Research Article

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IN VITRO STUDY TO EVALUATE THE WOUND HEALING EFFECT OF HAMSAPADI (*ADIANTUM LUNULATUM* BURM.F.) KALKA IN FIBROBLASTS AND MUSCLE CELL LINES

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ABSTRACT

Ulcer is one of the most common ailments that affect mankind. Acharya Sushruta has explained 60 different types of therapeutic measures which are used to treat any ulcer and one among them was Kalka (paste). Hamsapadi (*Adiantum lunulatum* Burm. f.) was selected as Vrana ropaka (wound healing). The cytotoxicity of Hamsapadi Kalka (paste) on HEK293, McCoy and L6 cell lines were carried out using cell lines.Further the wound healing of McCoy and L6 cell lines were carried out to check the potential of wound healing of Hamsapadi Kalka(paste).In between 3 cell lines, overall Hamsapadi Kalka (paste) showed more cytotoxic to HEK293 cell line, moderate to McCoy and less toxic to L6 cell line. Muscle (L6) cell line showed more wound healing rate compared to Fibroblast (McCoy) cell line with different doses of Hamsapadi Kalka (paste). Cytotoxic and wound healing activity of Hamsapadi showed less toxic to muscle cell line (L6). Thus, it is an external treatment found to be an efficient Vrana ropaka (wound healing). This can be one of the natural, sustainable non-invasive measures for wound healing, especially in deeper tissues.

Keywords: Hamsapadi, wound, vrana (wound), kalka (paste).

INTRODUCTION

Ferns are the first true land plants groups which lack of flowers and concept of invisible seed are responsible for their association with folklore, myth, magic and indigenous medicines. Hamsapadi (*A. lunulatum* Burm. f.) is a fern mentioned by Nighantukaras in wound management, also been used traditionally by folklore practitioners. As the name indicates, the plant is described as the one resembling the feet of swan[1]. The entire plant of this species is used as medicine in Ayurveda, Siddha and Unani[2]. With an attempt to reduce the usage of animals for study, also to reduce time and cost, as a pre-clinical wound healing study using the drug. Vrana (wound) is a disease which affects humans at all ages. The worldwide prevalence of chronic ulcers is around 1-2% of the world population and is growing at a rate of 3% annually and amounts to 3% of total health care expenditure[3]. In a study the prevalence of chronic wounds in the community was reported as 4.5 per 1000 population whereas that of Acute Wound was about 10.5 per 1000 population[4]. Acharya Sushrutha for the management of Vrana (wound) describes 60 different local and systemic therapeutic measures[5].

In 11-13th century, Shodhala Nighantu included Hamsapadi in Lakshmanadi varga, where the drug in Kashaya (decoction), Kalka (paste), Taila (oil) forms is stated to be beneficial in Vrana (wound), Nadi Vrana, Pootigandha.[6] Dhanvantari Nighantu, Madanapala Nighantu, Kaiyadeva Nighantu, Bhavaprakasha Nighantu mentions the use as Vranopaha. Hence, Hamsapadi (*A. lunulatum* Burm. f.), a freely available drug from family of fern has been selected for the evidence-based study to explore the local therapeutic measure of non-surgical debridement effectiveness in wound healing[7].

MATERIALS AND METHODS

Collection of Plant Materials

The fresh ferns Hamsapadi (*A. lunulatum* Burm. f.) authentic samples were collected from its natural habitat in the region of Udyavara, Udupi, Karnataka under the supervision of concerned subject expert and washed properly with double distilled water and dried. A known weight of plants was used for the preparation of Kalka (paste).

Taxonomical position of the plant

Scientific classification
Kingdom: Plantae
Division: Pteridophyta
Class: Pteridopsida
Order: Filicales
Family: Adiantaceae
Genus: *Adiantum*
Species: *lunulatum*

Preparation of Hamsapadi (*A. lunulatum* Burm. f.) Kalka (paste)

Dravyam aardram shilapistam shushkam va sajalam bhaveeth|
Prakshepa aavaapa kalkastu tanmanam karshasamhitham ||

A green drug is converted into a paste by rubbing it on a stone. If the drug is dry add a little quantity of water to make Kalka.

Prakshepa, Avapa-both of these are the synonyms of Kalka dravya.[8]

The fresh Kalka (paste) was prepared as per classical method from Rasashastra and Bhaishajya Kalpana laboratory of SDM Ayurveda College, Kuthpady, Udupi, Karnataka, India and used for cytotoxicity and wound healing activities using HEK293, fibroblasts and muscle cell lines.

Cytotoxicity screening of Hamsapadi (*A. lunulatum* Burm. f.) Kalka (paste) by MTT assays

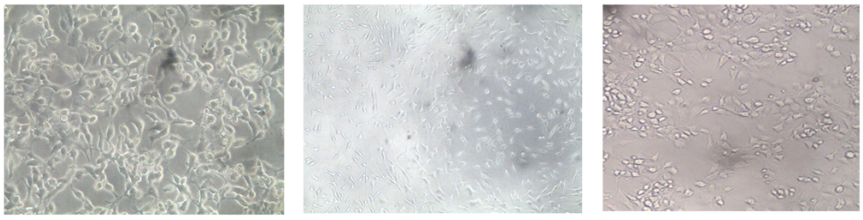
Human Embryonic Kidney 293 (HEK 293) cell line was procured from NCCS, Pune and subcultured at department of Biotechnology, SDM Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi. The confluent Human Embryonic Kidney (HEK) 293 cell line was selected and trypsinized the cells using 0.25 % trypsin. The cells were washed twice with Phosphate buffered saline (PBS) and centrifuge at 800 rpm speed. Further the cells were re suspended in suitable medium (medium with 10 % fetal bovine serum). The cells were counted using haemocytometer. Around 10,000 cells were plated to 96 well plate and incubated the plate at 37 ºC in CO_2 incubator for 24 hours. After 24 hours carefully old medium was discarded from 96 well plate. Different volumes of Hamsapadi (*A. lunulatum* Burm. f.) Kalka (paste) was mixed in serum free medium and added to the different test groups. The plates were incubated for 48 hours at 37 ºC in CO_2 incubator. After completion of incubation time the old medium was discarded and carefully washed the cells with Phosphate buffered saline. Add 20 µL of MTT dye (5 mg/mL in PBS) to all the wells and cover the plate with aluminum foil and incubate in CO_2 incubator for 4 hours. After 4 hours 100 µL of acidified Isopropanol was added to all the wells and mixed it by careful shaking. Using multiwell plate reader the OD was recorded at 540 nm (or 540 nm with reference to 630 nm). The percentage viable cells were calculated using following formula: [9]

% of viable cells = [(Test sample-blank) / (Control-blank)] x 100

Wound healing activity of Hamsapadi (*A. lunulatum*) Kalka (paste) using Fibroblast (McCoy) and Muscle (L6) cell lines (Cappiello *et al*., 2018)

Fibroblast (McCoy) and Muscle cell line (L6) were procured from NCCS, Pune and sub-cultured at department of Biotechnology, SDM Centre for Research in Ayurveda and Allied Sciences, Kuthpady, Udupi. Around 70-80 % confluent cell line were selected, and old medium was removed and washed twice with phosphate buffered saline (PBS) separately. The cells were treated with 100 µl of 0.25 % trypsin and incubated at 37 °C incubator for about 3 to 5 minutes. After incubation time, the excess trypsin was removed using pipette and the floating cells were transferred to new fresh 15 ml centrifuge tube containing fresh medium and centrifuged for 5 minutes at 800 rpm. After centrifugation the old medium was removed, and the cells were washed with PBS and centrifuged for 5 minutes at 800 rpm. After centrifugation the PBS was removed carefully, and cell pellet is re-suspended in fresh medium with 10% fetal bovine serum. The cells are counted using haemocytometer. Around 50,000 cells are transferred to 60 mm or 100 mm Petri dish and incubated for 24 hours supplemented with standard medium. After incubation time, a wound was created on cell line using a sterile needle and the old medium was removed and cells were washed with PBS. Different concentrations of selected drug Hamsapadi (*A. lunulatum* Burm. f.) Kalka (paste) was prepared using serum free medium and added to Petri dishes respectively and incubated for 48 hours to check the healing activity. One set of cells were treated with routine normal medium which was considered as the control group. After incubation time the old medium was removed, and the cells were washed twice with phosphate buffer saline. The cells were fixed with one ml of 4% formaldehyde followed by stained with 0.05% of Crystal / gentian violet solution and incubated for 20 minutes at room temperature. After incubation time the excess stain was carefully removed, and the Petri dishes are carefully washed with PBS and dried at room temperature. The area of wound before and area of healing after treatment is measured under inverted microscope using Motic software.

RESULTS



HEK293 cell line **Fibroblast (McCoy)** **Muscle cell line (L6)**

Figure 1: HEK293 cell line, Fibroblast (McCoy), Muscle cell line (L6)

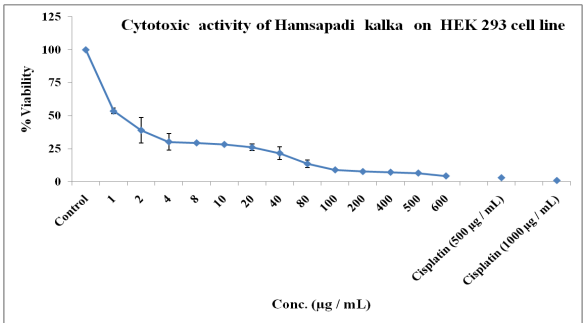


Figure 2: Cytotoxic activity of Hamsapadi Kalka (paste) on HEK293 cell line (IC50 = ≈1.173 µg)

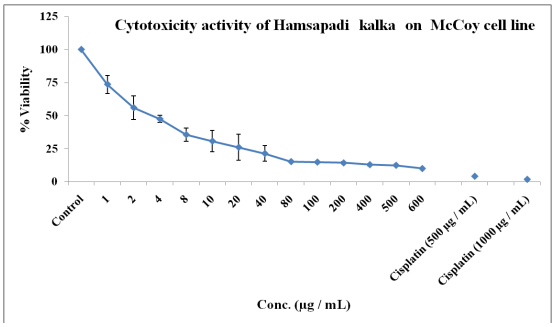


Figure 3: Cytotoxicity activity of Hamsapadi Kalka (paste) on McCoy cell line (IC50 = ≈3.66 µg)

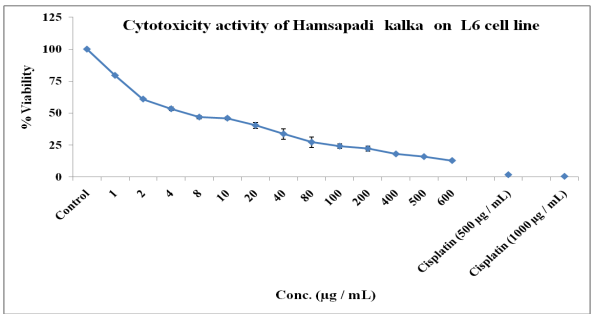


Figure 4: Cytotoxicity activity of Hamsapadi Kalka(paste) on Muscle (L6) cell line (IC50 = ≈6.307 µg)

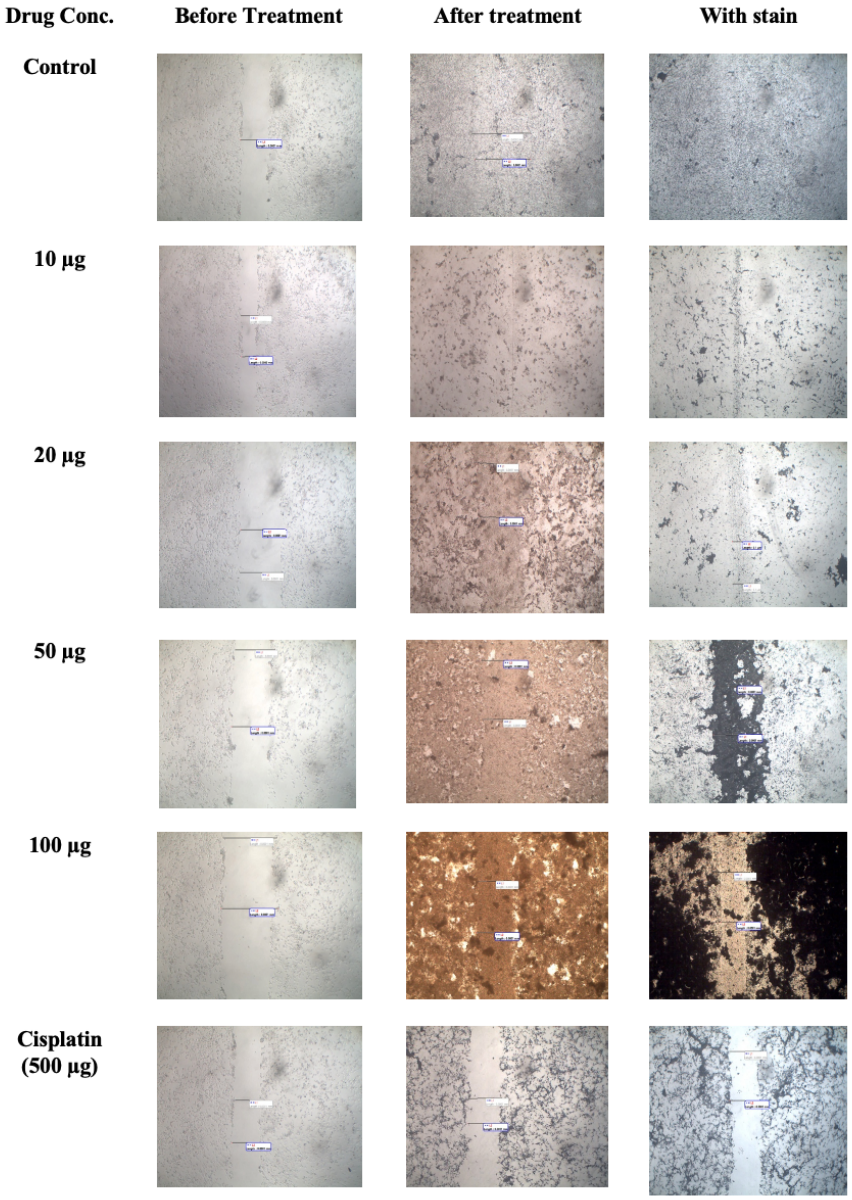


Figure 5: Wound healing activity of Hamsapadi Kalka (paste) on Muscle (L6) cell line

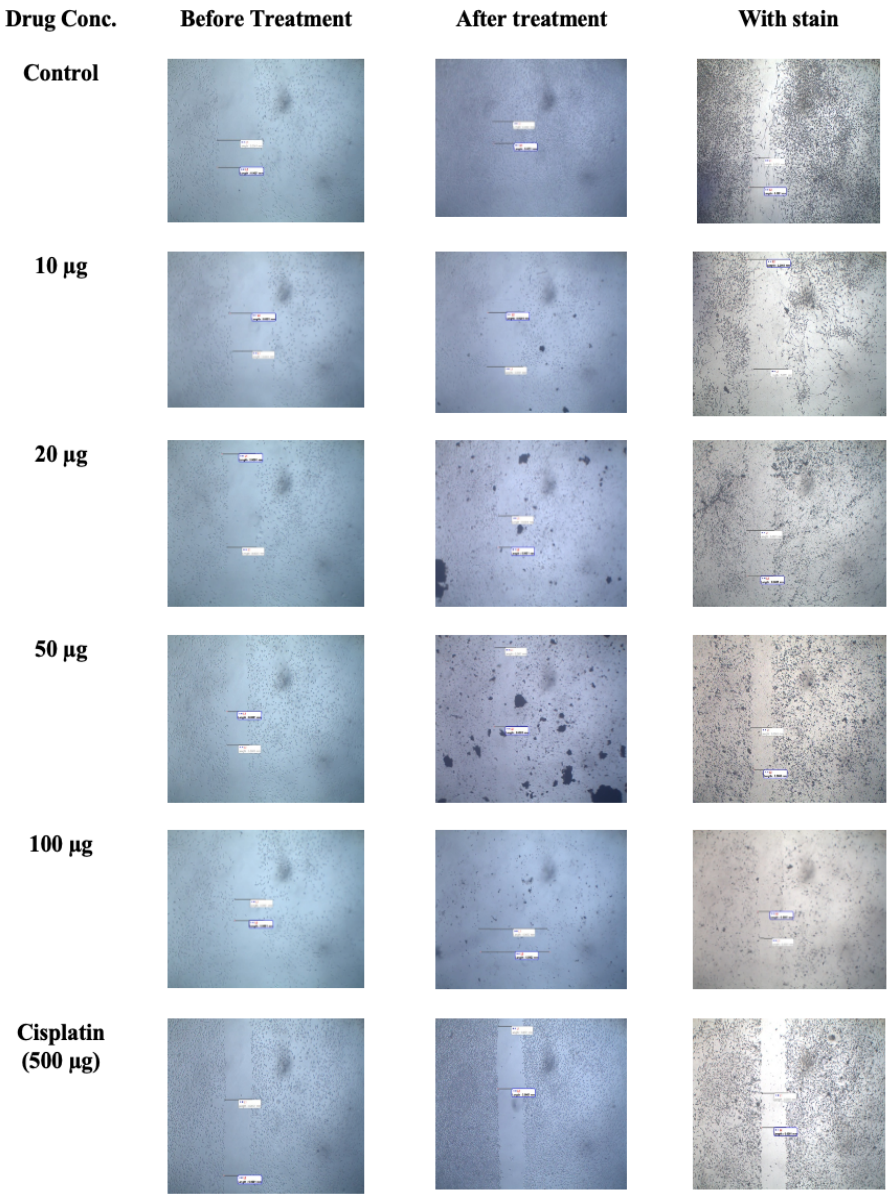


Figure 6: Wound healing activity of Hamsapadi Kalka (paste) on Fibroblast (McCoy) cell line

DISCUSSION

The cytotoxicity of Hamsapadi Kalka (paste) was screened using different doses of Hamsapadi Kalka (paste) (1 to 600 µg/mL) against HEK293 cell line (Figure 4). The IC_{50} value was 1.173 ug. As the concentration of Hamsapadi Kalka (paste) increased it showed increased toxicity to normal HEK293 cell line. The cytotoxicity of Hamsapadi Kalka(paste) was screened using different doses of Hamsapadi Kalka (paste) (1 to 600 µg/mL) against McCoy cell line (Figure 5). The IC_{50} value was 3.663 ug. As the concentration of Hamsapadi Kalka (paste) increased it showed increased toxicity to McCoy cell line. The cytotoxicity of Hamsapadi Kalka (paste) was screened using different doses of Hamsapadi Kalka (paste) (1 to 600 µg/mL) against L6 cell line (Figure 6). The IC_{50} value was 6.307 ug. As the concentration of Hamsapadi Kalka (paste) increased it showed increased toxicity to L6 cell line. In between 3 cell lines, overall Hamsapadi Kalka (paste) showed more cytotoxic to HEK293 cell line, moderate to McCoy and less toxic to L6 cell line.

Muscle (L6) and Fibroblast (McCoy) cell lines were used to check the wound healing activity of Hamsapadi Kalka (paste) with different concentrations ranging from 10, 20, 50 and 100 µg / mL. The cisplatin (500 µg/mL) was used as positive control which will inhibit the growth of cells. Muscle (L6) cell line (Figure 7) showed more wound healing rate compared to Fibroblast (McCoy) cell line (Figure 8) with different doses of Hamsapadi Kalka(paste). Healing of an ulcer is a complex phenomenon which depends upon multiple factors, by realizing this Acharya Sushrutha mentioned 60 Upakrama [modalities of treatment] which are targeted to facilitate these 'Phases of ulcer healing kalka [paste] is one of the important Upakramas [modalities of treatment], among the 60 Upakarmas[modalities of treatment] and kalka [paste] can be used for both vrana Sodhana [cleaning of ulcer] and Vrana Ropana [healing] depending upon the drug. Here Hamsapadi has the Karma [action] of both Sodhana [cleaning of ulcer] and Ropana[healing]. Hence, we have selected ropana [healing] concept in this study.

Hamsapadi having Tikta [bitter], Kashaya [astringent] and Madhura [sweet] Rasa, helps in Shamana [pacifying] of Pitta and Rakta with its Tikta [bitter] and Madhura [sweet] Rasa. It causes Kapha Shamana with its Tikta [bitter] and Kashaya [asrtringent] Rasa, hence helping to reduce the exudate. Madhura [rasa] Rasa helps in alleviating Vata Dosha thus maintaining moisture over the wound surface that promotes healing. Krimighna [anti-microbial] and Shothahara [anti-inflammatory] properties of the Hamsapadi should help in reducing microbial load, clearance of slough and favouring the inflammatory response towards optimized healing. In Hamsapadi the presence of Phyto steroids has been proven, which can help to reduce the pain and promote wound healing. The prolonged inflammatory stage is considered as one of the causes for the delay in ulcer healing. The neutrophils, monocytes, cytokines, proteases, will damage the newly forming granulation tissue. Previous analytical studies showed that the drug Hamsapadi has saponins, tannins, flavonoids as the active constituents. These helps in neovascularization and formation of collagen tissue helping in wound contraction and epithelialisation, thus causing Vrana Ropana.[10]

CONCLUSION

Hamsapadi is a drug that is easily available in nature, could be useful in non-surgical debridement and healing of ulcers with scientific evidence-based validation. This was an attempt to study the Shalayatantra exclusive concept of Vranaropana (wound healing), using a single drug Kalka (paste) formulation. Outcome of the research project can be used to heal DushtaVrana (Chronic non healing ulcers). The scientific experimentation with a proven efficacy will validate the ancient Ayurvedic Kalka (paste) Vrana Shodhanaropana Upakrama, boosts the confidence of Ayurveda, thus popularizing acceptability of Ayurvedic non-surgical management of ulcers to larger population of people. Overall Hamsapadi Kalka (paste) showed more cytotoxic to HEK293 cell line, moderate to McCoy and less toxic to L6 cell line. Muscle (L6) cell line showed more wound healing rate compared to Fibroblast (McCoy) cell line with different doses of Hamsapadi Kalka (paste) used.

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