# Chemical composition and wound healing activity of methanolic leaf extract of *Hydrolea zeylanica* Vahl. by *in vivo* excision and incision models

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## Abstract

**Background:** *Hydrolea zeylanica* L. leaves are used traditionally for burns, wounds, antiseptic, and callus ulcers. **Objective**: The objective of the study was to discuss chemical composition and to evaluate the wound healing property of *H. zeylanica* L. methanol extract (HZLME) on rats using excision and incision wound models. **Materials and Methods**: Wistar albino rats (150-200 g) were divided into four groups (n = 6). Group I negative control (Water-soluble ointment base), Group II standard (povidone iodine [5% w/w] ointment), Group III Test I - HZLME ointment (5% w/w), and Groups IV Test II - *H. zeylanica* leaf aqueous extract ointment (5% w/w) were applied topically. In incision wound models, the treatment was given for 10 days from the day of wound; skin breaking strength was estimated in incision wound model. In excision wound model, treatment continued until wound is completely healed. Rate of wound contraction and period of epithelization was evaluated thereafter. The data were analyzed by one-way ANOVA, followed by Dunnett's test. **Results:** Gas chromatography-mass spectrometry chromatogram analysis of the HZLME showed several peaks indicating the presence of various phytochemical constituents. HZLME increased the rate of wound contraction, decreased the period of epithelization and increased skin breaking strength. **Conclusion:** The use of *H. zeylanica* in various skin diseases has been proved by this work, as it showed a wound healing potential.

Key words: Epithelization, gas chromatography-mass spectroscopy analysis, *Hydrolea zeylanica*, skin breaking strength, wound healing

# INTRODUCTION

A disruption in the cellular and anatomical structure of body tissue which includes skin, mucous membrane, deeply lying tissues or surface of internal organs ranging from abrasion, incision, puncture, laceration and closed injuries (soft-tissue damage) such as contusion, hematoma, and crush injuries is known as "wound." Healing of wounds starts from the moment of injury and can continue for varying periods depending on the extent of wounding, and the process can be broadly categorized into three stages; inflammatory phase, proliferate phase, and the remodeling phase characterized by a wide range of chemically coordinate cellular processes as well as hormonal influences which ultimately determine the strength and appearance of the healed tissue.<sup>[1-3]</sup> Current estimates indicate that almost 6 million people are affected from chronic wounds worldwide.<sup>[4]</sup> In the community, the prevalence of chronic wounds was reported as 4.5 per 1000 population, whereas that of acute wounds was nearly double, at 10.5 per 1000 population.<sup>[5]</sup> The unhygienic conditions in some third world countries is the main cause of this problem. Besides that, most people in developing countries who are suffering from an infected wound cannot

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**Received:** 12-03-2017 **Revised:** 28-03-2017 **Accepted:** 08-04-2017 afford to purchase modern drugs, which are very expensive and might have side effects. Balick and Cox<sup>[6]</sup> reported that only 1-3% of drugs listed in the Western Pharmacopoeia are intended for use on the skin and on wounds; in comparison, at least one-third of herbal remedies are for such uses. Hence, plant products are seen as alternative solutions to the problem of wound treatment in developing countries. In India, a large number of plants are used by folklore traditions for the treatment of wounds, cuts and burns and largely preferred because of their widespread availability and effectiveness. There are several reports stating that the extracts of various plants are used for wound healing properties.<sup>[4,7-14]</sup>

*Hydrolea zeylanica* (L.) Vahl. family *Hydrophyllaceae* is found throughout India in moist and swampy places.<sup>[15]</sup> It is an annual herb with procumbent and branching stems up to 30 cm long. The leaves are dark green, narrow and pointed at the tip and are arranged alternately on the swollen, spongy stems. The stems growing above water are firmer and sturdier.<sup>[16]</sup> It is also known as Koliary and used for antiseptic<sup>[17]</sup> and antidiabetic.<sup>[18]</sup> In our previous study, we performed preliminary phytochemical screening,<sup>[19]</sup> acute toxicity test and conducted anthelmintic,<sup>[20]</sup> and antiulcer<sup>[21]</sup> studies of *H. zeylanica* (L) Vahl. leaf methanolic extract in earthworms (*Pheretima posthuma*) and rats, respectively.

The present investigation was undertaken to screen wound healing potential of *H. zeylanica* leaf extracts in Albino Wistar rats, as the plant is traditionally used for skin diseases and wound healing.<sup>[18,22-26]</sup>

# **MATERIALS AND METHODS**

#### **Plant Material**

The leaves of *H. zeylanica* (L) belonging to the family *Hydrophyllaceae* were collected from local area of Chittoor district Andhra Pradesh (India). The plant was identified and authenticated by Dr. K. Madhava Chetty, Plant Taxonomist (IAAT: 357), Department of Botany, Sri Venkateswara University Tirupati, Andhra Pradesh, India. The plant bearing voucher No. 1012 (10/12/2014) was deposited at Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

### **Preparation of Extracts**

The leaves of *H. zeylanica* (L.) were shade dried and reduced to coarse powder in a mechanical grinder. The powdered material obtained was then subjected to successive extraction by hot percolation method using petroleum ether, chloroform, methanol, and distilled water in a soxhlet extractor.<sup>[27]</sup> The different extracts obtained were evaporated using a rotary evaporator to obtained semisolid mass. The extracts thus obtained were subjected to phytochemical screening and the *H. zeylanica* leaf methanolic extract (HZLME) and *H. zeylanica* leaf aqueous extract (HZLAE) was used for further studies.

## Gas Chromatography-Mass Spectroscopy Analysis (GC/MS)

Nowadays, the study of the organic compounds from plants and their activity has increased. The combination of the best separation technique (GC) with the best identification technique (MS) made GC-MS an ideal technique for qualitative analysis for volatile and semi-volatile bioactive compounds.<sup>[28]</sup> The HZLME was subjected for GC-MS analysis at Indian Institute of Chemical Technology, Tarnaka, Uppal Road, Hyderabad, Telangana, for the determination of bioactive compounds. GC-MS analysis of the samples was performed using 6890 GC with 5973 I MSD. Helium was used as the carrier gas and the temperature programing was set with initial oven temperature at 400°C and held for 3 min, and the final temperature of the oven was 4800°C with rate at 100°C. A 2 µL sample was injected with splitless mode. Mass spectra was recorded over 35-650 amu range with electron impact ionization energy 70 eV. The total running time for a sample is 20 min. The chemical components from the methanolic extracts of plants were identified by comparing the retention times of chromatographic peaks using quadrapole detector with NIST library to relative retention indices. Quantitative determinations were made by relating respective peak areas to total ion chromatogram areas from the GC-MS.<sup>[29]</sup>

#### **Formulation of Drug**

Water-soluble ointment base was prepared by mixing polyethylene glycol (PEG) 4000 (40%) and PEG 400 (60%) and was employed as control drug. The test drug formulations of HZLME and HZLAE were prepared separately by incorporating 5 g of the test extracts in 100 g of water-soluble ointment base. Povidone iodine (betadine ointment 5% w/w) was used as a standard drug for comparing the wound healing potential of HZLME and HZLAE.

#### **Experimental Animals**

TheAlbinorats(maleandfemale)weighingbetween  $150 \pm 10$  g were used in this study. The animals were maintained under standard laboratory conditions in polypropylene cages under 12 h light/dark cycle, controlled temperature ( $24 \pm 2^{\circ}$ C), fed with commercial pellet diet (Nutrivet Life Science), and water *ad-libitum* in an animal house approved by committee for the purpose and supervision on experiments on animals (Reg. No. 1534/PO/a/11/CPCSEA). All the animals were acclimatized to the laboratory environment for 10 days before commencement of the experiments. The experimental protocol was approved by the Institutional Animal Ethical

Committee (IAEC) No. IAEC/AUCOP/2017/13, Anwarul Uloom College of Pharmacy, New Mallepally, Hyderabad, Telangana, India.

#### **Preliminary Phytochemical Screening**

The qualitative chemical tests carried out for the identification of the different phytoconstituents present in the powered crude drugs by standard procedures. They are usually tested for the presence of alkaloids, flavonoids, phenols, tannins, cardiac glycosides, triterpenes, steroids and saponins.<sup>[30-32]</sup>

## **Draize Test**

In Draize test for skin irritancy, Albino Wistar rats (Male) weighing (150-200 g) with n = 6 per group were used, and the test substance are applied to the skin that is shaved and abraded. Patch made from two layers of light gauze was dipped in solutions containing different concentrations - 0% (Control), 2.5%, 5% and 10% of test extract (HZLME) prepared in PG: EtOH (7:3). In the special holder, the animals were immobilized during the 24 h patch exposure. On removal of the patches, the animals were observed for any sign of erythema or edema for 72 h. The observations were repeated after 72 h.<sup>[33]</sup>

#### **Evaluation of Wound Healing Activity**

The wound healing activity was determined by two experimental models namely excision wound model and incision wound model.

#### **Excision Wound Model**

Animals were anesthetized before and during formation of the wounds with light ether. Experimental rats were inflicted with excision wounds as elucidated by Morton and Malone.<sup>[34]</sup> The dorsal fur of the rats was shaven with an electric clipper, and the anticipated area of the wound to be created were outlined on the back of the animals with methylene blue using a circular stainless steel stencil. The skin of impressed area was excised to the full thickness to obtain a wound area of about 300 mm<sup>2</sup> and 2 mm deep. The animals were divided randomly into four different groups of six rats each.

- Group I negative control (water-soluble ointment base)
- Group II standard (povidone iodine (5% w/w) ointment)
- Group III Test I HZLME ointment (5% w/w)
- Groups IV Test II HZLAE ointment (5% w/w).

The wound closure rate was evaluated postwounding by tracing the wounds on days 0, 2, 4, 8, 12 and 16 using transparent paper and a permanent marker. Change in wound area was measured, indicating the rate of wound contraction. The wound areas were calculated and recorded using graph paper. The day of scar falling without any residual raw wound were determined as a period of epithelialization.<sup>[13]</sup>

## **Incision Wound Model**

Rats were anesthetized with light ether before wound creation, and the back of the animal was shaved and washed with spirit and two paravertebral long incisions were made through the skin at the distance of 2 cm. Wounds are stitched using 4-0 number silk surgical thread using a bend needle (No. 11) in interrupted sutures, 1 cm apart. The given sutures were removed on the 7<sup>th</sup> day. Wound breaking strength was analyzed on 10<sup>th</sup> postwounding day. The breaking strength was measured with a manually operated instrument in terms of weight.<sup>[35]</sup>

#### **Statistical Analysis**

The values are expressed as mean  $\pm$  standard error of mean. P < 0.01 was considered significant and is denoted as "\*". The data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison *post-hoc* test using GraphPad Instat version 3.10 for Windows, GraphPad Software, San Diego California USA www.graphpad.com.

## RESULTS

The preliminary phytochemical screening carried out on HZLME revealed the presence of phytoconstituents such as glycosides, flavonoids, sterols, tannins, terpenoids, and alkaloids. The results of phytochemical screening are summarized in Table 1. No signs of allergy (allergic spots or redness of skin) were observed on rat's skin during the skin irritancy test. There were no cases of wound infection in all the treated groups.

GC-MS chromatogram analysis of the HZLME [Figure 1] showed several peaks indicating the presence of different phytochemical constituents. On comparison of the mass spectra of the constituents with the NIST library, the five

Table 1: Preliminary phytochemical screening of leaf   of HZLME			
Phytochemical screening	Results		
Test for glycosides	+		
Test for flavonoids	+		
Test for sterols	+		
Test for saponins	-		
Test for terpenoids	+		
Test for tannins	+		
Test for phenolic acids	+		

+: Presence of the constituents, -: Absence of the constituents, HZLME: *Hydrolea zeylanica* L. methanol extract phytocompounds were characterized and identified and given in Table 2. The mass spectra of all the five phytochemicals identified in the HZLME are shown in Figures 2-6, namely, trimethylsilyl ether of glycerol, hexopyranose, benzene, 2,4-dimethyl-1-(1-methylpropyl), D-glucose pentaacetate, and hexadecanoic acid.

In the excision wound model, topical application of HZLME and HZLAE test extracts in the form of 5% w/w ointments at wound site produced significant wound healing effect. Treated excision wounds showed an increase in rate of

Table 2: Phytocomponents identified in the   methanolic extract of <i>H. zeylanica</i> by GC-MS				
Name of the compounds	Molecular formula	Molecular weight (g/mol)		
Trimethylsilyl ether of glycerol	C <sub>12</sub> H <sub>32</sub> O <sub>3</sub> Si <sub>3</sub>	308.63718		
Hexopyranose	$C_{6}H_{12}O_{6}$	180.15588		
Benzene, 2,4-dimethyl-1-(1 methylpropyl)	C <sub>12</sub> H <sub>18</sub>	162.27132		
D-Glucose pentaacetate	$C_{16}H_{22}O_{11}$	390.33928		
Hexadecanoic acid	$C_{16}H_{32}O_{2}$	256.42408		

GC-MS: Gas chromatography-mass spectroscopy, *H. zeylanica: Hydrolea zeylanica* 

wound contraction, leading to faster healing as confirmed by the increased healed area when compared to the control group. The results of this study are shown in Table 3. It indicates that both the test extracts possess wound healing potential, and the effects are comparable to that of standard. Among the test extracts, HZLME (5% w/w) ointment was found to be more effective than HZLAE (5% w/w) ointment. In the incision wound model, breaking strength was found to be higher in HZLME treated group 708.19 g and it was equipotent to standard drug povidone iodine group 710.22 g [Table 4]. In incision wound model, all the test drugs shown to have significant wound healing activity.

# DISCUSSION

The chemical composition of leaves extract was analyzed by GC/MS. Analysis of leaves extracts determined that five compounds were identified, which represented 80.98% of total extract. The extract contains glycosides, flavonoids, sterols, tannins, terpenoids, and alkaloids.

This study reveals that topical application of *H. zeylanica* leaf methanolic extract could significantly enhanced the rate of wound healing. *H. zeylanica* plays an important role in the wound healing process and protect tissues from oxidative damage. Wound healing mechanisms can be contributed to

Table 3: Wound healing effect of HZLME and HZLAE on excision wound model					
Treatment groups	Day 2	Day 4	Day 8	Day 16	Period of epitheliazation
Negative control	4.92±1.01	4.67±0.33	3.88±0.22	2.19±0.71	27±0.22
Standard	4.90±1.14	3.44±0.85	2.11±0.14	1.11±0.67**	19±0.55**
HZLME 5% w/w	4.26±0.88	3.31±0.77	2.19±0.65	0.79±0.28**	20±0.45**
HZLAE 5% w/w	4.95±0.11	4.44±0.38	2.88±0.29*	1.03±0.22*	21±0.22**

Values in each column represent progressive change in wound area in mm as mean±standard error for six animals in each group. \*, \*\* indicate P<0.05, P<0.01 respectively when compared to the control group. HZLME: *Hydrolea zeylanica* leaf methanolic extract, HZLAE: *Hydrolea zeylanica* leaf aqueous extract



Figure 1: Gas chromatography mass spectrometry chromatogram of Hydrolea zeylanica leaf methanolic extracts

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Figure 2: Trimethylsilyl ether



Figure 3: Hexopyranose



Figure 4: Benzene, 2,4-of glycerol dimethyl-1-(1-methylpropyl)

stimulate the production of antioxidants in wound site and provides a favorable environment for tissue healing<sup>[36]</sup> and wound healing effects can be due to up-regulation of human collagen I expression,<sup>[37]</sup> and an increase in tensile strength of the wounds.<sup>[38]</sup> Enhancement of healing activity was attributed to increased collagen formation and angiogenesis.<sup>[39]</sup>



Figure 5: D-Glucose pentaacetate



Figure 6: Hexadecanoic acid

Table 4: Wound healing effect of HZLME andHZLAE on incision wound model				
Treatment groups	Incision breaking strength (g)			
Negative control	388.86±2.68			
Standard	710.22±2.44*			
HZLME 5% w/w	708.9±1.44*			
HZLAE 5% w/w	700.31±3.21*			

Values are mean±standard error \**P*<0.001 versus control *n*=6 animals in each group. HZLME: *Hydrolea zeylanica* leaf methanolic extract, HZLAE: *Hydrolea zeylanica* leaf aqueous extract

Angiogenesis in granulation tissues improves circulation to the wound site thus, providing oxygen and essential nutrients for the healing process with enhanced epithelial cell proliferation.<sup>[40]</sup>

# CONCLUSION

The use of *H. zeylanica* in Indian traditional systems of medicine for various skin diseases has been proved by this work, as it showed a wound healing potential. These findings

could justify, at least partially, the inclusion of this plant in the management of wound healing in folk medicine. Since the role of methanol extract in wound healing is clearly defined, wound healing potential of *H. zeylanica* may be partly due to the antioxidant activity of the plant. Further experiments are needed to test the effect of this plant in the treatment of chronic wounds.

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