described [25].

2.12 Dead space wound model

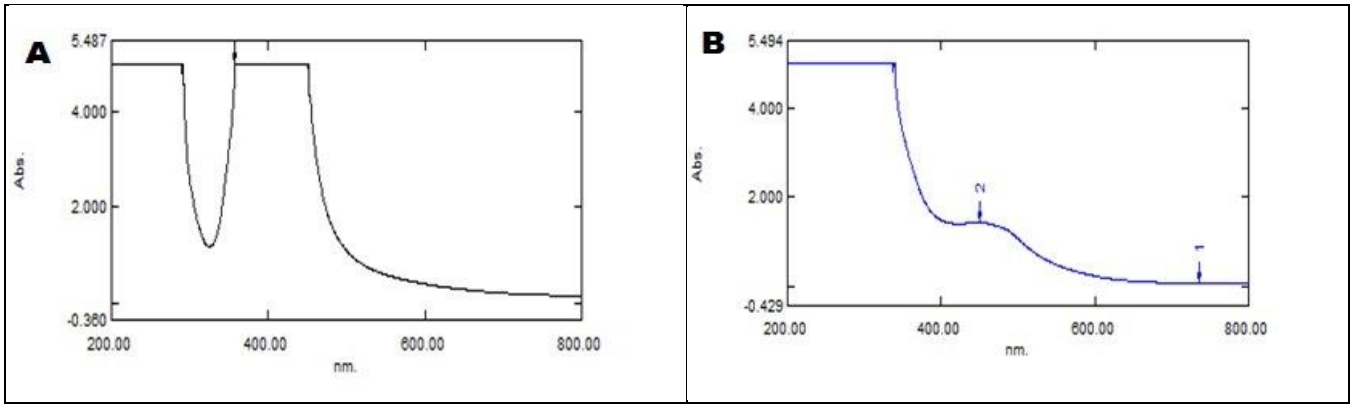
The predetermined area for wound infliction on the back was prepared by removing hair and swabbing with 70 % alcohol. The animals were anaesthesized and secured on operation table. A nick incision was given on either side of lumbar region and a subcutaneous pouch was created into which two sterile poly propylene tubes of size 2.5x0.5 cm^2 were inserted and the wound was sutured [26]. The respective treatments were applied topically as sterile surgical dressings on the wound. The dressings were changed daily for 10 daily. On the tenth post wound day, the granuloma surrounding tubes was collected and stored at -20°c for further analysis. The granulation tissue was used for the estimation of biochemical parameters like hydroxyproline [27], hexosamine [28], total protein [29] and antioxidant parameters like thiobarbituricacid reactive substances (TBARS) [30], superoxide dismutase (SOD) [31], catalase [32], reduced glutathione (GSH) [33] and ascorbic acid [34]. Granulation tissue was collected in 10% neutral buffered formalin for histological examination. Serial sections of granulation tissue were cut and stained with Hematoxylin and eosin (H & E) [35]. For better appreciation of collagen deposition, the tissues were stained with special stain i.e., Van Gieson [36].

2.13 Statistical analysis

The data for percent wound contraction measured on different days were analyzed using two way ANOVA with treatment and day as independent variables. The data for skin breaking strength, period of epithelialization, biochemical and antioxidant parameters measured at the end of the experiment were analyzed using one way ANOVA followed by *Tukey's posthoc* test using SPSS (17.0V) software.

3. Results

UV-Vis absorption spectra of chemical and phytogenic nanosilver showed absorption maxima between 350 to 450 nm and 450 nm (Fig 1 A, B), respectively. The results of FT-IR analysis indicated the involvement of hydroxyl and primary amine functional groups in the synthesis of chemical nanosilver and hydroxyl, carboxyl and primary amine functional groups in the synthesis of phytogenic nano silver (Fig 1C, D), respectively. SEM micrograph of chemical nanosilver showed an agglomerated morphology with size ranging from 374 nm to 718 nm whereas phytogenic nanosilver particles were spherical shape and monodispersive with size ranging from 192.8 nm to 346.1 nm (Fig 1E, F). The particle size as measured by the DLS technique of chemical nanosilver was 78.8 nm whereas that of phytogenic nanosilver was 273.5 nm.



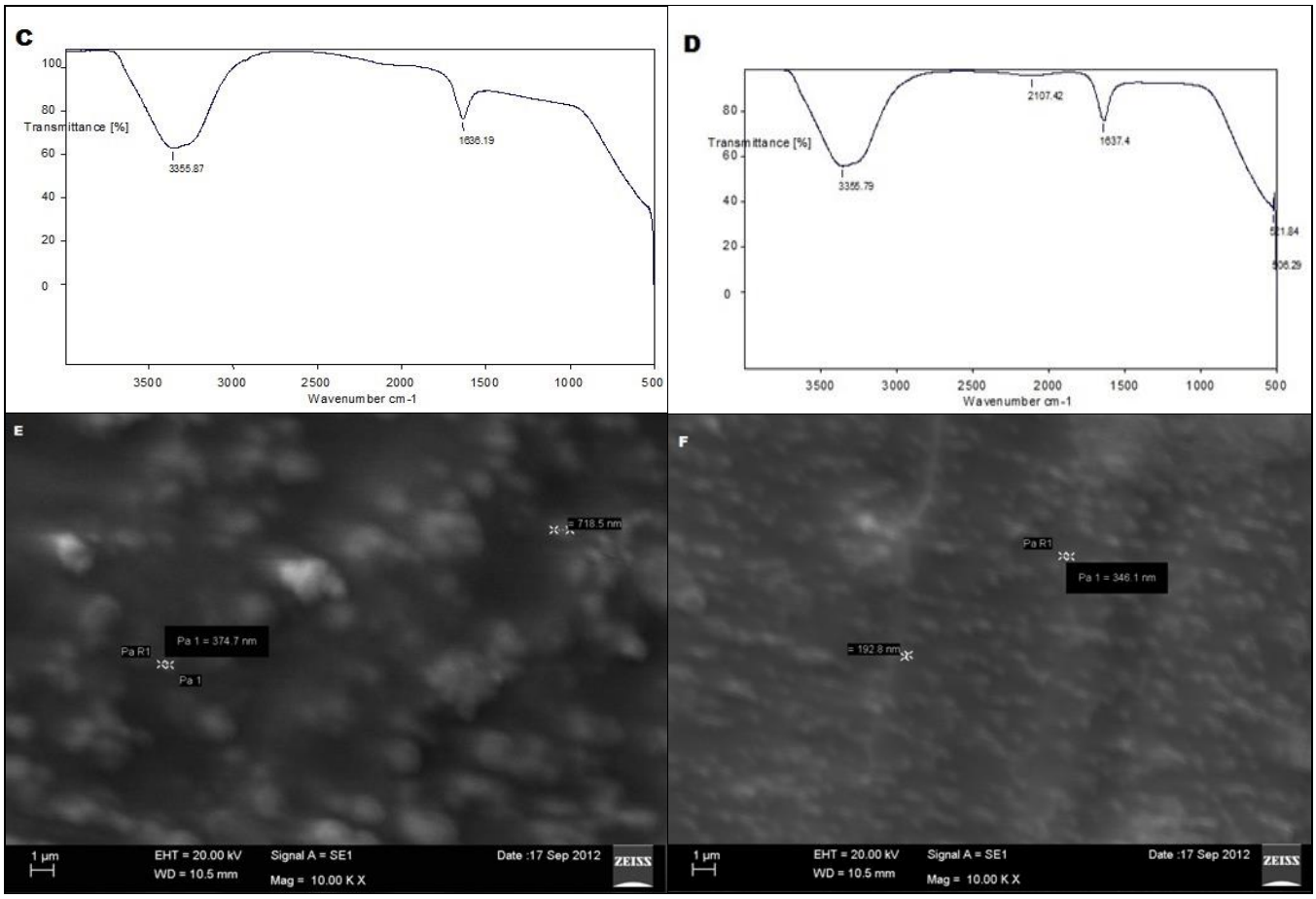


Fig 1: Characterization of Nano Particles **A.** UV Visible Spectrum of NS; **B.** UV Visible Spectrum of BENS; **C.** FT-IR Spectrum of NS; **D.** FT-IR Spectrum of BENS; **E.** SEM image of NS; **F.** SEM image of BENS; NS= Citrate coated nanosilver; BENS = *Boswellia ovalifoliolata* extract mediated nanosilver.

The results of *in vitro* studies indicated that phytogenic nanosilver showed better anti-bacterial properties against *Staphylococcus aureus* both in agar gel diffusion assay and tube dilution test as evidenced by the significantly (p<0.05) higher zone of inhibition (17.67±0.33mm) and lowest MIC value (1.77 µg/ml) when compared with both NS (10.33±0.33mm; 28.33µg/ml) and BE (15.00±0.58mm; 4.16mg/ml) (Table 1). Cytotoxicity studies revealed that phytogenic nanosilver showed more than 90 % viability at the doses studied i.e., 2-85 ppm while nanosilver produced 76.8% of cell viability at a concentration of 85 ppm. (Fig 2).

Table 1: Antibacterial activity of nanoparticles and Boswellia extract against *Staphylococcus aureus*

Group	Agar gel diffusion (Zone of inhibition, mm/100µL)	Tube dilution method (MIC)
NS	10.33 ± 0.33[a]	28.03 µg/mL
BE	15.00 ± 0.58[b]	4.16 mg/mL
BENS	17.67 ±0.33[c]	1.77 µg/mL
Df	(2, 8)	
F-Value	74.400	
Sig	0.000	

Values are Mean ± S.E. One way ANOVA followed by Tukey's *post hoc* using SPSS V.17.0 software. Means with different superscripts are significantly (p< 0.05) differ.

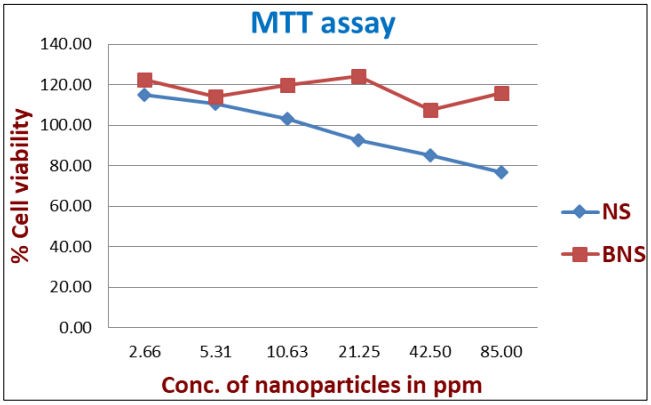


Fig 2: Cytotoxicity of nanoparticles

In excision wound model, phytogenic nanosilver showed significantly higher (p<0.05) percent of wound contraction (98%) when compared to control. There was a time dependent increase in percent wound contraction in BENS group from 51% to 98% from day 8 to day 16 as shown in (Table 2). The overall effect of BENS was significantly higher (p<0.05) when compared to BE. However the effect of povidone and NS are comparable. Further, complete re-growth of hair and normal epithelium was restored in the mentioned period in phytogenic nanosilver group (Fig 3). Histological section of

phytogenic nanosilver showed mild cellular infiltration with increased number of matured angioblasts and fibroblasts with increased amount of thick collagen deposition as compared to control with severe inflammatory cell infiltration in dermis with less number of angioblasts, fibroblasts and thin collagen deposition (Fig 4).

Table 2: Effect of nanoparticles on percentage wound contraction (%) in excision wound model

Group	4 day	8 day	12 day	16 day	20 day	Marginal means
Control	5.73 ± 0.64	25.30 ± 0.64	47.80 ± 0.64	75.37 ± 0.64	93.37 ± 0.64	49.51 ± 0.28[a]
Povidone	12.40 ± 0.64	42.80 ± 0.64	67.67 ± 0.64	86.83 ± 064	96.10 ± 0.64	61.16 ± 0.28[b]
AgNPs	13.10 ± 0.64	43.43 ± 0.64	67.60 ± 0.64	86.83 ± 0.64	96.17 ± 0.64	61.48 ± 0.28[b]
BE	15.03 ± 0.64	44.60 ± 0.64	70.36 ± 0.64	88.03 ± 0.64	95.87 ± 0.64	62.78 ± 0.28[c]
BENS	24.40 ± 0.64	51.17 ± 0.64	77.17 ± 0.64	95.16 ± 0.64	98.60 ± 0.64	69.30 ± 0.28[d]
Day marginal means	14.13 ± 0.28[a]	41.46 ± 0.28[b]	66.12 ± 0.28[c]	86.45 ±0.28[d]	96.02 ±0.28[e]	
	df	**F-value**	**Sig**	**Partial Eta Squared**		
day	(4, 125)	13824.22	0.000	0.998		
group	(4, 125)	629.97	0.000	0.953		
Day*group	(16,125)	35.040	0.000	0.818		

Values are Mean ± S.E. One way ANOVA followed by Tukey's *post hoc* using SPSS V.17.0 software. Means with different superscripts are significantly ($p < 0.05$) differ.

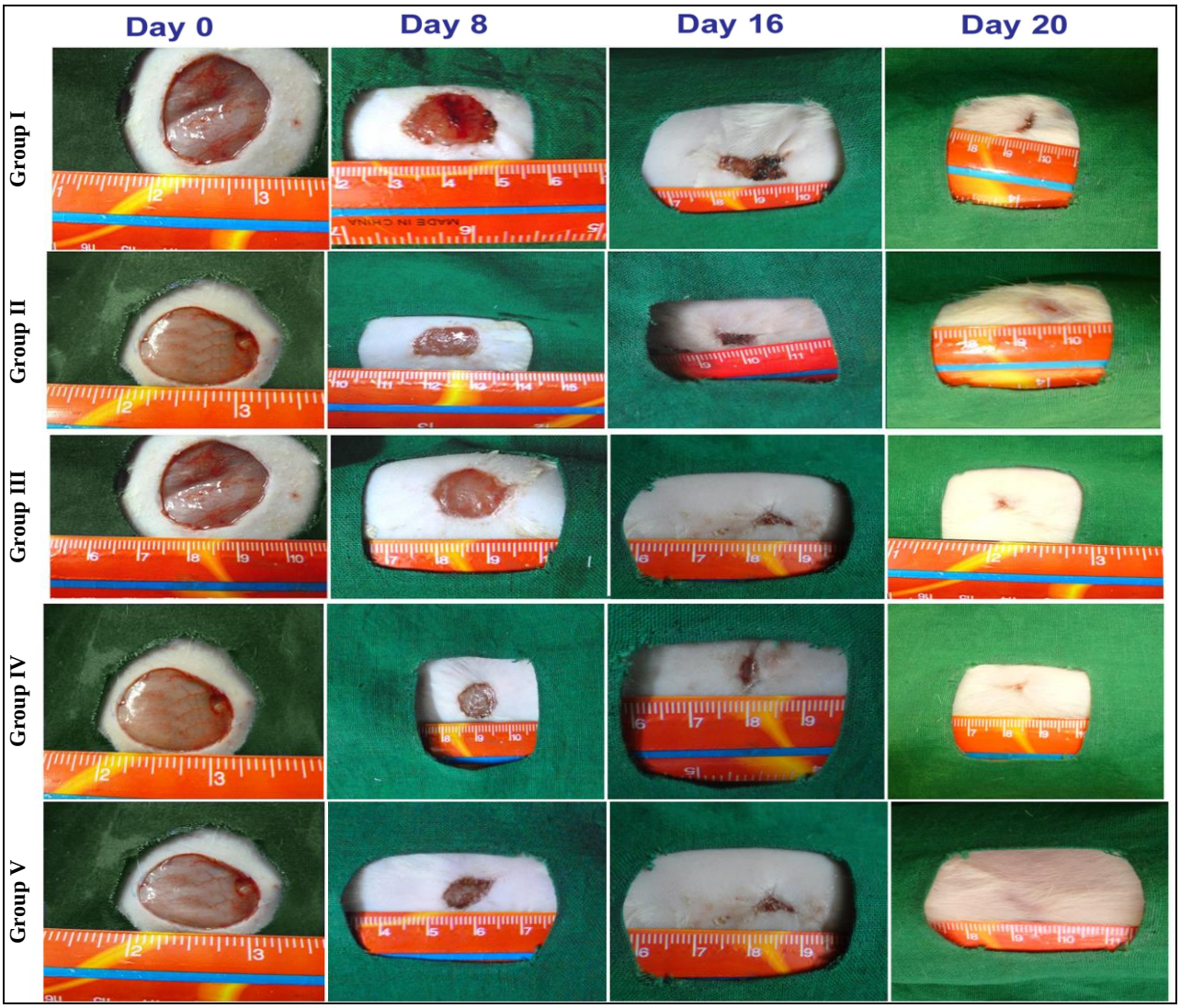


Fig 3: Wound contraction and epithelialization in excision wound model in different treatment groups.

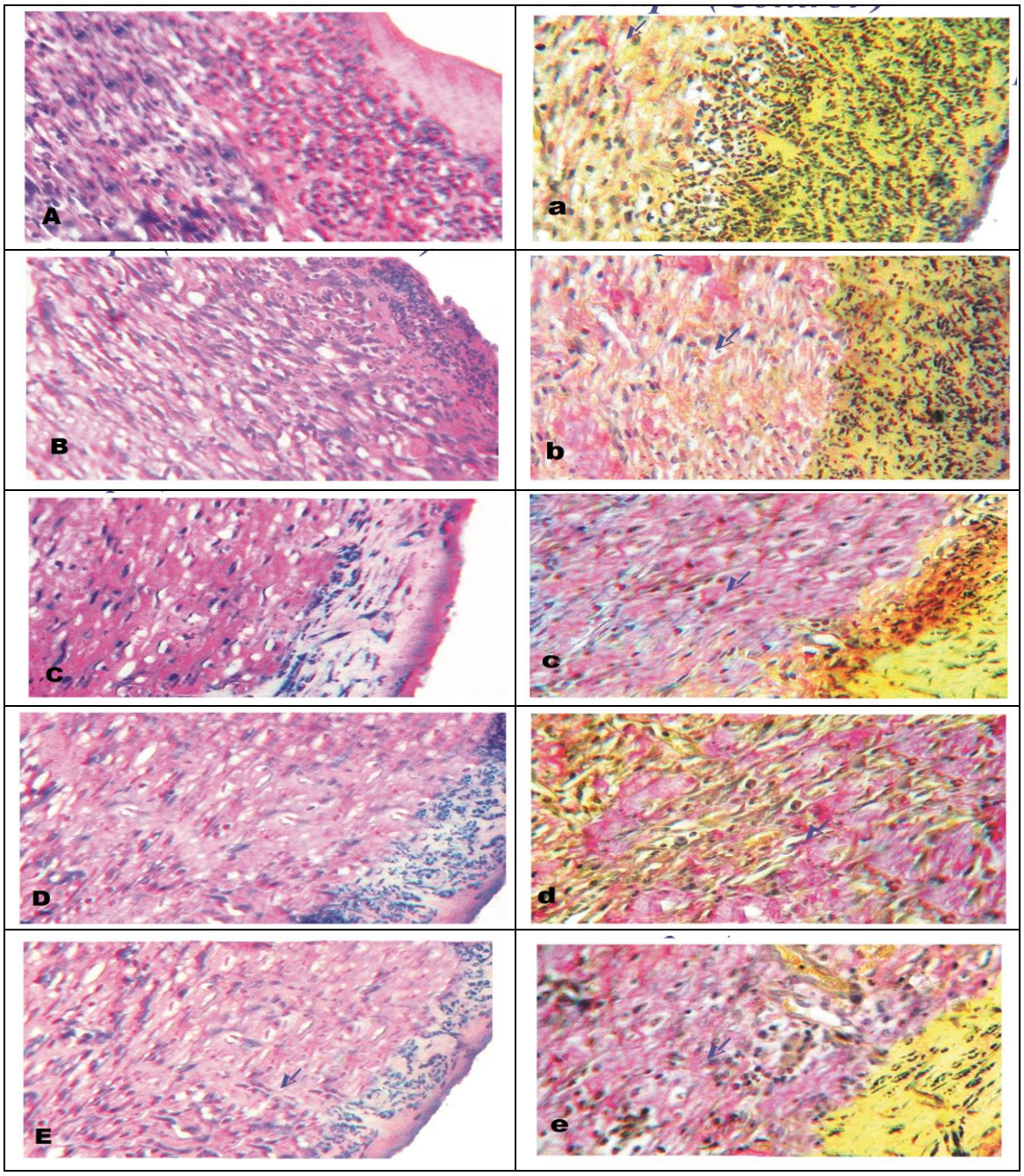


Fig 4: Histopathology of granulation tissue of various groups. **A-E:** Groups I to V Hematoxylin & Eosin (x280); **A:** Increased inflammatory cell infiltration with less number of fibroblasts and capillaries; **B:** Mild to moderate inflammatory cell infiltration with mild to moderate no. of fibroblasts and capillaries; **C:** Mild inflammatory cell infiltration with mild to moderate no. of fibroblasts and angioblasts; **D:** Moderate inflammatory cell infiltration with moderate no. of fibroblasts and few capillaries; **E:** Less inflammatory cell infiltration with increased no. of matured fibroblasts and capillaries**. a-e:** Groups I to V Vangieson stain (x280); **a:** Infiltration of cells and thin collagen deposit; **b:** Mild to moderate infiltration of cells and moderate amount of thick collagen; **c:** Mild to moderate amount of thick collagen deposit; **d:** Moderate to increased thick collagen deposit; **e:** Increased amount of thick collagen deposit.

In incision wound model, significantly higher (p<0.05) wound breaking strength was observed in BE and BENS when compared to control. However the period of epithelialization was significantly lower (p<0.05) in BENS (19.67 days) when compared to other groups with no significant difference between groups (Table 3).

Table 3: Effect of nanoparticles on wound breaking strength (g) and period of epithelialization (days)

Group	Wound breaking strength (g)	Period of epithelialization (days)
Control	386.83 ± 26.52^{a}	25.67 ± 0.37^{c}
Povidone	547.17 ± 26.24^{b}	22.00 ± 0.26^{b}
NS	525.83 ± 24.50^{ab}	23.00 ± 0.37^{b}
BE	699.50 ± 56.33^{c}	22.70 ± 0.21^{b}
BENS	717.50 ± 33.86^{c}	19.67 ± 0.33^{a}
df	(4, 29)	(4, 29)
F-Value	14.735	49.643

Sig	0.000	0.000

Values are Mean ± S.E. One way ANOVA followed by Tukey's *post hoc* using SPSS V.17.0 software. Means with different superscripts are significantly ($p< 0.05$) differ.

In dead space wound model, the biochemical profile of granulation tissue showed that all the treatment groups significantly ($p<0.05$) improved hydroxyproline, hexosamine and protein content when compared to control (Table 4). The value of hydroxyproline was significantly ($p<0.05$) higher in BENS group whereas the hexosamine content was significantly ($p<0.05$) increased by both NS and BENS group. The protein was significantly ($p<0.05$) increased by povidone, BE and BENS group.

Table 4: Effect of nanoparticles on biochemical parameters of granulation tissue in dead space wound model

Group	Hydroxyproline (mg/g tissue)	Hexosamine (µg/g tissue)	Total protein (g/g tissue)
Control	15.30 ± 0.77^{a}	15.30 ± 0.77^{a}	0.053 ± 0.003^{a}
Povidone	22.71 ± 0.79^{bc}	22.71 ± 0.79^{bc}	0.079 ± 0.002^{bc}
NS	21.44 ± 0.52^{b}	21.44 ± 0.52^{b}	0.069 ± 0.004^{b}
BE	25.10 ± 0.89^{c}	25.10 ± 0.89^{c}	0.081 ± 0.002^{c}
BENS	28.91 ± 1.06^{d}	28.91 ± 1.06^{d}	0.089 ± 0.02^{c}
df	(4, 29)	(4, 29)	(4, 29)
F-Value	36.877	36.877	23.811
Sig	0.000	0.000	0.000

Values are Mean ± S.E. One way ANOVA followed by Tukey's *post hoc* using SPSS V.17.0 software. Means with different superscripts are significantly ($p< 0.05$) differ.

The antioxidant profile of granulomatous tissue showed that all the NS, BE and BENS group significantly ($p<0.05$) lowered TBARS when compared to control (Table 5). The catalase activity was significantly ($p<0.05$) higher in BENS group as compared to povidone and NS groups. Vitamin C levels was significantly ($p<0.05$) higher in BENS group as compared to control. However, the treatments had no significant influence on the activity of SOD and GSH content.

Table 5: Effect of nanoparticles on antioxidant profile of granulation tissue in dead space wound model

Group	TBARS (mg MDA/g tissue)	Catalase (U/ mg protein)	SOD (U/ mg protein)	GSH (mg/g tissue)	Vitamin C (µg/g tissue)
Control	5.36 ± 0.36^{b}	0.030 ± 0.006^{a}	130.28 ± 48.47^{a}	2.62 ± 1.00^{a}	4.15 ± 0.99^{a}
Povidone	4.70 ± 0.73^{b}	0.059 ± 0.001^{ab}	147.79 ± 15.45^{a}	1.11 ± 0.35^{a}	5.73 ± 1.48^{ab}
NS	2.44 ± 0.48^{a}	0.054 ± 0.014^{ab}	151.78 ± 8.94^{a}	3.00 ± 0.69^{a}	11.46 ± 1.38^{ab}
BE	2.08 ± 0.19^{a}	0.070 ± 0.008^{bc}	139.02 ± 16.93^{a}	1.52 ± 0.19^{a}	10.90 ± 2.60^{ab}
BENS	0.73 ± 0.05^{a}	0.101 ± 0.05^{c}	166.71 ± 21.04^{a}	3.26 ± 1.01^{a}	15.00 ± 3.64^{b}
df	(4,29)	(4, 29)	(4, 29)	(4, 29)	(4, 29)
F-Value	19.844	7.672	0.278	1.653	3.934
Sig	0.000	0.000	0.890	0.192	0.013

Values are Mean ± S.E. One way ANOVA followed by Tukey's *post hoc* using SPSS V.17.0 software. Means with different superscripts are significantly ($p< 0.05$) differ.

5. Discussion

The clinical application of nanotechnology is the generation of silver nanoparticles which possess many beneficial properties for wound management including antibacterial, antifungal and anti-inflammatory properties [5]. Chemical reduction method is the most frequently employed method for the synthesis of silver nanoparticles. The disadvantages of this method such as use of harmful chemicals and aggregation of particles upon storage [37] has led to the use of biological methods for nanoparticle synthesis. Biological method involves synthesis at biological p^{H} and offer better manipulation and control over crystal growth and stabilization [38]. In this research, we synthesized *Boswellia ovalifoliolata* mediated phytogenic nanosilver and evaluated its wound healing activity.

A colour change of the solution from colourless to light yellow and eventually to yellow brown indicated the synthesis of nanoparticles. This colour change is due to surface plasmon resonance in silver nanoparticles [39]. The synthesis was further confirmed by UV Visible spectroscopy, SEM images, FT-IR spectrum and DLS technique. In UV Visible spectroscopy, sharp observation peak was observed around 380 to 450 nm for silver nanoparticles and around 450 for phytogenic nanosilver, which is the characteristic of silver nanoparticles [40]. FT-IR studies reported the involvement of hydroxyl, carboxyl and primary amine groups indicating flavonoids present in stem bark might have played an important role in the synthesis of *B. ovalifoliolata* mediated nanosilver.

Wound becomes infected as injured skin remains vulnerable to microbes of all kinds with subsequent sepsis. Therefore topical antimicrobial therapy is important in wound care and management. In this study both AgNPs and BENS possess potent antibacterial activity. The antibacterial property might be due to the bactericidal properties of silver nanoparticles [41]. A number of possible mechanisms for antibacterial actions of nanosilver have been proposed like alteration of permeability of cell membrane [42], release of lipopolysaccharides and membrane proteins [43], generation of free radicals responsible for the damage of membrane [44], dissipation of the proton motive force resulting in the collapse of membrane potential [45]. Phytogenic nanosilver was safer compared to chemical nanosilver in cytotoxicity assay on mouse spleenocytes. The cytotoxic effects of chemical mediated nanosilver was reported in HepG2 cell line and mice liver primary cell culture with IC_{50} value of 2.76 ppm and 121.7 ppm respectively [46]. The safety of phytochemically synthesized nanosilver using *C.verum* and aloin over chemical

mediated nanosilver was reported earlier [47, 48].

In wound healing, epithelialisation, contraction and connective tissue/matrix deposition are the various mechanisms involved. The matrix consists of collagen, elastin, fibronectin, laminin, hyaluronic acid and proteoglycans. These structures give strength and support, allow expansion and contraction, provide a surface for cell movement and help necessary chemical reactions to occur [49]. Skin breaking strength gives an indication of tensile strength of wound tissues and represents the degree of wound healing. The tensile strength of the wound is determined by the synthesis and maturation of collagen, where there is a covalent binding of collagen fibrils through inter and intra molecular crosslinking [50]. The results of the present study showed that the phytogenic nanosilver possess definite wound healing action. This is demonstrated by significant *($p<0.05$)* increase in rate of wound contraction, skin breaking strength and by enhanced epithelialisation. This enhanced epithelialisation might be due to increased collagen synthesis under the influence of flavonoids present in the *B. ovalifoliolata* mediated nanosilver. Flavanoids are known to promote wound healing process mainly due to their astringent and antimicrobial property, which is responsible for wound contraction and increased rate of epithelialization [51]. The increased tensile strength in phytogenic nanosilver may be due to an increase in collagen concentration and stabilisation of fibres and also due to its proper deposition and alignment facilitating wound healing. This was further supported by the presence of matured fibroblasts in the histological examination of granulation tissue and increased hydroxyproline, hexosamine content in the granulation tissue.

Hydroxyproline, an important aminoacid in collagen, is used as an index of collagen turn over [52]. Collagen is the predominant extracellular protein in the granulation tissue of healing wound. Hexosamine content increases in the early stages of wound healing and indicated that the fibroblasts actively synthesized, ground substances (mucopolysaccharides) on which the collagen can be laid on [53]. Increased hexosamine content reflects the stabilisation of collagen molecules by enhancing electrostatic and ionic interactions with it, which in turn reflects the remodelling of the new extracellular matrix produced [54]. The increase in hydroxyproline and hexosamine content increased collagen formation in phytogenic nanosilver and was further supported by the presence of matured fibroblasts in the histological examination of granulation tissue.

Free radicals and other reactive oxygen species (ROS) can induce severe tissue damage and are particularly encountered during connective tissue disorders like fibrosis as well as during wound healing [55]. Overproduction of ROS results in oxidative stress thereby causing cytotoxicity and delayed wound healing [56]. Therefore, elimination of ROS could be an important strategy in healing of chronic wounds. Hence, estimation of antioxidants like catalase, SOD and glutathione in granulation tissue is relevant because these antioxidants hasten the process of wound healing by destroying the free radicals. The studies on antioxidant parameters in granulation tissue of phytogenic nanosilver treated wounds revealed a significant increase in catalase activity and a significant decrease in MDA levels, which would have helped to prevent oxidative damage and promoted wound healing process. Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving cell vascularity [57]. The antioxidant effect could be attributed to its constituent flavonoid (-OH) mediated nanosilver in phytogenic nanosilver group which could have inhibited lipid peroxidation and increased the strength and viability of collagen fibrils by improving circulation and preventing the cell damage [58]. This was evidenced in histopathological examination of granulation tissue which showed an increased number of mature angioblasts and fibroblasts.

6. Conclusion

The better wound healing properties of *B. ovalifoliolata* mediated nanosilver might be due to its additive effect of antimicrobial, antioxidant and anti-inflammatory properties of phytoconstituents and silver nanoparticles. Further, small size of nanoparticles might have facilitated increased availability and penetration at wound site.

7. References

1. Rupesh T, Nikita J, Raghavendra P, Sandhu SS. Practices in wound healing studies of plants. Evidence-based Complementary and Alternative Medicine, 2011. Article Id: 438056, epublished. www.ecam.org.uk.
2. Udupa AL, Kulkarni DR, Udupa SL. Effect of *Tridax procumbens* extracts on wound healing. Interntional Journal of Pharmacognosy. 1995; 33(1):37-40.
3. Suh DD, Schwartz IP, Canning DA, Snyder HM, Zderic SA, Kirsch AJ. Comparison of dermal and epithelial approaches to laser tissue soldering for skin flap closure. Lasers Surgery Medicine. 1998; 22(5):268-274.
4. Thirumurgan A, Shaheedha SM, Dhanaraju MD. *In vitro* evaluation of antibacterial activity of silver nanoparticles synthesized by *Phytophthora infestans*. International Journal of Chemical Technology Research. 2009; 1(3):714-716.
5. Tian J, Wong KK, Ho CM, Lok CN, Yu WY, Che CM *et al*. Topical delivery of silver nanoparticles promotes wound healing. Chem Med Chem. 2007; 2(1):129-136.
6. Baker C, Pradhan A, Pakstis L, Darrin J, Shah S. Synthesis and antibacterial properties of silver nanoparticles. Journal of Nanoscience and Nanotechnology. 2005; 5(2):244-249.
7. Percival SL, Bowler PG, Dolman J. Antimicrobial activity of silver-containing dressings on wound microorganisms using an *in vitro* biofilm model. International Wound Journal. 2007; 4(2):186-191.
8. Wright JB, Lam K, Hansen D, Burrell D. Efficacy of topical silver against fungal burn wound pathogens. American Journal of Infectious Control. 1999; 27(4):344-350.
9. Sun RW, Chen R, Chung NP, Ho CM, Lin CL, Che CM. Silver nanoparticles fabricated in HEPES buffer exhibit cytoprotective activities towards HIV-1 infected cells. Chemical Communications. 2005; 40(40):5059-5061.
10. Nadworny PL, Wang J, Tredget EE, Burrell RE. Anti-inflammatory activity of nanocrystalline silver in a porcine contact dermatitis model. Nanomedicine. 2010; 4(3):241-251.
11. Shahverdi AR, Fakhimi R, Shahverdi HR, Minaian S. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. Nanomedicine. 2007; 3(2):168-171.
12. Protima Rauwel, Siim Kuunal, Stanislav Ferdov, Erwan Rauwel. A review on the green synthesis of silver

nanoparticles and their morphologies studied via TEM. Advances in Materials Science and Engineering, 2015. Article ID 682749, 9 pages. http://dx.doi.org/10.1155/2015/682749.
13. Kulkarni AP, Srivastava AA, Nagalgaon RK, Zunjarrao RS. Phytofabrication of silver nanoparticles from a novel plant source and its application. International Journal of Biological & Pharmaceutical Research. 2012; 3(3):417-421.
14. Savithramma N, Venkateswaralu P, Suhrulatha D, Basha SKM, Venkata Ramana Devi CH. Studies on *Boswellia ovalifoliolata* Bal and Henry- An endemic and endangered medical plant. The Bioscan. 2010; 5(3):359-362.
15. Niranjan Reddy VL, Ravinder K, Srinivasulu L, Venkateshwar Goud T, Malla Reddy S, Srujan Kumar D. Two macrocyclic diaryl ether heptanoids from *Boswellia ovalifoliolata*. Chemical Pharmaceutical Bulletin. 2003; 51(9):1081-1084.
16. Sakunthala Devi PR, Adilaxmamma K, Srinivasa Rao G, Srilatha Ch, Alpha Raj M. Evaluation of anti-inflammatory activity of stem-bark of Boswellia ovalifoliata in rats. Inventi Rapid; Ethnopharmacology. 2010; 1: ep159. Available from: http://www.inventi.in.[Google Scholar].
17. Arunbha M, Damodhar G, Hemant D, Satish N. Evaluation of *Boswellia serrata* oleo-gum resin for wound healing activity. Der Pharmacia Lettre. 2010; 2(2):457-463.
18. Supraja N, Prasad TVNKV, Krishna GT, David E. Effect of citrate coated silver nanoparticles on biofilm degradation in drinking water PVC pipelines. Advances in Nano Research. 2015; 3(2):97-109.
19. Oboh IE, Akerele J, Obasuyi O. Antimicrobial activity of the ethanol extract of the aerial parts of the *Sida acuta* burm. f. (Malvaceae). Tropical Journal of Pharmaceutical Research. 2007; 6(4):809-813.
20. Geert Huys. SOP (Standard Operating Procedures). Antibiotic susceptibility testing of aquaculture-associated bacteria with the broth macrodilution method (MIC determination). Labaratory of Microbiology, Gent, Belgium, 2002.
21. Mossman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983; 65(1-2):55-63.
22. Mortan JJ, Malone MH. Evaluation of vulnerary activity by an open wound procedure in rats. Archives of International Pharmacodynamics and Therapeutics. 1972; 196(1):117-126.
23. Werner S, Breededen M, Hubner J, Greenhalgh DG, Longaker MT. Introduction of keratinocyte growth factor expression is reduced and delayed during wound healing in the genetically diabetic mouse. Journal of Investigative Dermatology. 1994; 103(4):469-473.
24. Ehrlich HP, Hunt TK. The effects of cortisone and anabolic steroids on the tensile strength of healing wounds. Annals of Surgery. 1969; 170(2):203-206.
25. Lee KH. Studies on mechanism of action of salicylates II. Retardation of wound healing by aspirin. Journal of Pharmaceutical Sciences. 1968; 57(6):1042-1043.
26. Turner RA. Screening methods of pharmacology.1, 2, Academic Press, New York, 1965, 122.
27. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Archives of Biochemistry and Biophysics. 1961; 93(2):440-447.
28. Elson LA, Morgon WTJ. A colorimetric method for the determination of glucosamine and chondrosamine. Biochemical Journal. 1933; 27(6):1824-1828.
29. Gronall AG, Bardawill CJ, David MM. Determination of serum proteins by biuret reaction. Journal of Biological Chemistry. 1949; 177(2):751-756.
30. Subramanian KA, Manohar M, Mathan VI. An unidentified inhibitor of lipid peroxidation in intestinal mucosa. Biochemica et Biophysica Acta. 1988; 962(1):51-58.
31. Mirsa HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. The Journal of Biological Chemistry. 1972; 247(10):3170-3175.
32. Beer RF, Sizer RW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. The Journal of Biological Chemistry. 1952; 195(1):133-140.
33. Moron MS, Depierre JW, Mennervik B. Levels of glutathione, glutathione reductase and glutathione-S-transferase in rat lung and liver. Acta. Biochemistry and Biophysics. 1979; 582(1):67-78.
34. Omayer ST. Selected methods for determination of ascorbic acid in animal tissues and fluids. Methods in Enzymology. 1979; 62:3-8.
35. Singh UB, Sulochana S. A practical manual of Histological and Histochemical techniques. Kothari Publications, Bombay, 1997, 1-41.
36. Mallory FB. Pathological techniques. WB Saunders Co, Philadelphia, 1942, 152.
37. Mukherjee P, Roy M, Mandal BP, Dey GK, Mukherjee PK and Ghatak J. Green synthesis of highly stabilized nanocrystalline silver particles by a non pathogenic and agriculturally important fungus T.asperellum. Nanotechnology. 2008; 19(7):075103. http://dx.doi.org/10.1088/0957-4484/19/7/075103
38. Elumalai EK, Prasad TNVK, Venkata K, Nagajyothi PC, David E. Green synthesis of silver nanoparticles using *Euphorbia hirta* Land their antifungal activities. Archives of Applied Science Research. 2010; 2(6):76-81.
39. Pallavi Singh C, Vikas S, Prasad GBKS, Rajesh Singh T. Effect of silver nanoparticle-mediated wound therapy on biochemical, hematological and histological parameters. Asian Journal of Pharmaceutical and Clinical Research. 2018; 11(3):251-258.
40. Prusty AK, Prassana P. Green synthesis of silver nanoparticles using *Eichhornia crassipes* and study of in-vitro antimicrobial activity. Scholars Academic Journal of Pharmacy. 2014; 3(6):504-509.
41. Maribel G, Jean D, Stephane G. Synthesis and antibacterial activity of silver nanoparticles against gram positive and gram negative bacteria. Nanomedicine: Nanotechnology, Biology and Medicine. 2012; 8(1):37-45.
42. Sondi I, Sondi BS. Silver nanoparticles as antimicrobial agent; a case study on E.coli as a model or gram negative bacteria. Journal of Colloid and Interface Science. 2004; 275(1):177-182.
43. Amro NA, Kotra LP, Wadu-Mesthrige K, Bulchevy A, Mobashery S, Liu GY. High resolution atomic force microscopy studies of the *Escherchia coli* outer

membrane: The structural basis of permeability. Langmuir. 2000; 16(6):2789-2796.
44. Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ *et al*. Antimicrobial effects of silver nanoparticles. Nanomedicine: Nanotechnology, Biology and Medicine. 2007; 3(1):95-101.
45. Chun-Num L, Ho CM, Chen R, He QY, Yu WY, Sun HP *et al*. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. Journal Proteome Research. 2006; 5(4):916-924.
46. Faedmaleki F, Shirazi FH, Rastegari H, Salarian A. Toxicity effect of silver nanoparticles on HepG2 cell line and mice liver primary cell culture. Research in Pharmaceutical Sciences. 2012; 7(5):1.
47. Sreevani P, Adilaxmamma K, Alpharaj M, Muralidhar Y, Eswara Prasad P, Prasad TNKV. Evaluation of wound healing efficacy of chemical and phytogenic silver nanoparticles. IET Nanobiotechnology, 2016, 1-9. Doi:10.1049/iet-nbt.2015.0084.www.ietdl.org.
48. Chaitanya Kumar TV, Muralidhar Y, Eswara Prasad P, Prasad TNVKV. Evaluation of therapeutic potential of nanosilver particles synthesized using plant active principle aloin in experimental murine mastitis model. IET Nanobiotechnology, 2013, 78-82. Doi:10.1049/iet-nbt.2012.0045.www.ietdl.org.
49. Sharma Datta H, Shankar Kumar M, Patwardhan B. Wound healing activity of topical application forms based on ayurveda. Evidence-based Complementary and Alternative Medicine, 2011. Article Id: 134378, epublished. www.ecam.uk.
50. Malviya M, Jain S. Wound healing activity of aqueous extract of Radix paeoniae root. Acta Poloniae Pharmaceutica Drug Research. 2009; 66(5):543-547.
51. Jayasheelan V, Uma Maheswari A, Shanmugarathinam A, Puratchikody A. Wound healing potential of *Psammaplysilla purpurea*: A pharmacological and biochemical evaluation. Journal of Pharmacy Research. 2010; 3(6):1325-1329.
52. Nayak S, Pereira P. *Catharanthus roseus* flower extract has wound healing activity in sprague dawley rats. BMC Complementary and Alternative Medicine. 2006; 6(4):41-46.
53. Karthikeyan J, Rani P. Enzymatic and non-enzymatic antioxidants in related piper species. Indian Journal of Experimental Biology. 2003; 41(2):135-140.
54. Priya KS, Arumugam G, Rathinam B, Wells A, Babu M. *Celosia argentea* Linn. leaf extract improves wound healing in a rat burn wound model. Wound Repair and Regeneration. 2004; 12(6):618-625.
55. Senel O, Cetinkale O, Ozbay G, Ahcioglu F, Bulan R. Oxygen free radicals impair wound healing in ischaemic rat skin. Annals Plastic Surgery. 1997; 39(5):516-523.
56. Gupta A, Singh RL, Raghubir R. Antioxidant status during cutaneous wound healing in immunocompromised rats. Molecular and Cellular Biochemistry. 2002; 241(1-2):1-7.
57. Getie M, Gebre MT, Reitz R, Neubert RH. Evaluation of the release profiles of flavanoids from topical formulations of the crude extract of the leaves of *Dodonea viscosa* (Sapindaceae). Pharmazie. 2002; 57(5):320-322.
58. Mahesh SP, Patil MB, Ravi Kumar, Patil SR. Evaluation of aqueous extract of leaves of Ocimum Kilimandscharicum on wound healing activity in albino wistar rats. International Journal of Pharm Tech Research. 2009; 1(3):544-550.