Resveratrol - Loaded Microsponge Gel for Wound Healing: In vitro and In vivo Characterization

RUNNING TITLE: Resveratrol Microsponges Hydrogel for Wound Healing

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Abstract
Objectives: The study was aimed to formulate Resveratrol (RSV) loaded microsponges to deliver drug at the wound site and incorporate it in the Moringa oleifera gel base to provide an appropriate moist environment for wound management. RSV, a stilbenoid activates Sirtuins (SIRTs), cell-signaling regulators involved in the process of wound healing.

Materials and Methods: Microsponges were prepared by oil in oil emulsion solvent diffusion method by optimizing the independent variables; drug: polymer ratio and volume of internal phase solvent and their effects on entrapment efficiency and particle size. Formulation batches were evaluated for drug content, production yield, entrapment efficiency and in-vitro drug release. The microsponges were further incorporated into Moringa oleifera gum gel, which was then evaluated for spreadability, viscosity, ex vivo diffusion study and in vivo studies using excision wound model in rats.

Results: SEM revealed spherical and porous nature of the microsponges In vitro release study of the of optimized batch of RSV microsponges showed 80.88 % drug release within 8 h. DSC results revealed no drug and polymer interaction during formation of microsponges. Ex vivo diffusion study through goat skin revealed sustained release of RSV through porous microsponges embedded in the gel base at the wound site. In vivo study performed using excision wound model showed wound healing and closure within day 8. Histopathology showed increased re-epithelization and reduced ulceration in RSV microsponge gel treated group compared with Sham operated.

Conclusion: RSV microsponge gel delivered the drug at the wound site and the
gel base provided the moist environment and influenced cell adhesion thereby promoting faster wound healing.

**Keywords:** Resveratrol, Microsponges, Wound healing, *Moringa oleifera* gum, Excision wound model.

**Introduction**

Microsponges are polymeric drug delivery system composed of porous structure.\(^1,2\) These are tiny porous, sponge-like spherical particles with a surface area of 5 to 150 mm. The major advantages of microsponges are good entrapment efficiency with good stability at high pH and temperature. Due to their porous structure, they can extend the drug release.\(^3\) Emulsion solvent diffusion, suspension polymerization or oil in oil emulsion solvent diffusion methods are used for the formulation of microsponges.\(^4\) Microsponges encapsulate the drug and this technique of microencapsulation helps to control drug release rates and prolong the release time.\(^5\)

To formulate microsponges, one of the preferred polymer is Eudragit RL 100 to control the drug release of the formulation. Eudragit RL 100 is methacrylic acid esters possessing hydrophilic properties due to the presence of more amounts of quaternary ammonium groups as compared to Eudragit RS 100. This nature of Eudragit RL 100 helps to improve the water uptake capacity which is required for the rapid absorption of exudates from wound, maintaining its ability to preserve water required for wound healing.\(^6\) The cationic nature of Eudragit RL helps it to interact strongly with the negatively charged mucins via electrostatic attraction increasing its bio-adhesivity.\(^7\) Also, Eudragit RL 100 is reported to permit water vapour and oxygen permeation which is required for wound healing.\(^8\)

RSV (3,5,40-trihydroxy-trans-stilbene), a natural polyphenolic compound present in grape skin, peanuts and red wine.\(^9,10\) It belongs to Biopharmaceutical Classification System (BCS) class II and exhibits low solubility and high permeability.\(^11\) It is a non-flavonoid polyphenolic compound.\(^12,13\) The compound was first isolated from the root of Polygonum cuspidatum, a plant used in traditional Chinese and Japanese medicine.\(^14\) RSV was studied for its different activities viz. anti-inflammatory, immunomodulatory, cardioprotective, antioxidant, anticancer and also for promoting vascular endothelial function.\(^15,16\) It stimulates endothelial NO synthase (eNOS) activity and facilitate Vascular endothelial growth factor (VEGF) expression, thus providing vascular protection and improving the blood supply.\(^17\)

Lakshmanan R and co-workers reported RSV loaded nano-fibrous scaffolds accelerated wound healing process by regenerating dermal tissue.\(^18\) In a study carried out by Balan P, composite nano-fibrous scaffolds loaded with RSV and ferulic acid showed 100% wound closure within 15 days.\(^19\) RSV potentiates the activity of antioxidant enzymes Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) and thus have positive influence on wound healing.\(^20\) RSV loaded microparticles distributed in collagen laminin matrix scaffold showed improved wound healing without serious inflammatory response.\(^21\) Zhao P and co-workers reported in their study that the direct local application of RSV in dorsal skin wound bed tissue activated AMP-activated protein kinase (AMPk) signaling pathway resulting in rapid wound healing through effective vascularization.\(^22\) Considering the beneficial effects of RSV in the wound healing process, the study was carried out to formulate RSV microsponges to promote wound healing.\(^9\)
Moringa oleifera gum is natural gum obtained from exudates of Moringa oleifera plant. It is hydrophilic plant polymer acting as emulsifier, gelling agent, suspending agent, thickener and stabilizer. Being a natural gum, it is biocompatible, nontoxic, environmental friendly and economic to cost and biodegradable in nature. It has antimicrobial activity against various strains of bacteria. Arabinogalactan, a polysaccharide present in the Moringa oleifera gum has been reported to stimulate cell proliferation, which in turn promotes tissue re-epithelialization and reorganization of the tissues at the wound site.

The current scenario focuses on treating the wound at economical cost with easy applicability and no pain to patient. Most of the microorganisms are resistant towards the current synthetic drug and therefore a need arise to explore natural drug with minimum dose and maximum effect. The synthetic gel formulations for wound healing cause burning sensation produce rashes on the skin and even damage the skin around the wound area. Effective wound healing requires that the active ingredients should be delivered in high concentration at the target site or the contact time of active ingredient on the surface of skin or within epidermis should be increased, thereby preventing its penetration inside the skin as well eliminating the skin ailments associated with the synthetic agents. To achieve this, porous microsponges loaded with active ingredient prove to be the best choice, which can be incorporated into gels, creams, lotions and powders. Microsponges also help to modify the drug release rate, thus reducing the frequency of application of dosage form and improving patient compliance. Controlling the rate of moisture at the wound site locally, through the application of gel is important criteria required for faster wound healing. Hence, the work was carried out with an aim to formulate RSV-loaded microsponges incorporated into the gel base of Moringa oleifera gum to make the drug available at the wound site.

The hypothesis of the research work is to achieve the dual advantage of sustained release of drug due to its entrapment, in the porous structure of the microsponges as well as the benefits of Moringa oleifera gel base for efficient wound healing without any irritation and damage to the skin around the wound site.

Materials and Methods
Materials
Herbo Nutra Chemical Supplier, New Delhi, India, supplied RSV. Evonik India Pvt. Ltd. Mumbai, India gifted Eudragit RL-100. Moringa oleifera gum was collected from local market. Magnesium stearate, acetone, n-hexane, methyl paraben, propyl paraben, propylene glycol, triethanolamine were purchased from Loba chemie, Mumbai, India

Methods
Preparation of microsponges
Oil in oil emulsion solvent diffusion method was used for the preparation of microsponges. The internal phase consisted of RSV, Eudragit RL100, magnesium stearate (Loba chemie, Mumbai, India) and acetone (Loba chemie, Mumbai, India). Appropriate ratio of drug and polymer were dissolved in acetone, magnesium stearate (3% w/v of solvent) was added to it, which was then sonicated in ultrasonic bath 70 kHz frequency for 20 to 25 min (Bio-Techniques, Mumbai, India) to get homogenous dispersion. Magnesium stearate in appropriate concentration acts as a droplet stabilizer in the formulation of microsponges, it reduces interfacial tension between light liquid paraffin and formed microparticles of Eudragit RL 100 and thus prevents flocculation resulting in the formation of stable discrete microsponges. The obtained internal phase solution was then poured drop wise into cold liquid paraffin (external phase) and stirred at 800 rpm for 1 h using overhead mechanical stirrer (EMTEK Instruments, Mumbai, India). Lastly, the solidified
microsponges were filtered and washed with n-hexane to get the porous rigid structure, air dried at room temperature for 12 h and stored in desiccators for further study.29,30

Selection of formulation parameters
In the preliminary trials, the effect of formulation parameters; ratio of drug: polymer (1:2, 1:3 and 1:4), volume of acetone (5, 7.5 and 10ml), volume of light liquid paraffin (15ml and 30ml), volume of magnesium stearate (1.5, 3 and 5% w/v of internal phase solvent) and stirring speed (600 and 800 rpm) for a time period of 90 min were evaluated on the formulation aspect of the microsponges.

Experimental design
Based on the preliminary results, it was found that the concentration of drug: polymer ratio (X1) and volume of internal phase solvent (X2) were the critical parameters governing the drug entrapment efficiency (Y1) and particle size (Y2). To further optimize these parameters, 3² full factorial design (Design Expert 11.0., Stat-Ease Inc., Minneapolis) was adopted to optimize the microsponge formulation (Table 1).

Determination of λmax in Ultra violet spectroscopy (UV)
For preparation of stock solution (1000µg/ml), 10 mg resveratrol was dissolved into 10 ml of methanol. 0.1 ml stock solution was further diluted with methanol to get 10µg/ml. The spectrum was scanned over the range of 200-400 nm. The standard calibration curve for RSV was then plotted at different drug concentrations (0.2-10 µg/ml) absorbance was measured using UV spectrophotometry

Characterization of microsponges
Production yield
Production yield was calculated by using equation (1) and carried out in triplicate.31

\[
\text{Production yield} = \frac{\text{Practical mass of microsponges}}{\text{Theoretical mass (polymer + drug)}} \times 100 \ldots (1)
\]

Drug content and entrapment efficiency
RSV microsponges (10mg) were dispersed in methanol (5 ml), followed by shaking for 10 min using vortex mixer and the final volume made up to 10 ml using methanol. The resulted solution was filtered, diluted and the concentration of RSV was determined spectrophotometrically using a UV spectrophotometer (1800, Shimadzu, Japan) at λmax 305.80 nm against blank (methanol), and tests were performed in triplicate using equation (2) and (3):

\[
\text{Drug content} = \frac{\text{Amount of drug in microsponges}}{\text{Amount of Microsponges}} \times 100 \ldots (2)
\]

\[
\text{Drug entrapment efficiency} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \times 100 \ldots (3)
\]

Particle size
Particle size of RSV loaded microsponges was evaluated by optical microscope and repeated three times to calculate the mean particle size. Approximately 50 microsponge particles were randomly measured. Edmondson’s Equation was used to estimate the average particle size of the microsponges

\[
D_{\text{mean}} = \frac{\sum_{n}^{nd}}{n} \ldots (4)
\]

Where n= number of microsponges counted
\ d = mean size range
**In vitro drug release study**

In vitro release studies of RSV loaded microsponges were carried out in USP dissolution test apparatus (Type II paddle) (Electrolab India PVT.LTD). An accurately weighed amount of RSV loaded microsponges (100mg) were placed in the dissolution test apparatus containing 900ml phosphate buffer pH 7.4 maintained at 37±0.5°C. An aliquot of 2 ml sample was withdrawn at definite time intervals for a predetermined time upto 8 h. The samples were analyzed for the drug content spectrophotometrically at $\lambda_{max}$ 305.80 nm. Each time after withdrawal of sample, the aliquots were replaced with same buffer solution pH to maintain sink condition.

**Scanning electron microscopy and Differential scanning calorimetric analysis of RSV microsponges**

The morphology of RSV microsponges was assessed by scanning electron microscopy (JEOL, JSM-6360-A, Tokyo, Japan) and the thermal analysis was carried out using Differential Scanning Calorimeter (Mettler Star SW 12.10, Mumbai, India).

**Formulation of microsponges loaded gel**

Moringa oleifera gum (4%) was soaked in water for a period of 24 h, followed by drying at room temperature. The dried gum was washed with acetone to remove any impurities and then passed through sieve number 45. The purified powdered Moringa oleifera gum was soaked in water and the mixture was stirred at 600 rpm to obtain a uniform dispersion. To, the obtained homogenous dispersion, methyl paraben (1%), propyl paraben (0.05%), and propylene glycol (5%), were added. The pH of the Moringa oleifera gel base was adjusted with slow addition of triethanolamine, followed by the incorporation of RSV microsponges into the gel base. Microsponges equivalent to 4% w/w of RSV were added into all the three batches of the gel base.

**Characterization of microsponges loaded gel**

The gel formulations were characterized for visual appearance, color, odor and pH.

**Measurement of pH**

The pH of the RSV microsponge gel was evaluated with digital pH meter (Digital Instrument Corporation, Ahmedabad, Gujrat). The readings were recorded as the mean of three readings.

**Measurement of viscosity**

Brookfield viscometer (Brookfield Engineering Corporation, Ametek, Mumbai, India) was used to measure the viscosity of the RSV microsponge gel. The gel was placed in a beaker and viscosity was measured using spindle number 64 at 10 rpm after a time period of three minutes. The readings were recorded as the mean of three readings.

**Texture profile analysis**

Texture Analyzer (CTX, Brookfield, Ametek, Mumbai, India) was used to carry out the texture profile analysis of RSV loaded microsponge gel. The sample holder of a cone analytical probe (TA3/100) (30 mm diameter, 60°) was completely filled with gel, followed by forcing the cone down into sample holder (1 mm/s and depth of 10 mm). Once, the trigger force of 5 g was achieved, the cone started to pierce the sample at speed of 2 mm/s to depth of 25 mm. After achieving the penetration upto a specified distance, the probe (cone) departed from sample. The obtained force -time plot was used to determine the hardness and the adhesiveness of the gel.

**Ex vivo diffusion study**

The goat skin membrane was obtained from local slaughter house and washed with water, scalpel was used to remove non-dermatome skin. Then the skin membrane was soaked in phosphate buffer pH 7.4 for 24 h and then placed on the Franz diffusion cell (DBK diffusion cell, Peliyagoda, India). A predetermined amount of microsponges loaded gel (1 g) was placed on the
The receptor medium was filled with phosphate buffer pH 7.4, maintained at 37±0.5°C and continuously stirred at 300 rpm. To assess the amount of drug diffused, samples (2ml) were collected from the receiver compartment at specific time intervals. An equal volume of fresh phosphate buffer pH 7.4 solution was used to replace the solution to maintain the sink condition. Collected samples were evaluated by UV spectrophotometer at $\lambda_{max}$ 305.80 nm. The readings were recorded as the mean of three readings.

**In vivo studies**

Healthy Albino Wistar rats of either sex weighing between 180-220 g were used for study. The study protocol was approved by the Institutional Animal Ethics Committee (Reg. no. 1239/PO/Re/S/09/CPCSEA) and IAEC protocol number RSCPR/IAEC/2019/06. Animals were procured from National institute of bioscience, Pune and house in animal house of JSPM’s Rajarshi Shahu College of Pharmacy and Research, Tathwade, Pune, maintaining at 10-12 h light and dark cycle, provided with 23°C±2°C temperature and 44-50% humidity with food and water ad libitum during the study.

**Excision wound model**

The rats were divided into four groups containing 6 rats each ($n=6$). Group I Sham operated group, Group II represents placebo control group received *Moringa oleifera* gel base, Group III is treatment control group received RSV loaded microsphere gel and Group IV represents standard control group received Megaheal gel- colloidal silver (ARISTO pharmaceuticals Pvt. Ltd, Mumbai, India)

The dorsal furs of animals were removed using depilatory cream (Veet hair removal cream, Reckitt Benckiser (India) Ltd.) and anesthetized using pentobarbital sodium (40 mg/kg, i.p., body weight). An impression was made and wound approximately of 12 mm (and 2 mm depth of full thickness was marked and created using forceps, surgical blade and scissors).

Treatments were started immediately after creating wound on day 0, by daily application of above mentioned gel formulations on wounded area. Day 0 considered to be a wounding day when the wound was created first. One g of each, of formulated gels and marketed gel mentioned above was applied once daily from day 0 until complete healing to the respective Groups.

**Measurement of Wound Contraction**

The wounded area was monitored and measured using vernier caliper and percent contraction after every 4th day was calculated. The initial size of the wound was considered as 100% using the following equation (4).

$$\% \text{Wound contraction} = \frac{\text{Initial wound area} - \text{Specific day wound area}}{\text{Initial wound area}} \times 100 \ldots (5)$$

**Histology of wound granulating tissue**

All the rats were anesthetized and specimen sample from healed wound tissues were collected from each group and stored in 10% v/v formalin solution to carry out the histological examination.

**Statistical analysis**

All results of wound closure were presented as mean± standard deviation (SD) and analyzed using two way analysis of variance (ANOVA) followed by Bonferroni post hoc test using Graph Pad Prism 5.0 to determine the statistical significance ($P \leq 0.001$) were considered as statistically significant.

**Results and Discussion**

**Preparation of microsponges**
Oil in oil emulsion solvent diffusion method was used for formulating the microsponges. The results from the preliminary trial batches pointed out the appropriate parameters required for the formulation of microsponges were the concentration of light liquid paraffin (30 ml), concentration of magnesium stearate (3%w/v) and stirring speed of 800 rpm for a time period of 90 min. The critical parameters affecting the drug entrapment efficiency (Y1) and the particle size (Y2) of the microsponges were identified as drug: polymer ratio (X1) and the volume of solvent (acetone) as an internal phase (X2) which were further optimized using the $3^2$ full factorial design. Optimization technique was utilized to get desired concentration of drug: polymer ratio and volume of internal phase solvent to formulate microsponges with desired characteristics as shown in (Table 1).

**Determination of $\lambda_{max}$ in UV**
A solution of 10μg/ml of resveratrol in methanol was scanned in the range 200-400 nm. The UV scan of the drug is shown below (Fig. 1). The $\lambda_{max}$ of the resveratrol was found to be 305.80 nm. The calibration curve for RSV was linear in the range of 2-10 μg/ml confirming the Beer-Lambert's law with the regression co-efficient value (0.9994)

**Drug entrapment efficiency**
The drug entrapment efficiency of RSV loaded microsponge formulations ranged from 59.36±1.35% to 91.75±1.69%.

Formulation F9 showed entrapment efficiency of 91.75%. The ratio of polymer concentration and the volume of acetone in the formulation F9 was 1:4 and 10 ml respectively. With the increase in concentration of polymer, entrapment efficiency was increased due to more amount of polymer available to entrap the drug. Also, as the volume of internal phase was increased, viscosity was found to be decreased, resulting in uniform mixing of drug and polymer, forming a matrix which in turn enhanced drug entrapment efficiency of the microsponges. As the concentration of polymer and the volume of internal phase decreased, entrapment efficiency also decreased and was found to be 59.36%.

The statistical analysis by design expert software (Design expert 11.0., Stat-Ease Inc., Minneapolis) indicated the effect of factors influencing the entrapment efficiency. The effect of both independent variables (X1 and X2) on entrapment efficiency (Y1) was given by following equation:

$$Y_1 = 30.48944 + 2.80X_1 + 5.00867X_2 \ldots (5)$$

Linear regression equation was obtained for the response of drug entrapment efficiency (Y1) which indicated positive effect of X1 and X2 on production of microsponges with good fit R$^2$ value 0.979 and significant F value 103.63 and p value <0.0001.

From equation 5 and Fig.2 (a); the factor X1 showed positive value indicating high drug entrapment efficiency in microsponges with the higher concentration of polymer. Also, with increase in the polymer concentration, the diffusion rate of internal solvent from the microsponges decreased. This in turn led to formation of concentrated solution resulting in more time for droplet formation with increased precipitation of drug in microsponges leading to increase entrapment efficiency.

The positive effect of X2 was observed on entrapment efficiency. The high volume of internal solvent attributed to better solubilization of drug in internal solvent, resulting in enhanced entrapment efficiency of the microsponges.

**Particle size**
Polymer concentration and volume of internal phase solvent are the major attributes determining the particle size of microsponges. The particle size of the microsponge formulation ranged from 432 to 586 μm.
Decrease in particle size could be attributed to the viscosity of emulsion formed during processing. With the increase in the volume of the internal phase, the viscosity of emulsion decreased, resulting in reduction in globule size of emulsion droplets, leading to the formation of smaller particles and vice versa.\(^4\)

The effect of (X1) and (X2) on particle size of microsponges was explained by following equation:

\[
Y_2 = +549.11 + 22.33X1 - 58.53X2 + 6.75X1X2 - 0.6667X_1^2 - 32.17X_2^2 \quad \ldots (6)
\]

The equation (6) is a quadratic regression equation for the response Y2 with \(R^2\) value 0.9899 and significant F value 59.08, \(p\) value <0.0034.

According to equation (6), and Fig. 2; X1 showed positive influence on particle size due to higher concentration of polymer, resulting in increased particle size due to more amount of the polymer available to entrap the drug. X2 exhibited negative influence on particle size indicating that with the increase in the volume of internal phase, the viscosity of emulsion decreased, resulting in reduction in globule size of emulsion droplets, leading to the formation of smaller particles. The interaction terms X1 and X2 showing the combined effect on concentration of polymer and volume of internal solvent, have positive effect on particle size. The reason for this could be increase in the viscosity of internal solvent. It was observed that with the increase in the concentration of polymer, the emulsion globules can hardly be subdivided into smaller particles.\(^3\) The individual and the combined effect of the factors X1 and X2 on the response Y1 and Y2 are explained in the 3D surface graphs. Fig.2

Optimization of microsponge formulation

The numerical optimization method was used to optimize the microsponge formulation. The desirability plot obtained indicates the optimum conditions needed for the formulation of microsponges with desired attributes. The optimized formulation (F5) showed minimum particle size (460 μm), higher drug entrapment efficiency (87.93% ± 2.369) with desirability value of 0.915577.

Scanning electron microscopy (SEM) of RSV microsponges

Scanning electron microscopy of optimized batch of RSV microsponge (F5) showed uniform, spherical shape particle at 35X magnification and porous surface at 3000X magnification Fig.3.

Differential Scanning Calorimetry

RSV exhibited single sharp endothermic peak at 263°C. RSV loaded microsponges of batch F5 showed a broad endothermic peak at 215.90°C and physical mixture of RSV and eudragit RL100 at 247°C respectively Fig.4. RSV peak was not observed in RSV loaded microsponge formulation due to encapsulation of drug in matrix form of microsponges.\(^4\) This results demonstrated no interaction between drug and polymer during formation of microsponges.

In vitro drug release study of microsponges

In vitro drug release of formulation F4, F6 and F9 containing high polymer concentration showed delayed release of drug (84.54%, 82.25% and 79.05% respectively within 8 h); Due to high amount of polymer, the escape of drug required more time to escape from the pores of microsponge. Low concentration of polymer in formulation F2, F3 and F7 resulted in rapid release of drug (96.87%, 94.59% and 90.48% within 8 h) from microsponges. This fast drug release at wound site was not satisfactory as sustained release of the drug is desirable for wound healing.\(^4\) Formulation F1, F5 and F8 released 90.02, 80.88 and 86.82% drug within 8 h from the microsponges. Formulation F5 showed sustained drug release thus, selected for further study Fig.5. As the formulation F5 has higher amount of drug: polymer ratio. The more number of binding sites on the surface of Eudragit RL100 were available due to presence of ammonium
groups leading to the stronger interaction of the drug with polymer, which in turn prolonged the drug release.\textsuperscript{48}

\textbf{Evaluation of RSV microsponge loaded Moringa oleifera gel}

RSV microsponge loaded Moringa oleifera gel was smooth, free from grainy particles, with a pH close to 7.2 similar to wound pH,\textsuperscript{49} indicating easy applicability of gel on the wound site without any discomfort and irritation to the patient after application on wound area. The appropriate viscosity 15392±5.567 exhibited by the gel indicated the easy of applicability and retention of gel at the wound site. Texture profile analysis of gel showed hardness (firmness) 273 g, adhesiveness 1.4 mJ and adhesive force 79 g Fig.6 indicating the easy spreadability of RSV loaded microsponge gel. The \textit{in- vitro} drug diffusion studies of RSV microsponge loaded Moringa oleifera gel showed 23.17\% release in 3 h, 57.97\% in 6 h and 68.98\% in 8 h respectively, thus indicating sustained release of RSV through the gel matrix of the polymer. The reason for this could be the swelling of the gel which caused an increase in the diffusional path length, thereby sustaining the drug release.

\textbf{In vivo study}

Wound closure and contraction were assessed by image analysis visually Fig.7. The wound area and % contraction after topical application of all gel formulations (Moringa oleifera gel base, RSV loaded microsponge gel and Standard gel) are presented in (Table 2).

During the course of treatment the formulations was found to show its preliminary effect from day 4 to day 16 Fig.7. Group I (Sham operated) showed slight wound healing even after 16 days of study. Percent wound closure of group I on day 0, 4, 8, 12 and 16 was found to be 0\%, 4.41\%, 6.08\%, 10.25\% and 17.66\%, respectively. Group II referred as placebo treated group received (Moringa oleifera gel base) showed significant (P<0.001) increase in % wound closure on day 4, 8, 12 and 16 which was found to be 14.83\%, 30.41\%, 37.41\% and 45.83 respectively when compared with sham operated group. After a wound, the physiological reparative process of the body initiates which involves movement of adjacent epithelial cells to the injured area. Arabinogalactan, a polysaccharide present in Moringa oleifera is reported to influence this integrins recognition, thus influencing cell adhesion and affecting the healing process. The cell adhesion and the higher replication ability of the epithelial cells led to a faster stratification of the tissue and faster wound healing. Hence, on day 16, the percent wound closure in Group II (Moringa oleifera gel base) was 45.83\% as compared to Group I (Sham operated) which was 17.66\%. (P<0.001)

Gel containing RSV microsponges (Group III) showed its maximum significant effect (P<0.001) by wound contraction with respect to sham operated group that proportionally confers the healing process which showed similar contraction as that of standard control (Group IV) on day 12 and day 16. As per the rate of epithelization concerned, the RSV loaded microsponge gel showed its contributory role in accelerating epithelization (P<0.001) as compared to sham operated Group I and standard control Group IV.

Many researchers reported the role of polyphenolic compounds in promoting wound healing. The important events involved in the wound healing process are inflammation; cell proliferation and cell migration. Sirtuins (SIRTs) are NAD+ -dependent histone deacetylase which are reported to exhibit anti-inflammatory activity and stimulate cell proliferation and cell migration.\textsuperscript{50} RSV acts as an activator of SIRTs, which act as one of the therapeutic strategy to enhance, wound healing. The activation of SIRTs by RSV suppress the stimulation of TNF-α, which is an important cytokine causing inflammation and inactivates nuclear factor kappa B (NF-κB), a transcription factor which is major regulator of proinflammatory cytokine expression thus exhibiting anti-inflammatory effects.\textsuperscript{51,52} The activation of SIRTs by RSV enhances the production of nitric
oxide which is involved in re-epithelialization, neo-vascularization and collagen synthesis.\textsuperscript{53} The NO production also accelerates wound closure by recreation of keratinocyte proliferation.\textsuperscript{54} Group III showed significant (\textit{P}<0.001) increase in percent wound closure at day 4, 8, 12 and 16 which was found to be 70.58\%, 98.33\%, 99.33\% and 99.41\% respectively when compared with sham operated and standard control group. Reason for this could be the \textit{Moringa Oleifera} gel base which provided suitable moist environment required for wound management. In the moist environment the epithelial cells migrate more readily as compared to dry ones; also the growth factors are active, readily available and synthesized in moist environments.\textsuperscript{4} Arabinogalactan, a polysaccharide present in the \textit{Moringa oleifera} gum has been reported to stimulate cell proliferation, which in turn promotes tissue re-epithelialization and reorganization of the tissues at the wound site thus promoting faster wound healing.\textsuperscript{26,27} The prolonged release of RSV through gel and further through microsponges formulated with Eudragit RL 100 indicated the retention of drug in the porous structure of microsponges to slowly release it at the target site to heal wound. The mucoadhesive and the hydrophilic properties of Eudragit RL 100 permitted the retention of the RSV microsponges at the wound site ensuring the availability of higher concentration of drug at the wound area of skin as well as maintaining its ability to preserve the moisture required for wound healing. The solubility of RSV in phosphate buffer pH 7.4 might have also favored faster drug diffusion in wound area to achieve faster healing. Hence, the topical application of gel containing RSV microsponges in excision wound model in rats successfully closed and healed wound within day 8. It was observed that on day 4, drying of the wound was observed and the wound area was reduced to 0.355±0.009 in Group –III. It was further observed that on day 8 the wound was completely dried and the wound area was further reduced to 0.020±0.009. In control Sham Group I the area of the wound was 1.127±0.040 even on the day 8. In present study, RSV loaded microsponge gel showed prolonged drug release and retains on skin to accelerate wound healing process followed by wound contraction within 8-12 days, by reducing scar formation. Thus fulfilling the hypothesis of the use of microsponge technology, where higher concentration of entrapped RSV was available at wound area of skin with sustain release as well as the benefits of \textit{Moringa oleifera} gel for efficient wound healing with no irritation and damage to the skin around the wound.

\textit{Histopathology}

Histopathological study was done to observe any pathological changes in rat skin during application of formulated gels. The tissue samples of excision wound was given for histopathological studies on day 16 Fig.8a-8d. Platelet coagulation, cytokine and pro-inflammatory mediators, red blood cells, blood vessels, fibroblast, mast cell, fibrin thread, ulceration and development of newly formed epithelium in sham-operated group (Group I) was observed Fig.8(b). In the placebo treated skin (Group II) showed slight ulceration and inflammation. Start of fibroplasia- collagen synthesis and deposition and arrival of neutrophils macrophages and slight formation of epithelial bridge with newly formed epithelium were observed Fig.8(c). Group III showed rapid reduction in ulceration with reduced inflammatory cells, fibroplasia- collagen synthesis, granulation tissue formation, matrix formation and collagen fiber deposition, regenerating epithelium. Integrity of basement membrane was preserved. Healing by a process of epithelization was observed in Group III Fig. 8(c). In the standard Group IV Fig.8 (d) reveals, epithelization with angiogenesis, regeneration of epidermis, dermis, hair follicle and reduced inflammatory cells with no ulceration. Collagen fibrin cross linking and scar maturation was seen.
These results is in accordance with the previous studies, stated that RSV increases synthesis of collagen fibers, increased granulation by enhancing angiogenesis, scar formation and improvement in wound healing.\textsuperscript{9,55,56}

**Conclusion**

In conclusion, RSV loaded microsponge gel was successfully formulated and developed for wound healing. Microsponges were prepared by oil in oil emulsion solvent diffusion method using Eudragit RL100. Microsponges released drug in sustained manner at wound site through pores and *Moringa oleifera* gum gel provided moist environment in the later stages of wound healing. This unique combination of RSV loaded microsponges in *Moringa oleifera* gel demonstrated rapid wound healing and could be considered as easy to apply with no pain dosage form as well as a potential alternative to the current synthetic agents used in the treatment of wound healing thus improving the patient compliance and reducing the global burden of wound care.

**Ethical Approval**

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

**Acknowledgement**

Authors thank Herbo Nutra chemical suppliers, New Delhi, India for providing RSV and Evonik India PVT.LTD, Mumbai, India for providing gift sample of Eudragit RL100.

**Conflict of interest**

Authors declared ‘no conflict of interest’.

**References**

Figure 1. UV Spectrum of Resveratrol
Figure 2. Three dimensional surface plots of (a) Entrapment efficiency, (b) Particle size
Figure 3. Scanning electron microscopy of resveratrol microsponges (a) 200x magnification, (b) 3000x magnification
Figure 4. DSC thermograms of a. Resveratrol, b. Physical mixture and c. Resveratrol loaded microsponges
Figure 5. In vitro drug release of resveratrol loaded microsponges

Figure 6. Spreadability of resveratrol loaded microsponge gel
Figure 7. Photographs of wounds in rats in group I (sham operated): a wound created on day 0, b wound on day 4, c wound on day 8, d wound on day 12 and e wound on day 16; group II (Placebo): f wound created on day 0, g wound on day 4, h wound on day 12, i wound on day 16.

Figure 8. Histopathological sections of wound on day 16 of a. Sham operated, b. Placebo, c. Resveratrol loaded microsponge gel, d. Standard gel.

Table 1: Formulation of Microsponges Using 3² Factorial Design
<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Drug: Polymer (X₁)</th>
<th>Acetone volume (ml) (X₂)</th>
<th>Magnesium stearate (mg)</th>
<th>Actual drug content (%)</th>
<th>Production yield (%)</th>
<th>Entrapment efficiency (%) (Y₁)</th>
<th>Particle size (µm) (Y₂)</th>
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<tr>
<td>F1</td>
<td>3</td>
<td>7.5</td>
<td>225</td>
<td>35.12±1.43</td>
<td>66.58±1.41</td>
<td>72.71±1.92</td>
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<td>F2</td>
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<td>5</td>
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<td>27.12±1.43</td>
<td>56.11±0.76</td>
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<td>F3</td>
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<td>F4</td>
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<td>7.5</td>
<td>300</td>
<td>36.95±0.96</td>
<td>74.55±2.48</td>
<td>79.55±1.29</td>
<td>581±1.94</td>
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<tr>
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<td>150</td>
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<td>68.26±1.34</td>
<td>586±1.03</td>
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<td>75.2±1.50</td>
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<tr>
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<tr>
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<td>91.75±1.69</td>
<td>481±0.90</td>
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</tbody>
</table>

Each value is the mean of three observations

Table 2: Effect of Resveratrol Loaded Microsponge Gel on Wound Closure and % Wound Contraction in Excision Wound Model

<table>
<thead>
<tr>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
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</thead>
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<tr>
<td>Area (cm)</td>
<td>% contraction</td>
<td>Area (cm)</td>
<td>% contraction</td>
</tr>
<tr>
<td>Day 0</td>
<td>1.200±0.08 0</td>
<td>1.200±0.007 0</td>
<td>1.200±0.003 0</td>
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<tr>
<td>Day 4</td>
<td>1.147±0.32 4.41</td>
<td>1.022±0.008 14.83</td>
<td>0.353±0.009 70.58</td>
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<td>Day 8</td>
<td>1.127±0.40 6.08</td>
<td>0.835±0.009 30.41</td>
<td>0.020±0.009 98.33</td>
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<tr>
<td>Day 12</td>
<td>1.077±0.61 10.25</td>
<td>0.751±0.024 37.41</td>
<td>0.008±0.009 99.33</td>
</tr>
<tr>
<td>Day 16</td>
<td>0.988±0.15 17.66</td>
<td>0.650±0.007 45.83</td>
<td>0.007±0.008 99.41</td>
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</tbody>
</table>

All results of wound closure were presented as mean± standard deviation (SD). *** (P<0.001) to determine statistical significance