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ORIGINAL ARTICLE

Dioscorea villosa Leaf Extract Enhances *in vitro* Wound Healing and Expression of Extra Cellular Matrix Factors Transforming Growth Factor-Beta 1 and Collagen-1 in L929 Cell Lines

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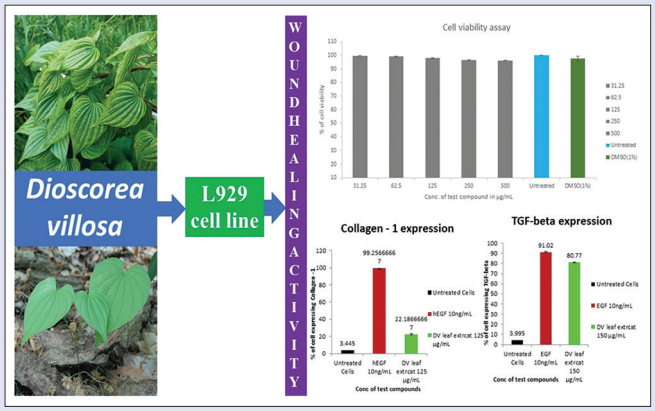
ABSTRACT

Background: Easy availability, relatively low cost with fewer side effects, has made the herbal extracts/fractions/pure compounds as prominent source of medicinally important molecules. *Dioscorea villosa* L. commonly known as wild yam belongs to the family *Dioscoreaceae* and has been used in various parts of India to treat joint pain, arthritis, and various other diseases. However, its role in wound healing has not been documented so far. In the current study, the *in vitro* wound healing capabilities of *D. villosa* were examined using L929 cells. **Materials and Methods:** Methanolic extraction of *D. villosa* leaves was prepared by applying inexpensive maceration method. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to analyze the cytotoxicity of *D. villosa* extract and *in vitro* wound healing capabilities were investigated by applying scratch assay. The qualitative measurement of different secondary metabolites was determined by standard biochemical assays. Gas chromatography-mass spectrometry (GC-MS) was performed to identify the possible wound healing components present in the methanolic leaf extract of *D. villosa,* and the antioxidant properties of the plant extract were evaluated by α,α-diphenyl-β-picrylhydrazyl and ferric reducing antioxidant power assays. Furthermore, the possible molecular factors involved in the proliferation and migration of fibroblast in the presence of *D. villosa* extract was determined by flow cytometry technique. **Results:** The experiments to analyze the cytotoxic effect of *D. villosa* on L929 cells revealed that at the highest concentration used, i.e., 500 µg/mL after 48 h of incubation, 96.06% ± 0.42% of the cells were viable. The results of the scratch assay revealed that 125 µg/mL of plant extract induced the migration in 88.58% of fibroblast cells. Through GC-MS analysis, antioxidant and anti-inflammatory molecules such as 1 H-Indole-2,3-dione (Isatin) and Dexamethasone have been identified. In addition, flow cytometry data showed the influence of plant extract on the expression of Collagen-1 and transforming growth factor (TGF)-beta, which play a major role in the wound healing processes. 125 µg/mL of plant extract induced Collagen-1 in 22.18% cells and TGF-beta in 80.77% of cells, respectively. **Conclusion:** The presence of potent antioxidant and anti-inflammatory molecules and capability to induce the expression of fundamental wound healing molecular factors TGF-beta and collagen-1 in fibroblast cells, endorsed *D. villosa* as a potential wound healing agent.

Key words: Collagen-1, *Dioscorea villosa*, gas chromatography-mass spectrometry, scratch assay, transforming growth factor-beta, wound healing



SUMMARY

- The *in vitro* wound healing capabilities of *Dioscorea villosa* were examined using L929 cells
- 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was employed to analyze the cytotoxicity of *Dioscorea villosa* extract and *in vitro* wound healing capabilities were observed by scratch assay
- The experiments to analyze the cytotoxic effect of *Dioscorea villosa* on L929 cells revealed that the higher concentration after 48 h of incubation reduced the viability of cells to 96.06% ± 0.42%
- The results of scratch assay revealed that 125 µg/mL of plant extract induced the migration in 88.58% of fibroblast cells
- α,α-diphenyl-β-picrylhydrazyl and ferric reducing antioxidant power experiments showed the potential antioxidant activity of *Dioscorea villosa*

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- Gas chromatography-mass spectrometry data revealed the presence of chemical constituents with antioxidant properties
- In addition, flow cytometry data showed the influence of plant extract on the expression of Collagen-1 and transforming growth factor-beta, which play a major role in the wound healing
- 125 µg/ml of plant extract induced Collagen-1 in 22.18% cells and transforming growth factor-beta in 80.77% of cells, respectively
- *Dioscorea villosa* extract may induce the wound healing in transforming growth factor-beta mediated pathway and could be recommended as potential wound healing agent.

Abbreviations used: DA: *Dioscorea villosa*; LDH: Lactate dehydrogenase; ECM: Extra Cellular matrix; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate. D-PBS: Dulbecco's phosphate-buffered saline; FACS: Fluorescent activated cell sorter; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; µg: Micrograms; ng: Nanogram; mL: milliliter; SD: Standard Deviation; hEGF: Human epidermal growth factor.

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INTRODUCTION

In this modern era, synthetic drugs are being employed widely to treat various diseases but due to the side effects and emerging resistance to available drugs, new therapeutic sources are needed. Medicinally important plants are affluent resource of discrete phytochemicals with multi-complex remedial potential.[1] Ever since the ancient times, the indigenous plant parts have been used against different ailments including chronic wound healings.[2] *Dioscorea villosa* L. is rare medicinal plant in the family of *Dioscoreaceae* which is acknowledged by its significance in conventional medicine throughout the world. Conventionally, the plant has been used to treat nausea and spasms during pregnancy, in the treatment of bilious colic and as a muscle relaxant relieving gas and pains of bowels.[3]

Cell migration is a fundamental process for an array of pathophysiologic conditions such as wound healing, cancer, and inflammation. The mechanism of cell migration is a synchronized process for various cell types such as fibroblasts, endothelial cells, mesenchymal stem cells, and neural stem cells.[4-7]

Several studies have been demonstrated to understand the important role of fibroblasts in complex wound healing mechanism. It has been found that these cells release a variety of growth factors which are employed as stimulants to facilitate wound healing due to their regulatory activities on all phase of wound healing. Various growth factors and cytoskeleton proteins such as transforming growth factor (TGF)-beta, fibroblast growth factor (FGF), epithelial growth factor (EGF), platelet-derived growth factor-BB, collagens, vimentin, and other cytokines which play a pivotal role in inflammation.[8] Collagens are the most predominant factors released by fibroblasts cells on stimulation by ECM factors immediate after wound creation in the skin. These collagens initially act as chemotactic agents to attract the other fibroblasts, keratinocytes, and immune cells toward wound area which initiate re-epithelialization.[7,8] In later stages, on degradation of the collagens by the action of the enzymes - matrix metalloproteases, collagen derivatives stimulate the proliferation of vascular endothelial cells which release a variety of growth factors that initiate angiogenesi.[9,10]

TGF-beta is another multifunctional and most important growth factor released by fibroblast cells which regulates all phases of the wound healing mechanism. Other than being a main component of ECM, TGF-beta also stimulates a variety of processes such as proliferation, migration, differentiation of keratinocytes, fibroblasts, macrophages, and endothelial cells.[11,12] On the other hand, studies on the interactions among growth factors released by fibroblasts cells revealed that the reciprocal interactions between TGF-beta and collagen-1 are crucial to enhance the wound healing.[13,14] Hence, many researchers focused on the identification of drugs which modulate the functions of fibroblasts cells through TGF and collagen mediated pathways to enhance the wound healing.

The objectives of this study were to explore the wound healing properties of *D. villosa* leaf extract by means of fibroblast proliferation, migration and TGF-beta and collagen-1 expression in fibroblasts cells. In addition, gas chromatography-mass spectrometry (GC-MS), for the determination of organic biochemical components of *D. villosa* leaf extract with wound healing capabilities.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM) high glucose (#AL219A, Himedia, India), fetal bovine serum (FBS) (#RM10432, HiMedia, India), Dulbecco's Phosphate-Buffered Saline (D-PBS) (#TL1006, HiMedia, India), Mouse Anti-Human Collagen Type I-fluorescein isothiocyanate antibody (#FCMAB412F, Merck, USA), Mouse anti-Human TGF-β1-PE (#562339, BD Biosciences, USA) Cipladine (Cipla Ltd., India), human EGF (hEGF) (#11376454001, Roche, USA) 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (#4060 HiMedia, India), dimethyl sulfoxide (DMSO) (#PHR1309, Sigma-Aldrich, USA), α,α-diphenyl-β-picrylhydrazyl (DPPH) (#D9132, Sigma-Aldrich, USA) Fluorescent Activated Cell Sorter (FACS) Calibur (BD Biosciences, USA), Microplate reader (#EC800, Biotek), GC-MS (#Clarus 500, PerkinElmer Life and Analytical Sciences, USA).

Collection of plant material

The leaves of D. villosa were collected at Dehradun, Uttarakhand, India. The plant material was identified and authenticated by Dr. Rama Rao, Research Officer (Scientist-2), Botany, Regional Ayurveda Research Institute for Metabolic Disorders (RARIMD). The specimen was deposited in the herbarium of the RARIMD, Bangalore, with an ithenticate number RRCBI-mus 245.

Methanolic extraction of leaves

The polar solvent system, methanol which liberates a high amount of medicinally valuable secondary metabolite from plant sample was used for plant extraction. Briefly, the collected *D. villosa* leaves were chopped,

air-dried, and milled by grinder. 50 g of leaf powder was mixed with 200 mL of methanol and continuously stirred using magnetic stirrer for 24 h. Whatman no. 1 filter paper was used to filter the leaves mixture solution, and the filtrate was concentrated and dried using rotary evaporator at 40°C. The stock concentration of 1 mg/mL was prepared. 1 mg of the extract was dissolved in medium that contained 50 µL of 99.99% DMSO and 950 µL of DMEM with 10% FBS and the solution was stored at room temperature.[15]

Cell culture and maintenance

The mouse fibroblast cell line L929 was obtained from NCCS, Pune, and cultured in DMEM with 4.5 g/L of glucose, premixed with 10% FBS and 1% pen/strep antibiotics in a humidified 5% CO_2 incubator at 37°C. After reaching 80% of density, cells were detached by using 0.025% trypsin and 0.01% EDTA (in D-PBS) solution. Cell viability and count were performed using hemocytometer. The appropriate density of cells was seeded in T25 flasks and cultured until further usage of cells to conduct cell-based assays.

Cell viability analysis by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The cytotoxicity effect of *D. villosa* leaf extract was analyzed on L929 cells by performing MTT assay. MTT is a tetrazolium salt which is converted into insoluble purple-colored formazan crystals by the action of lactate dehydrogenase enzyme released by mitochondria.[16] Briefly, L929 cells in 200 µl of DMEM and at a density of 20,000 were plated in a 96-well plate and incubated at 37°C for overnight. After attachment of cells to the surface of cell culture plate, the spent medium was replaced with DMEM having various working concentrations of plant extract (31.25, 62.5, 125, 250, and 500 µg/mL). After drug addiction, L929 cells were incubated for 48 h at 37°C and 5% CO_2. After incubation, cells were treated with 20 µL of MTT (0.5 mg/mL) was added and incubated for 2 h at 37°C. DMSO (100 µL) was used to dissolve the formazan crystals, and purple color was measured at 570 nm using microplate reader. The viability of cells treated with DMEM alone was considered as 100%, and the percentage of cell viability was calculated using the formula below:

$$\% \text{ of viability} = \frac{\text{OD of test sample treated cells} - \text{OD of blank}}{\text{OD of untreated cells} - \text{OD of blank}} \times 100$$

In vitro assessment of cell migration and wound closure

Scratch assay is an inexpensive and widely applied *in vitro* method to estimate the wound healing potential of various therapeutic agents.[7,17] In the current study, 2×10^5 cells/mL of L929 cells were seeded in a 6-well tissue culture plates for overnight at 37°C and 5% CO_2. The culture medium was removed and washed with sterile PBS pH 7.4 to remove floated cells. The wound was created using sterile 200 µL tip and detached cells were washed with D-PBS twice. 125 and 5 µg/mL of plant extract and positive controls were added to respected wells and incubated for 48 h. Inverted microscope was used to observe the cell migration and wound closure at different time intervals and images were taken by digital camera. The gap filled due to migration of cells at different time intervals was measured by Image J software. The scratch assays were performed in triplicates ($n = 3$).

Phytochemical screening

The qualitative analysis of secondary metabolites including phenolics, tannins, flavones, saponins, alkaloids, and glycosides of *D. villosa* was performed based on standard biochemical reactions. The presence of phenolic compounds in the extract was identified by using few drops of alcoholic ferric chloride which gives bluish-green or bluish-black spots. Tannins were identified using the lead acetate solution through the formation of orange-red spots. Shinoda test was performed for identification of flavones. Saponins were identified by copious lather formation in water. Alkaloids were identified through the Draggendroff's test and formation of orange-red precipitates. Glycosides were identified with the anthrone reagent with dark green coloration.[18-21]

Anti-oxidant assays

α,α-diphenyl-β-picrylhydrazyl antioxidant assay

The DPPH-free radical scavenging potential of *D. villosa* leaf extract was analyzed to determine its antioxidant activity. 150 µL of 100 µM methanolic solution of DPPH was mixed with I50 µL of different concentrations of *D. villosa* leaf extract and ascorbic acid in a microtiter plate. The mixture was shaken vigorously and was incubated at room temperature for 30 min, the change in the color from deep violet to light yellow were read at 517 nm. Control and blank solutions were prepared, control consisted of methanol, and DPPH radical solution except the leaf extract and blank consisted only methanol. The capability of scavenging the DPPH radical was calculated using the following formula:[22]

Inhibition (%) = ([Absorbance of Control – Absorbance of sample]/ Absorbance of Control) × 100

Here, ascorbic acid was used as standard antioxidant. The IC_{50} values were calculated by applying linear regression Y = Mx+C, and the IC_{50} value represents the concentration of an antioxidant required to reduce the initial concentration of DPPH by 50%.

Ferric reducing antioxidant power assay

The antioxidant activity of the *D. villosa* leaf extract was determined using ferric reducing antioxidant power (FRAP) assay by following modified protocol Benzie and Strain.[23] The FRAP reagent was freshly prepared by mixing 1 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution with 300 mM acetate buffer in 10 mL of 40 mM hydrochloric acid and 1 mL of 20 mM ferric chloride hexahydrate. To the freshly prepared 1 mL of FRAP reagent, 40 µL of leaf extract and 60 mL of water was added. The reaction mixture was incubated at 37°C for 15 min, and the absorbance was measured at 593 nm against blank which contained only 50% methanol. Using different concentrations of standard ascorbic acid, a calibration curve was prepared and expressed as milligram of ascorbic acid equivalents per gram dry weight of leaves.

Gas chromatography-mass spectrometry analysis

Perkin Elmer Clarus-500 GC was used to perform the GC-MS analysis of methanolic extract of *D. villosa* along with the MS. For separating the components, fused silica capillary column of 30 m × 0.32 µm was used. The temperature was programmed as 50°C initially for 10 min followed by 350°C for 5 min with a ramp of 20°C min 1. The sample was injected using helium as a carrier gas. Mass spectra were taken at 70eV. The National Institute of Standards and Technology library was used as a reference for identifying the compounds by comparing the recorded spectra with the existing spectra in the library.

Intracellular growth factors expression studies by flow cytometry

The effect of plant extract on most pivotal molecular factors of wound healing, TGF-beta, and collagen-1 expressions was analyzed by flow cytometry. L929 cells at a density of 2 million cells/mL were seeded in a 6-well plate and cultured for overnight. Later, cells were exposed to 125 and 10 ng/mL of plant extract and positive control, hEGF for 48 h. The

cells were trypsinized and washed with D-PBS. Seventy percent ice-cold methanol was used to fix the cells by incubating for overnight at −20°C. Cells were stained with antibodies against TGF-beta1 (1:1000) and collagen-1 (1:1000) and incubated under dark conditions for 30 min at room temperature. Flow cytometric measurement of protein expressions was assessed by FACS Calibur and data were analyzed by Cell Quest Pro software.

Statistical analysis

All the results in this study are presented as mean ± standard error of mean. Data analysis was performed using the Graph Pad Prism Ver. 5 (Graph Pad Software, San Diego, CA, USA). One-way analysis of variance followed by Bonferroni *post hoc* test was used for multiple comparisons. $P < 0.05$ was considered as statistically significant.

RESULTS

Viability of L929 cells was not affected by methanolic extract of *Dioscorea villosa* leaf

The cytotoxicity effect of *D. villosa* leaf extract was analyzed colorimetrically. After 48 h of exposure of L929 cells to *D. villosa* leaf extract, MTT assay was performed and absorbance values were measured at 570 nm. The results revealed that the test compound did not affect the viability of L929 cells and 99.60 ± 0.24% and 96.06 ± 0.42% of cells are viable at 31.25 and 500 µg/mL of *D. villosa* leaf extract, respectively. The concentrations of the plant compound used to treat the cells, and respective % of cell viability are shown in Table 1 and Figures 1, 2.

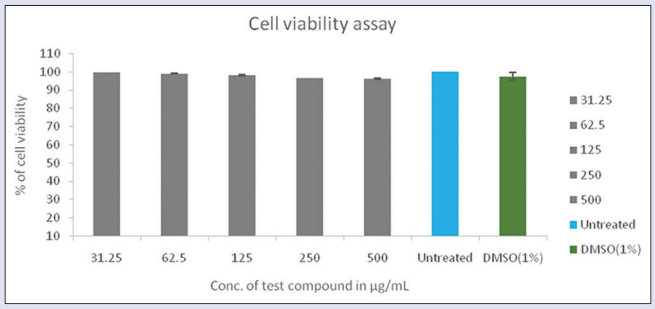


Figure 1: The effect of *Dioscorea villosa* on L929 cell line viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay method. Each bar graph represents % viability of L929 cell line against 31.25, 62.5, 125, 250, and 500 µg/ml concentrations of *Dioscorea villosa* extract after 48 h exposure. The data were shown as mean ± standard deviation of triplicate experiments

Dioscorea villosa leaf extract accelerate the migration of fibroblast cells

In vitro scratch assay is a less expensive and well-defined model to assess the wound healing effect of herbal extract and other therapeutic agents. It involves the formation of a scratch on a thick monolayer of suitable cells and measuring the cell migration and wound closure by microscopic observations.[17] The analysis of wound healing effect of *D. villosa* leaf extract was studied by applying 125 µg/mL concentration of *D. villosa* leaf extract and 5 µg/mL standard Cipladine on L929 cells for 48 h and cell migration was captured at 0, 12, 24, 48 h and wound closure distance was calculated by Image J software. Developed by Wayne Rasband (retired from NIH) at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin), Madison (town), Wisconsin, USA. The results indicated that 125 µg/mL concentration of plant extract induced 44.50%, 74.15%, and 88.58% of cell migration at 12, 24, and 48 h, respectively. Untreated cells were used as negative control and 5 µg/mL of Cipladine as a positive control [Figures 3 and 4].

Qualitative analysis of phytochemical compounds

The preliminary screening of the *D. villosa* leaf was showed to the presence of different metabolic compounds such as phenols, tannins,

Table 1: Cell viability effects of *Dioscorea villosa* extract in L929 cell line

Culture conditions	Percentage of cell viability
Vehicle control	97.50±2.80
31.25	99.60±0.24
62.5	98.98±0.37
125	97.94±0.50
250	96.55±0.08
500	96.06±0.42

Table 2: The preliminary screening data of phytochemical compounds from *Dioscorea villosa* leaf extract

Dioscorea villosa leaf extract	Qualitative measurement of phytochemicals
Phenols	+++
Tannins	++
Flavonoids	++
Saponins	++
Alkaloids	++
Glycosides	+++

+: indicates the presence of secondary metabolites

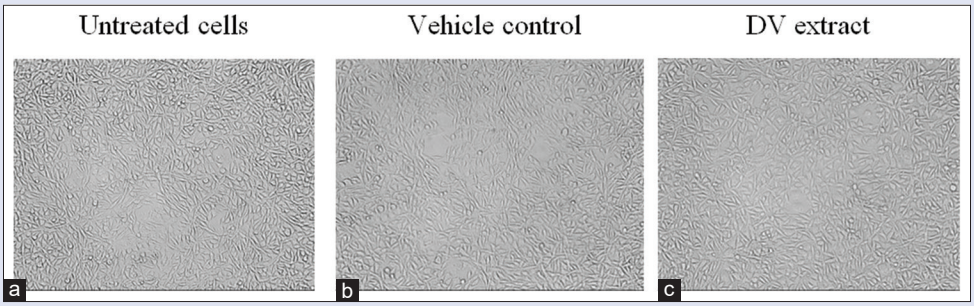


Figure 2: The effect of *Dioscorea villosa* leaf extract on the morphology and viability of L929 cells. Images were taken by inverted light microscopy after exposing the cells to different concentrations of *Dioscorea villosa* leaf extract for 48 h. From "A" to "C" where (a) untreated control (b) vehicle control and (c) 500 µg/mL concentration of *Dioscorea villosa* leaf extract

flavonoids, saponins, alkaloids, and glycosides. Compared to all other metabolites phenols, and glycosides were observed to be the highest levels in the methanolic extract of *D. villosa* leaf [Table 2].

Gas chromatography-mass spectrometry analysis of bioactive metabolites from *Dioscorea villosa* leaf extracts

The metabolites with a physiological role in wound healing were assessed by metabolic profiling of the methanol leaf extract of *D. villosa* using GC-MS.

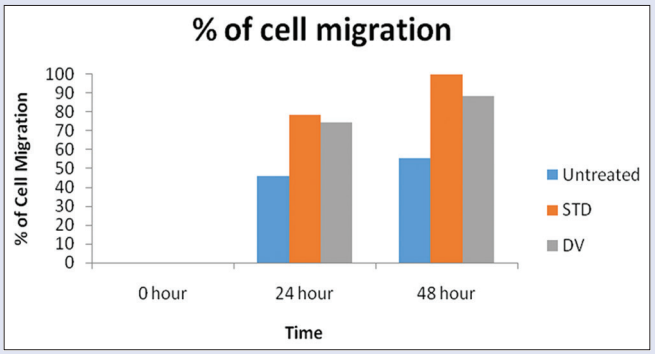


Figure 3: Results of the scratch assay: Percentage of cells migrated toward the wound and involved in wound closure. Migration of cells in the absence or presence of *Dioscorea villosa* leaf extract and cipladine. Blue: Cells with culture medium alone; orange: 5 µg/mL of standard drug cipladine. Gray: 125 µg/mL of *Dioscorea villosa* leaf extract

The result of GC-MS analysis of *D. villosa* leaf extract showed the presence of 38 peaks [Figure 5]. 36 out of 38 were identified based on their structure and molecular mass. The retention time, percentage of concentration of peak area, and their biological activities of the compounds were presented in Table 3.

Dioscorea villosa has potent antioxidant activity

α,α-diphenyl-β-picrylhydrazyl assay

DPPH is a dark-colored crystalline powder composed of stable free-radical molecules which produce free radicals in methanolic solution. The molecule having antioxidant potential can scavenge the free radicals produced by DPPH when incubated together. The change of visible color from purple to straw color indicates the radical scavenging. Data suggested the strong antioxidant potential of *D. villosa* in a dose-dependent manner with more than 90% inhibition of free radical production at 100 µg/mL [Figure 6]. Ascorbic acid was used as standard antioxidant.

Ferric reducing antioxidant power assay

The antioxidant potentials of the methanol extracts of the leaves of *D. villosa* was estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). It was observed antioxidant activity increased in a dose-dependent manner. The results of the reducing power of the *D. villosa* extract and of ascorbic acid showed that at 100 µg/mL concentration of extract, maximum optical density of 1.3 and at minimum concentration of 6.25 µm/mL, it was found to be 0.23 indicating that reducing the power of *D. villosa* increases with increasing concentration. Ascorbic acid which was used as a standard showed a value of 2 in optical density at 100 µg/mL concentration [Figure 7].

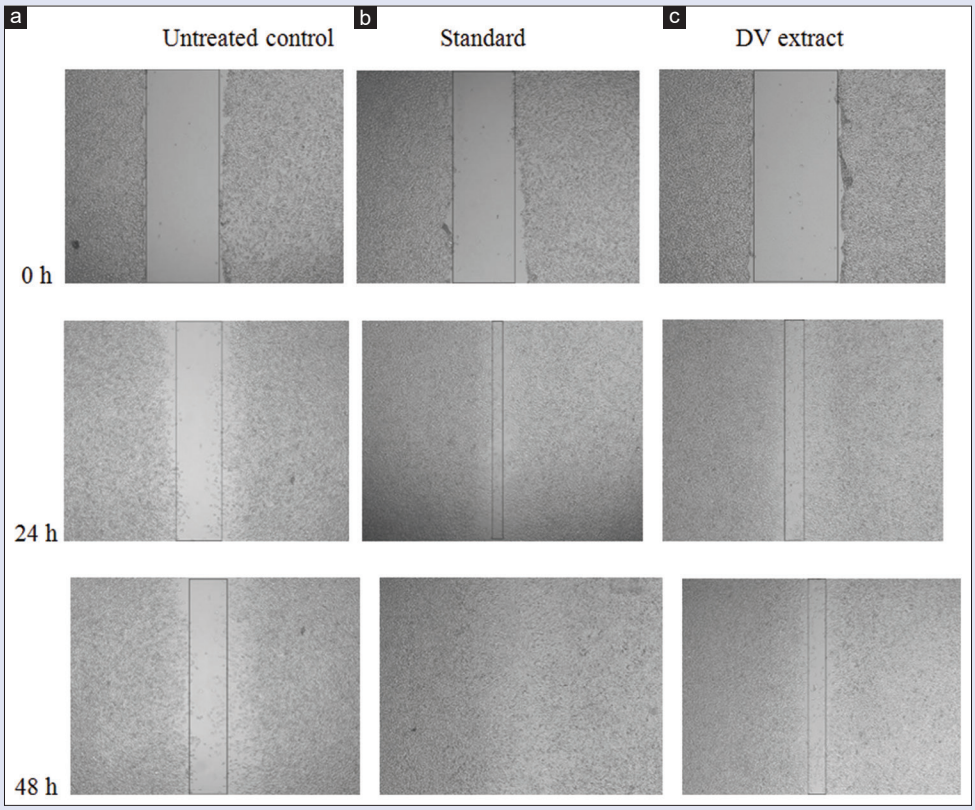


Figure 4: *In vitro* wound healing activity of methanolic extract of *Dioscorea villosa* leaf: L929 cells were incubated in the presence or absence of *Dioscorea villosa* leaf extract and standard drug cipladine and images were captured at 0, 24 and 48 h. (a) Negative control, (b) 5 µg of positive control cipladine (c) 125 µg/ml of *Dioscorea villosa* leaf extract

Dioscorea villosa leaf extract enhanced the extracellular matrix factor, collagen-1 expression

One of important proteins of extracellular matrix is collagen-1 which plays a prominent role in the formation of extracellular matrix in the process of wound healing. In the present study, the expression of collagen-1 was evaluated by flow cytometry in L929 cells treated with 125 µg/mL of or hEGF. Flow cytometry data showed increased the expression of collagen Type 1 in *D. villosa* leaf extract-treated cells. The percentage of cells that expressed collagen Type 1 in the extract-treated cells was 22% and in hEGF-treated cells was 99.25% of cells [Figures 8 and 9].

These results indicate that the extract enhances collagen Type 1 expression in L929 cells, possibly thereby enabling the wound healing process.

Flow cytometric assessment of transforming growth factor-beta expression in L929 cells

TGF-β is released in wound areas following tissue damage, through platelet degranulation which plays a key role in the process of wound healing. Herein, when % frequency of cells expressing TGF-β was assessed by flow cytometry in post-treated cells with 125 µg/mL concentrations of *D. villosa* leaf extract and treatment naïve cells, it was observed that more than 80% cells posttreatment expressed TGF-β when compared with untreated control. EGF was used as positive control and it was observed that more than 90% of cells treated with EGF were positive for TGF-β [Figures 10 and 11].

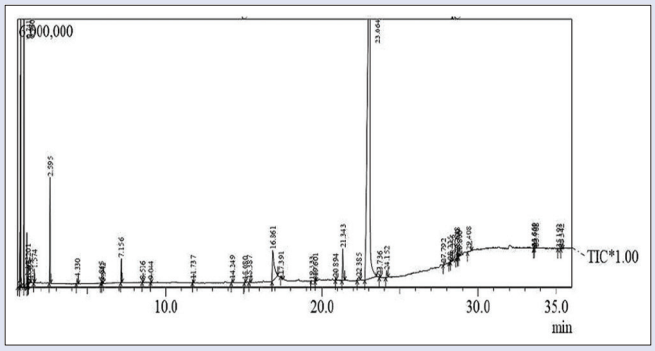


Figure 5: Gas chromatography-mass spectrometry spectrum of methanolic extract of *Dioscorea villosa* leaves

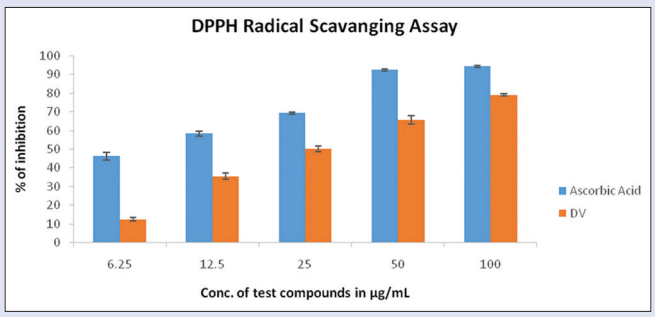


Figure 7: Ferric reducing antioxidant power assay of methanolic extracts of *Dioscorea villosa*. Ascorbic acid was used as standard, and the results are expressed as mean ± standard deviation of triplicate experiments

DISCUSSION

Finding of cost-effective drugs with less side effects has become a challenging task to the medical researchers to treat various diseases including wound healing. In this direction, researchers have sought after plant-derived extracts that have lesser adverse effects as a potential source for drug designing. Although *D. villosa* is traditionally known to treat joint pain, arthritis, etc.,[24] its wound-healing ability was undocumented. Herein this study, the wound healing capabilities of *D. villosa* were examined by using *in vitro* cell culture models.

Proliferation and migration of the fibroblasts are key cellular events in wound healing. Our assessment of the same using *in vitro* scratch assay on L929 cells, an important measure of the wound healing capacities of a compound, revealed that the leaf extract of *D. villosa* enhanced the migratory ability of the fibroblasts. For this assay, a scratch was made using 200 µL sterile microtip on monolayers of L929 cells were exposed to 125 µg/mL of plant extract and 5 µg/mL of standard drug. The untreated cells were considered as negative controls. Our results 125 µg/mL of methanolic crude extract of *D. villosa* leaves induced 88.58% L929 cells to proliferate and migrate toward the wound after 48 h of incubation. These results are comparable with standard drug which stimulated 100% of cells to migrate [Figures 3 and 4].

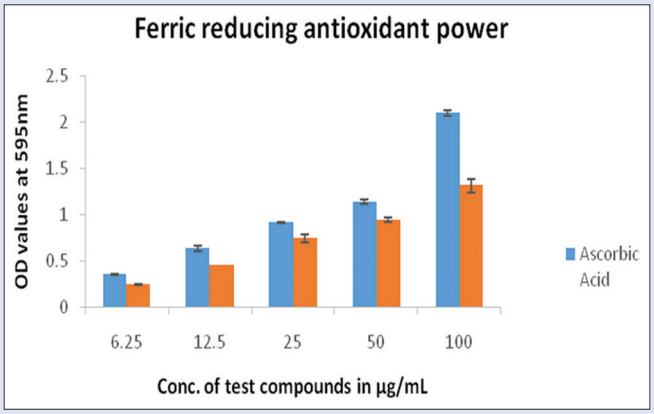


Figure 6: α,α-diphenyl-β-picrylhydrazyl free radical scavenging activities of methanolic extracts of *Dioscorea villosa*. Ascorbic acid was used as standard, and the results are expressed as mean ± standard deviation of triplicate experiments

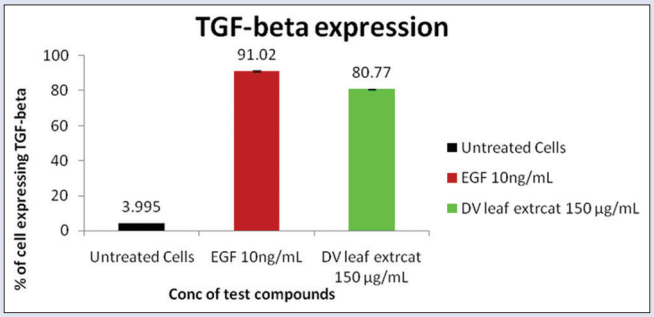
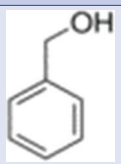
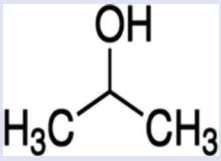
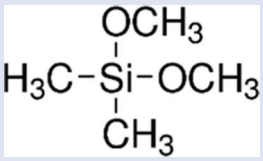
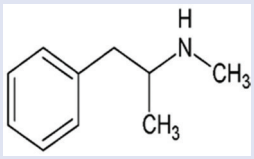
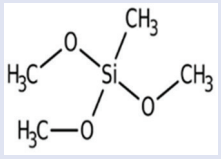
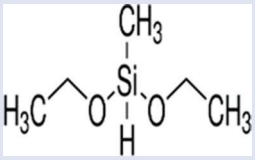
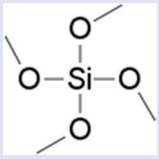
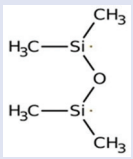
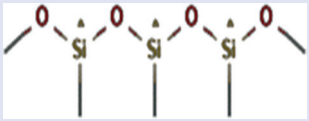
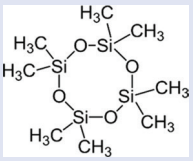


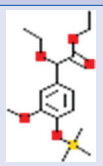
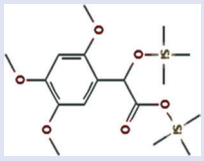
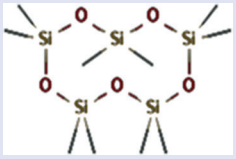
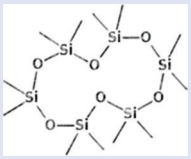
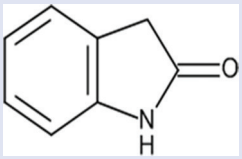
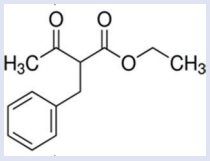
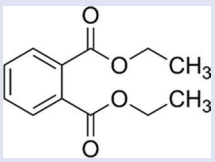
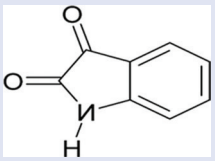
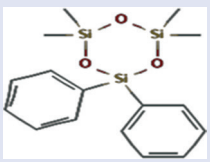
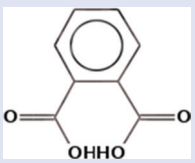
Figure 8: The effect of *Dioscorea villosa* leaf extract on transforming growth factor-beta expression. L929 cells were exposed to 125 µg/ml of *Dioscorea villosa* leaf extract and 10 ng/ml of human epidermal growth factor for 48 h. (a) Black- untreated cells; (b) green – *Dioscorea villosa* leaf extract; (c) red - human epidermal growth factor. The studies were conducted in triplicates and 10,000 events were measured for each flow cytometry run

Table 3: Total compounds identified in the methanolic crude extract of *Dioscorea villosa* leaves using gas chromatography-mass spectrometry analysis with its retention time, peak area and reported biological activities

Name of the chemical	Molecular formula	Molecular weight	R-time	Peak area (%)	Structure	Biological activity
Benzenemethanol	$C_9H_{13}N0$	151	0.567	0.79		Cosmetic-hair and scalp treatment
2-Propanol	C_3H_80	60	0.741	9.27		Disinfectant and sanitizing agent
Silane, di-methoxydimethyl	$C_4H_{12}O_2Si$	120	0.94	13.5		
Amphetamine	$C_9H_{13}N$	135	1.142	0.29		Activate neurotransmitters
Silane, tri-methoxymethyl	$C_4H_{12}0_3Si$	136.22	1.201	0.37		
Silane, di-ethoxymethyl	$C_5H_{14}O_2Si$	134	1.339	0.22		
Silicic acid, tetramethyl ester	$C_4H_{12}O_4Si$	152	1.574	0.22		
Sym- tetramethyldimethoxydisiloxane	$C_6H_{18}O_3Si_2$	194	2.595	3.18		Cosmetics- skin conditioning agent
1,1,2-trimethoxy-1,2-dimethyldisilane	$C_6H_{18}O_4Si_2$	210	4.33	0.23	Not found	Data is not available
1,5 - dimethoxy-1,3,5-trimethyltrisiloxane	$C_5H_{18}O_4Si_3$	226	5.865	0.07		
Cyclotetrasiloxane, octamethyl	$C_8H_{24}O_4Si_4$	296	5.942	0.03		Cosmetics

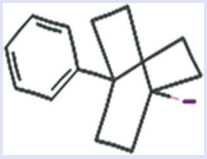
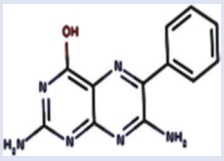
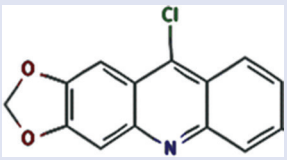
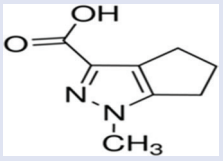
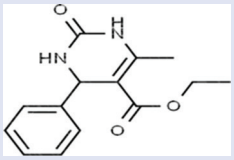
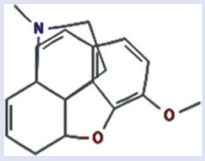
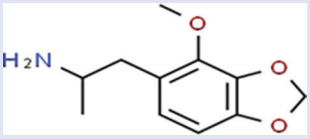
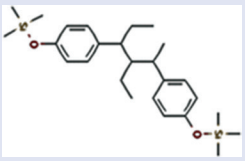
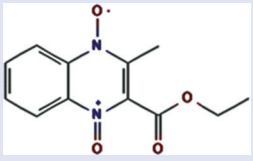
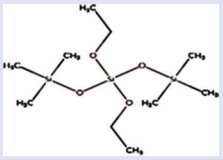
Contd...

Table 3: Contd...

Name of the chemical	Molecular formula	Molecular weight	R-time	Peak area (%)	Structure	Biological activity
1-(4-hydroxy-3-methoxyphenyl)-1-ethoxy acetic acid ethylester	$C_{16}H_{26}O_5Si$	326	7.156	0.81		Data is not available
2,4,5- Trimethoxymandelic acid	$C_{17}H_{30}O_6Si_2$	386	8.516	0.04		Data is not available
Cyclopentasiloxane, decamethyl	$C_{10}H_{30}O_5Si_5$	370.76	9.044	0.04		Data is not available
Cyclohexasiloxane dodecamethyl	$C_{12}H_{36}O_6Si_6$	444.92	11.737	0.08		Data is not available
2H-Indole-2-one, 1,3-dihydro	C_8H_7NO	133.14	14.249	0.11		Data is not available
Ethyl 2-benzylacetoacetate	$C_{13}H_{16}O_3$	220.26	15.08	0.07		Data is not available
Diethyl phtalate	$C_{12}H_{14}O_4$	222.24	15.387	0.09		Data is not available
1 H-Indole-2,3- dione	$C_8H_5NO_2$	147.13	16.861	3.99		Antitumor, Antiinflammatory, Antioxidants
1,3-Diphenyl- 1,3,5,5-tetramethyl-cyclotrisiloxane	$C_{16}H_{22}O_3Si_3$	346.6	17.391	0.05		Data is not available
1,2-benzenedicarboxylic acid,	$C_{16}H_{20}O_4$	276.33	19.333	0.04		Data is not available

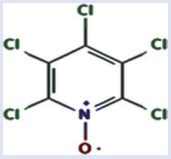
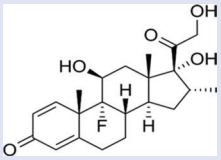
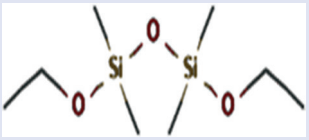
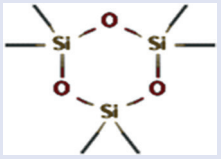
Contd...

Table 3: Contd...

Name of the chemical	Molecular formula	Molecular weight	R-time	Peak area (%)	Structure	Biological activity
Bicycle (2.2.2) octane 1-iodo-4-phenyl	$C_{14}H_{17}I$	312.194	19.601	0.13		Data is not available
4-hydroxy-2,7-diamino-6-phenylpteridine	$C_{12}H_{10}N_6O$	254.24	20.894	0.07		Data is not available
1,3- dioxolo (4,5-b) acridine-10 Chloro	$C_{14}H_8ClNO_2$	257.67	21.343	1.38		Antitumor, Antiinflammatory, Antioxidants
Methyl 1,4,5,6- tetrahydro-cyclopentapyrazole-3carboxylic acid	$C_8H_{10}N_2O_2$	166.18	22.385	0.33		Data is not available
Ethyl 1,4- dihydro- 2- hydroxy- 6-methyl-4-phenyl-5-pyrimidine carboxylate	$C_{14}H_{16}N_2O_3$	260.11	23.064	63.15		Data is not available
7 a, 9c-(iminoethano) phenanthro (4,5-bcd) furan, 4a alpha, 5-dihydro-3-methoxy-12-methyl	$C_{18}H_{19}NO_2$	281.355	23.736	0.07		Data is not available
N- allyl anilne	$C_9H_{11}N$	133.194	24.152	0.34		Anti-fungal activity
N- (2-methoxy-3,4-methylenedioxyamphetamine	$C_{11}H_{15}NO_3$	209.245	27.792	0.04		Data is not available
Benzestrol di- TMS derivative	$C_{26}H_{42}O_2Si_2$	442.78	28.356	0.09		Data is not available
2- Ethoxycarbonyl-3-methylquinoxaline 1,4 dioxide	$C_{12}H_{12}N_2O_4$	248.23	28.648	0.3		Data is not available
2.alpha- isopropyl-3-epi-gibberellin	Not available	Not available	28.7	0.12	Data is not available	Data is not available
silicic acid, diethyl bis (trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$		29.408	0.05		Data is not available

Contd...

Table 3: Contd...

Name of the chemical	Molecular formula	Molecular weight	R-time	Peak area (%)	Structure	Biological activity
2-Bromo-5,8-dimethoxy -3-methylnaphthalene	$C_{13}H_{13}BrO_2$		33.55	0.03	Data is not available	Data is not available
Pyridine, pentachloro-,1-oxide	C_5Cl_5NO	267.311	33.601	0.05		Data is not available
Dexamethasone	$C_{22}H_{29}FO_5$	392.46	33.708	0.06		Anti-inflamatory
Disiloxane, 1,3-diethoxy-1,1,3,3-tetramethyl	$C_8H_{22}O_3Si_2$	222.42	35.192	0.08		Data is not available
Cyclotetrasiloxane, hexamethyl	$C_{11}H_{28}O_4Si_4$	336.68	35.342	0.05		Data is not available

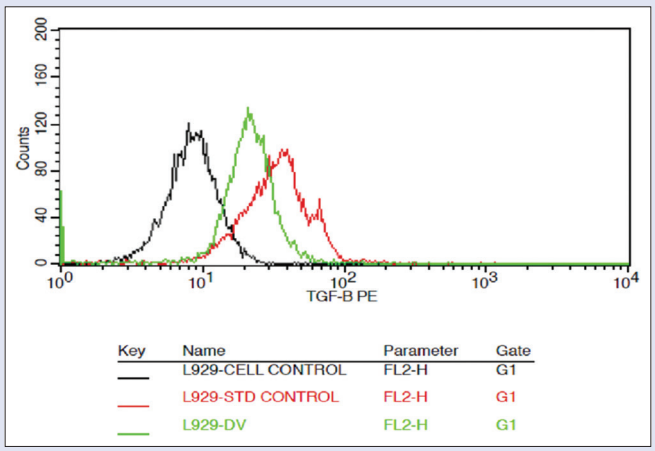


Figure 9: Overlay of relative fluorescence intensities of cells expressing transforming growth factor-beta: L929 cells were treated with 125 µg/mL and 10 ng/ml of *Dioscorea villosa* leaf extract and human epidermal growth factor respectively for 48 h. (a) Black- untreated cells; (b) green – *Dioscorea villosa* leaf extract; (c) red- human epidermal growth factor

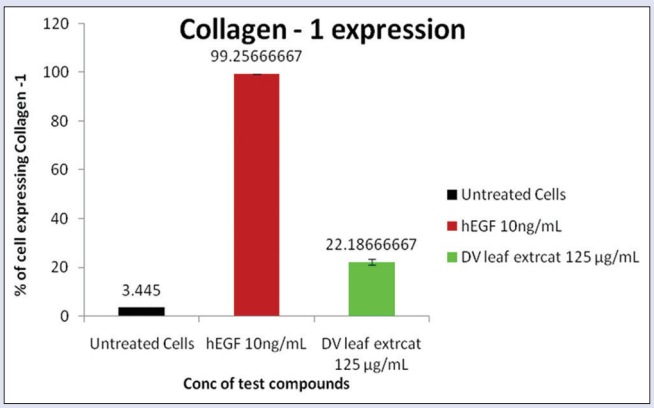


Figure 10: Effect of *Dioscorea villosa* leaf extract on Collagen-1 expression. Flow cytometry-based studies revealed that 22.18% and 99.25% of L929 cells expressed collagen -1 upon treatment with 125 µg/mL of *Dioscorea villosa* leaf extract and human epidermal growth factor respectively. (a) Black-untreated cells; (b) green – *Dioscorea villosa* leaf extract; (c) red- human epidermal growth factor

Preliminary studies on qualitative phytochemical screening revealed the presence of phenols and glycosides in higher amounts when compared to other secondary metabolites [Table 2]. Several studies proved phenolic compounds as one of the most significant groups of free radical terminators and primary antioxidants in various naturally occurring medicinal plants.[25] Further, to support this preliminary screening and to identify the bioactive compounds present in the *D. villosa* leaf extract, GC-MS was performed and 38 chemical constituents have been identified [Figure 5]. The structure, molecular formula, and molecular weights for 36 compounds are represented in Table 3. Of 36 compounds identified, the most prominent compounds were ethyl 1,4- dihydro-2-hydroxy-6-methyl-4-phenyl-5-pyrimidinecarboxylate (63.15%), silane, di-methoxydimethyl (13.5%), 2-Propanol (9.27%), 1 H-Indole-2,3-dione (3.99%), Sym-tetramethyldimethoxydisiloxane. I have removed the space. (3.18%), 1,3- dioxolo(4,5-b) acridine- 10 Chloro (1.38%), 1-(4-hydroxy-3-methoxyphenyl)-1-ethoxy acetic acid ethylester (0.81%), and benzenemethanol (0.79%). Biological activities of 9 compounds out of 36 are known. 1H-Indole-2,3-dione and 1,3- dioxolo (4,5-b) acridine-10 Chloro possess antioxidant, anti-inflammatory, and antitumor activity.[26-28] Benzenemethanol is used in cosmetics-hair and scalp treatment.[29] 2-Propanol acts as a disinfectant and sanitizing agent. Identification of bioactive compounds which are responsible for

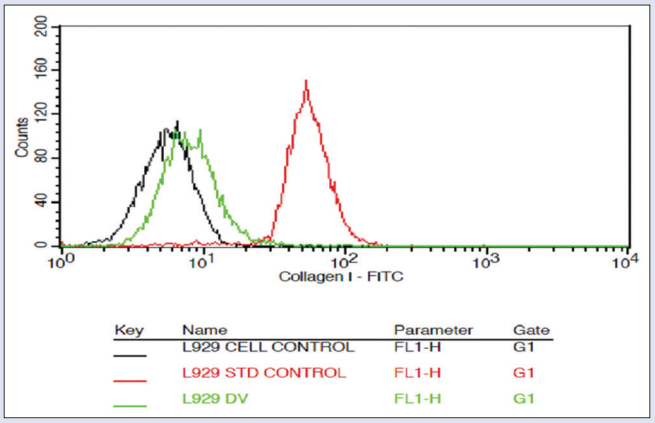


Figure 11: Overlay of relative fluorescence intensities of cells expressing collagen-1: L929 cells were treated with 125 µg/mL and 10 ng/ml of *Dioscorea villosa* leaf extract and human epidermal growth factor respectively for 48 h. (a) black-untreated cells; (b) green – *Dioscorea villosa* leaf extract; (c) red- human epidermal growth factor

the wound-healing capacity infers that the treatment with extract of *D. villosa* has a significant medicinal importance in the wound healing applications. The mechanism involved in wound healing activity still remains unknown.

Further analysis of the possible molecular mechanism behind the wound healing potential of plant extract was determined by studying the stimulatory effect of plant extract on TGF-beta and collagen-1. TGF-beta family members play a crucial role in all phases of wound healing starting from initial anti-inflammatory to scar formation.[29] TGF-beta is the predominant factor which is produced by keratinocytes, fibroblasts, and macrophages immediately after wound creation. Several investigations have been made to find potential therapeutic drugs which show wound healing nature by modulating TGF-beta expression.[30] The main functions of TGF-beta include stimulation of fibroblast cells proliferation and act as a chemotactic agent to make necessary cells to migrate toward the wound. It also involves in the cross-talk between keratinocytes and fibroblasts and finally in the synthesis of collagen, fibronectin, and other factors needed to form ECM.[31] In the current study, L929 cells were treated with 125 µg/mL of the plant extract for 48 h and TGF-beta expression was observed by flow cytometry. Experimental results suggested that the plant extract enhanced the expression of TGF-beta over the untreated control. With the untreated cells considered as control, 80.77% of the plant extract (125 µg/mL) treated cells expressed TGF-beta. This was relative to 90.2% of the cells expressing TGF-beta on induction with EGF (10 ng/mL). These results clearly indicated the ability of *D. villosa* leaf extract to effectively induce the expression of TGF-beta, a key molecular player in wound healing. In other words, the possible mechanism of this plant extract for wound healing might be the induction of the expression of TGF-beta and thereby enhancing the proliferation and migration of the fibroblasts.

Collagen-1, a major component in wound healing mechanisms that is involved in the ECM production by coordinating with other molecular factors, is produced by fibroblast cells which are near to the wound edges and its controlled synthesis is regulated by growth factors such as TGF-β1 and FGF.[14] Our experimental results not only showed an increased expression of TGF-beta on treatment with plant extract but also showed an enhanced expression of collagen-1 in the plant extract treated samples. We studied the stimulatory effect of the extract on collagen-1 synthesis using L929 cells. The cells treated with 125 µg/mL of plant extract expressed collagen-1 as 22.81% of the cells were positive for it. This suggests that *D. villosa* leaf extract enhanced the expression of collagen-1.

In all, the methanolic leaf extract of *D. villosa* enhanced the expression of TGF-beta, which possibly by regulating collagen-1 expression increased proliferation and migration of fibroblasts to the wound site so as to close the wound suggesting the wound healing potency of *D. villosa* leaf extract.

CONCLUSION

Current studies revealed that the *in vitro* wound healing activities of *D. villosa* scratch assay stimulatory effect of plant extract on cell migration and 125 µg/mL of *D. villosa* leaf extract induced 88.58% of cell migration. The same concentration *D. villosa* leaf extract showed a positive effect on the expression of TGF-beta 1 and collagen-1 in L929 cells. Hence, the above results indicated that, *D. villosa* leaf extract may induce the wound healing mechanism by regulating the production of TGF-beta 1 and collagen-1 from fibroblasts. Increased production of reactive oxygen species leads to oxidative stress thereby causing cytotoxicity and delayed wound healing. Therefore, scavenging the ROS can be a therapeutic strategy to heal wounds. With this viewpoint, we had evaluated the antioxidant potential of *D. villosa* extract using DPPH and FRAP assay. Data from both the experiments showed the potential antioxidant activity of *D. villosa* which might have helped to prevent oxidative damage and promote wound healing process. This could be one possible mechanism for wound healing potential. Furthermore, the non-toxic nature of the plant extract made this plant to propose as a potential herbal drug for wound healing.

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Conflicts of interest

There are no conflicts of interest.

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