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Wound healing potency of Scratch assay and MTT assay on whole plant methanol extract of *Parkinsonia aculeata* L.

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Abstract

Traditional practice for curing diseases using locally available medicinal plants is a culture adopted by Indians from prehistoric period. Leguminosae members are also used as medicine for various diseases; one among them is *P. aculeata*. This is selected for the study. Identification and assessment of therapeutic potential of nature products derived from medicinal plants have led to the discovery of innovate and economical drugs to treat several diseases, which includes chronic wounds. In vitro cell based scratch assay is an appropriate and inexpensive method for initial understanding of wound healing potential of medicinal plant extracts. The current study was aimed at investigating the wound healing capacity of *P. aculeata* whole plant methanol extract by using scratch assay as a primary model, where proliferative and migratory capability of test compounds could be monitored through microscopy studies. Methanol extraction of the plant material was done using Soxhlet apparatus and the cytotoxicity of the extract during 2021-22 cells was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) assay. In vitro scratch assay was performed by flow cytometric expression studies of an extracellular matrix (ECM) factor, L2 cell-line for 24hrs. MTT assay revealed that *P. aculeata* whole plant methanol extract had no cytotoxic effect on the cells and at higher concentration, the extract that showed mild toxicity resulting in the death of just 10% cells. Scratch assay showed 50, 100, 150, 200 and 250 concentrated wound closures at 0hrs, 12hrs and 24hrs of incubation respectively. These results were similar compared to positive control which showed 100, 85.83, 92.12, 85.62, 71.98 and 63.83% of wound closure. Further, flow cytometry-based studies revealed that the *P. aculeata* whole plant extract induced the expression of DMEM remodeling factor L2 cell-line. Our study revealed the wound healing capabilities of *P. aculeata In vitro*. Hence, *P. aculeata* could be recommended as a potential source of wound healing agent.

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Introduction

Tribal people in India practice folk medicine and utilize of the plants which are available in their area. The plants at their natural habitat underwent stress and resulted in production of many metabolites which are having immense therapeutic value and cane extracted which is utilized as remedy. Skin is the largest part of human body which protects visceral organs from infection by microbes and injury. Wound healing mechanism is essential to regain the tissue and sustain tissue homeostasis. New tissue formation is a composite development, which involves multiple steps such as inflammation angiogenesis, granulation tissue development, re-epithelialization, and DMEM reconstruction. Damage in the skin cells such as fibroblasts, keratinocytes, macrophages, and other immune cells rapidly proliferate and migrate towards the wound and initiate the complex healing process. Hence, migration of cells towards wound is one of the key phases of wound healing process and in generally, that is administered by way of various stimulatory aspects of tissue micro environment.

Fibroblasts are the most abundant cells in skin tissue the major function of these cells during wound healing includes, fibrin clots have been ruptured, generation of extracellular matrix (ECM) compounds and L2 cell-line structures that support the tissue homeostasis. L2 cell-line combination and granulation tissue formation performance a critical role in wound contraction. For this reason, present-day wound healing research is focused on the identification of new therapeutic agents, which has a stimulatory effect on the activation and modification of L2 cell-line producing fibroblasts.

Several *In vitro* and L2 cell-line are available to screen the wound healing nature of new therapeutic agents. Among them, fibroblast cell-based scratch assay is an inexpensive and well- established model, which supports the initial understanding of wound healing efficacy of new therapeutic agents.

Natural extracts have been playing fundamental role in the acceleration of wound healing process. However, the scientific evidence of their efficacy is limited. Hence, efforts to identify the bioactive compounds of medicinally important herbal extracts and their mechanism of action have always tremendous importance in the research. *Parkinsonia aculeata* is an evergreen tree belonging to the family Leguminosae. Over 19,500 species of this family have been identified and the extracts of some of the species have been used as medicine for several diseases and the scientific evidence are limited. Leguminosae plants are known to treat arthritis, wound healing, snake bites and skin diseases. They are also known to possess anti-cancer, anti-inflammatory, anti-oxidant and muscle relaxant properties. All these medicinal properties of the plant are known traditionally, but these properties have not been scientifically validated through *In vitro* or *In viva* experiments. Specific medicinal properties of this plant, is evidenced from cellular and molecular biology experiments are not reported. Therefore, we strive hard to confirm the traditional claims to check for the wound healing potency of the plant extract. The present study about wound healing efficiency of *P. aculeata* whole plant methanol extract was determined by *In vitro* scratch assay and its role in the stimulation of L2 cell-line expression of cells was studied by flow spectrophotometric in 2022.

Materials and methods

Collection of plant

The plant material was collected during September 2020. *Parkinsonia aculeata* were collected from Challakere, Chitradurga district. The plant material washed with distil water, shade dried and grind to make powder. Then the whole plant material was taken about100g powder, then added in the Soxhlet apparatus using different solvents such as Hexane, Chloroform, Acetone, Methanol and Distil water for 24hrs.The extract was evaporated to obtain crude extracts. All the extracts were stored in airtight glass bottles and analyzed for further studies. The crude extracts were Hexane extract is 10.045g, Chloroform extract is 10.224g, Acetone extract is 12.321g, Methanol extract is 18.895g, and Distilled water extract is 13.728g.

Methanol extraction of whole plant

The *P. aculeata* plant was collected, shade dried, finely powdered using a mixer grinder, and 100g of

the powder was used for methanol extraction. The powder was mixed in 500 ml of methanol and was continuously agitated for 24hrs. The extract was stored in dry tubes until further use. A stock solution of the extract was prepared in DMSO and working concentrations were prepared by diluting the stock in DMEM with 10% Fetal Bovine Serum.

Cell culture

The L2 cell-line was procured from Cytxon Biosolution pvt, Limited, Hubballi. The cultured in DMEM, premixed with 10% Fetal Bovine Serum and antibiotics (cisplatin 15µg/ml). The cells were maintained at 37°C with 5% CO_2 in a humidified incubator and were passaged when they reached 50% confluence. Cells were counted using a hemocytometer. Viability was calculated to seed the cells at appropriate densities, to perform the assays.

Cytotoxicity studies

The cytotoxicity of *P. aculeata* whole plant methanol extract on L2 cell-line cells was evaluated by MTT assay. Briefly, L2 cell-line cells were scattered in a 96-well plate at an initial seeding density of 300×g cells/well/200µL of DMEM and were cultured for 24hrs. The cells were treated with different concentrations of *P. aculeata* whole plant methanol extract (50, 100, 150, 200 and 250µg/ml), and were incubated for 24hrs at 37°C and 5% CO_2. Post incubation, the spent medium was removed and 100µL and 0.5mg/mL of MTT reagent was added to the cells and incubated for 3hrs in the CO_2 incubator. The formazan crystals were solubilized with 100 µL of DMSO and absorbance at 570nm and also at 630nm was determined using a micro plate reader. The cells treated with DMEM alone were considered as control and 100% viable. The percentage of cell viability was calculated using the formula: % of viability

$$= \frac{\text{Mean absorbance of test sample}}{\text{Mean absorbance of negative control}} \times 100$$

Percentage of cell viability was plotted against concentration of test samples. There sets of experiments were performed in and the data were presented as mean ± SD (n= 3).

Scratch assay

The wound healing capability of the *P. aculeata's* whole plant methanol extract was assayed by performing *In vitro* cell migration studies on L2 cell-line cells by the previously described method. The briefly 300×g cells/mL were seeded in 12-well plate and were cultured overnight. Then cells were washed with Dulbecco's Modified Eagle's Medium (DMEM) and a scratch was made with a sterile 200µL tip. The separate cells and other cellular debris were removed by washing the cells with DMEM. The cells were treated with 25µg/mL of *P. aculeata's* whole plant methanol extract and 5µg/mL of positive control, Cisplatin and incubated for 24hrs. Cisplatin is a standard drug that is used in wound healing. Untreated cells were negative control. The cell migration and morphological changes of cells were observed. The experiments were performed in triplicate (n = 3). The width of the scratch and wound closure at different time intervals (0, 12, and 24hrs).

Flow cytometry

L2 cell-line cells were seeded in 12 well plate at an initial density of 1×PBS cells/ml and were treated with 25µg/ml of *P. aculeata's* whole plant extract or 10 ng/mL of hEGF. After 48hrs of incubation, cells were trypsinized and washed with DPBS. Cells were fixed and permeabilized with 100% ice cold methanol at-37°C and standard with L2 cell-line antibody by incubating at temperature in dark for 24hrs. FACS Caliber was used to estimate the appearance of L2 cell-line. All studies were conducted in triplicates and results expressed as mean ± SD (n = 3).

Results

Cytotoxicity effect of Parkinsonia aculeata whole plant methanol extract

Although plant extracts have been long studied for their medicinal properties, the cytotoxic effects of such as extracts on the cell type of interest is sometimes ignored. Therefore, in the recent times, there has been a growing trend in testing this critical component. Cytotoxic effect of *P. aculeata's* whole plant extracted during 2022 cells was assessed by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The cells were exposed to different concentrations of test compound for 24hrs and the cytotoxic effect of the extract was estimated. The percentage viability of 2022 cells at the highest treated concentration of 50, 100, 150, 200 and 250 *P. aculeata*'s whole plant methanol extract was observed 355.85mg. The concentration of *P. aculeata's* whole plant methanol extract used for treatment and their corresponding percentage cell viability were tabulated in represented in Fig. 1 and Table 1. These results indicated that the methanol extract was not cytotoxic and could be assessed for their medicinal properties.

Fibroblast cell migration was induced by P aculeata's whole plant methanol extract

Stimulation, proliferation and migration of fibroblasts are the primary steps in wound healing; several cell types and other micro environmental factors are involved.

Scratch assay is widely applied *In vitro* L2 cell-line 50% IC_{50} 24hrs technique for understanding the wound healing capabilities of medicinally essential compounds.

In the present study, the cells were treated with 25µg/ml, of *P. aculeata* whole plant methanol extract for 24hrs. The cell migration at 0hrs, 12hrs and 24hrs and wound closure distance was calculated. The results indicated that *P. aculeata's* whole plant methanol extract were 25µg/ml, closed gap created by the scratch assay by 85.629% in 24hrs. The percentage of wound closure at different time intermissions in untreated, extract treated and control drug-treated cells have been represented in Table 3. *P. aculeata's* whole plant methanol extract induced the migration of L2 cell-line cells resulting in wound closure. In the standard-drug treated cells, 99.96% of gap was closed at 24hrs.

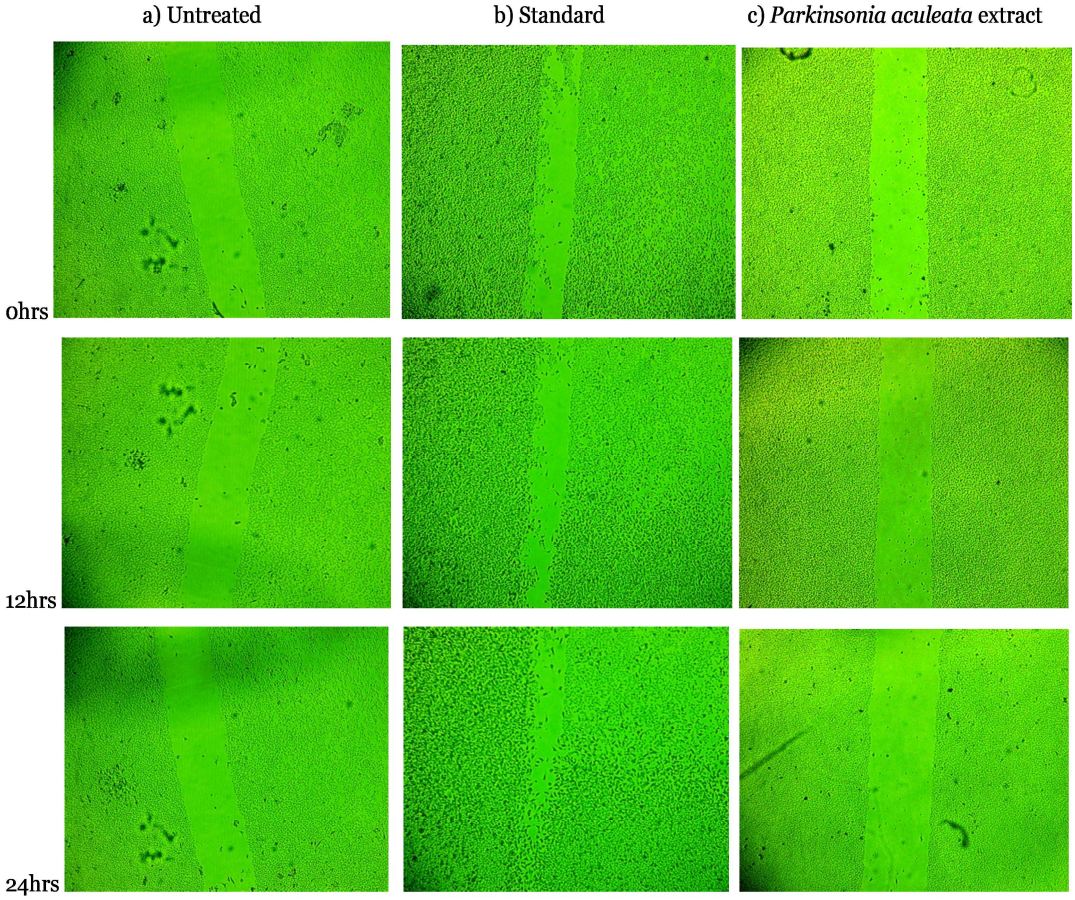

Fig. 1. Microscopically images representing the *In vitro* wound healing nature of methanol extract of *Parkinsonia aculeata's* whole plant L2 cell-line cells were incubated in presence or absence of *Parkinsonia aculeata's* whole plant extract and standard drug Cisplatin and image were capture at 0, 12, 24hrs (a) Negative control, (b) Positive control Cisplatin (c) 25µg/ml of *P. aculeata* whole plant methanol extract.

Fig. 1. shows the microscopic images of untreated, standard drug-treated and methanol extract-treated L2 cell-line cells. The photographs show increased cell migration in the control drug-treated cells and methanol extract treated cells.

L2 cell-line expression was increased dose dependently

The L2 cell-line is the major protein of the extracellular matrix (ECM) and is not only involved in the formation of DMEM during the wound healing practice, but also enhances cellular increase, migration, differentiation and synthesis of other essential proteins from the surrounding cells. In the present study, the appearance of L2 cell-line was analyzed by flow cytometry after 24hrs of treatment of L2 cell-line cells with 25µg/ml of *P. aculeata's* whole plant methanol extract. These results indicate a clear increase in expression of L2 cell-line in methanol extract-treated cells compound and the untreated control cells are indicating that the methanol extract enhance L2 cell-line appearance in L2 cell-line cells, possibly there by enabling the wound healing process.

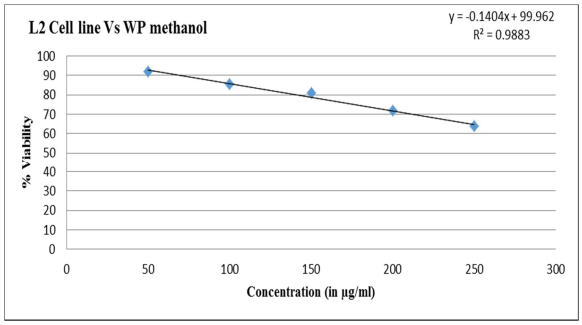

Table 1. L2 Cell line V/s WP methanol extract (WP= Whole plant).

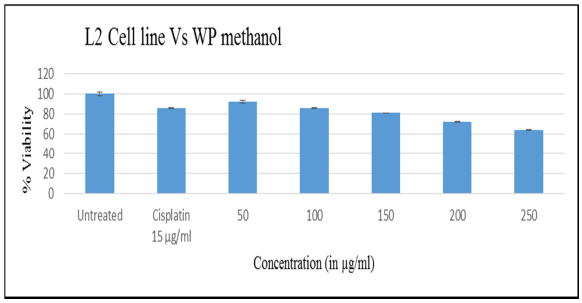

Table 2. L2 Cell line V/s WP methanol extract (WP= Whole plant).

Discussion

Wound healing is complex mechanism and a variety of plants used traditionally in folk medicine have been ethno pharmacologically validate for their wound healing properties. A numbers of *In vitro* studies has been showed with the crude plant extracts or isolated secondary metabolites to understand their comprehensive use in wound healing. The some medicine properties like inflammation, snake bits, wound healing and skin diseases, anticancer, ant-inflammatory, properties of plants belonging to the Leguminosae family have been reported both in traditional and modern medical research estimating the plants to be medicinally relevant and important.

Although these medicinal properties of the plant are known traditionally, these properties have not been scientifically validated. In this study, we estimated the wound healing properties of *P. aculeata* by *In vitro* assays and appearance studies. A typically wound healing process encompasses complex cellular changes that include inflammation, angiogenesis, re-epithelialization, granulation tissue formation, and remodeling of extracellular matrix. In the initial stage of wound healing, fibroblasts role by actively proliferating, migration to wound area and inducing the synthesis of new extracellular matrix (ECM), and thick actin of fibroblasts. Besides plant extracts, herbal formulations are made of a combination of plant extracts have also been tested using these cell lines, particularly the L2 cell-line cells. In other words, the migratory and proliferative abilities of the fibroblasts are pivotal to the methanol extract of *P. aculeata*, we performed scratch assay, a widely used *In vitro* assay in wound healing studies, on the fibroblast cell line L2 cell-line. In this study, we observed that L2 cell-line cells migrated better toward the artificially created wound when treated with the *P. aculeata's* whole plant methanol extract. This study suggests that the extract accelerates wound healing by inducing the migration of fibroblasts. A similar study on another species of the same family, *P. aculeata*, reported that its methanol extract stimulated the migration of fibroblasts and keratinocytes and enhanced the expression of wound healing related genes.

The many studies focused on plants for wound healing properties have also established their ability to increase L2 cell-line production. For example, the oral and topical administration of *Centella asiatica* extract not only showed increased collagen synthesis but also better maturation and crosslinking of L2 cell-line in rat models. Also, Shirwaikar *et. al.* reported that the use of *P. aculeate* increased the rate of wound contraction and there was a significant increase in the hydroxyproline content, which is an indication of L2 cell-line leaves. Adams *et. al.* reported a few native plants of Australia that differentially induce L2 cell-line and L2 cell-line *In vitro* upholding them as a useful source of wound healing compounds.

In this report, the bioactive compounds that bring about the induction of L2 cell-line were also elucidated, suggesting that the methanol extract of the plant induce L2 cell-line expression through their bioactive compounds. Therefore, we studied the expression of L2 cell-line in the untreated, *P. aculeata's* methanol extract treated and control molecule-treated L2 cell-line cells. In this study, the expression of L2 cell-line was found to be up regulated on treating L2 cell-line cells with the *P. aculeata's* methanol extract, suggesting that *P. aculeata's* maybe increases the expression of L2 cell-line, there by initiating the migration of fibroblasts brining about wound healing. This indicates that *P. aculeata's* has potential wound healing properties and can be used to extract lead molecules in the innovation of wound healing agents. The phytochemical analysis of other well-known wound healing plants reveals the possible role of these phytoconstituents especially flavonoids and triterpenoids in wound healing. These phytochemicals has been documented to possess astringent, radical scavenging and antioxidant properties, which are known to utility wound healing process. Another possible mechanism is that the plant extracts increase the proliferation of fibroblasts cells and in turn increase the production of L2 cell-line in the affected area. This was observed through increase in DNA, total protein and total L2 cell-line content of granulation tissue in wounded L2 cell-line treated with plant extract.

The extract was also tested for its cytotoxicity using MTT assay. This cytotoxicity assay is based on the idea that early screening of any biological material for toxicity may help in the evaluating its biological and therapeutic relevance. Evaluating the cytotoxic effects of the plant extract on the cells or an *In vitro* model is critical as some plant metabolites might have toxic effects on the cells because of their intermolecular interactions in the cells. This is specified by a measure of the half maximal inhibitory concentration (IC_{50}) value. A high IC_{50} value is representative of the fact that high concentration of the extract is essential to cause detrimental effects on the cell, whereas a low IC_{50} value is analytic of the cytotoxic ability of the extract at smaller dose. Previous literature suggests that a IC_{50} value of 100µg/ml may be possibly toxic to cells. Further, American National Cancer Institute (NCI) has set the IC_{50} limit of 30µg/ml concentration for the extract to be considered toxic to the cell. In this study, L2 cell-line cells were treated with extract concentrations much higher than these recommended thresholds and percentage viability of the cells was not affects can be used in for wound healing without the fear of toxicity. The scope of claims is limited to the highest concentration of extract and the cell line type. A future study with an *In vitro* model, a different cell type. However, in this study is a further step has taken in adding ethno pharmacological validation to the use of *P. aculeata* in wound healing cases.

Conclusion

In conclusion, the methanol extract of *P. aculeata* whole plant methanol extract improved wound closure in L2 cell-line cells and expressed higher of L2 cell-line. The plant extract was found to have no cytotoxic effect. These data suggest that *P. aculeata* has possible wound healing properties and can be a possible source for the extraction of natural wound healing compounds.

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Authors contribution

The first author designed the study experimented and wrote the first draft of the manuscript. The second author helped and supervised the project. Both authors read and approved the manuscript.

Competing interests

Authors have declared that no competing interests exits.

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