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Wound Healing Activity of *Hyptis suaveolens* (L.) Poit (Lamiaceae)

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ABSTRACT: *Hyptis Suaveolens* (L.)Poit is a traditional pubescent annual herb found throughout India. On the basis of its traditional use and literature references, this plant was selected for the screening of wound healing property. The leaves of *Hyptis Suaveolens* were exhaustively extracted by soxhlet apparatus with different solvents like petroleum ether, solvent ether, chloroform, alcohol and chloroform water in ascending order of the polarity. All the five extracts were subjected to antibacterial screening by using the cup plate method. The petroleum ether, alcoholic and chloroform water extract showed maximum zone of inhibition. So these extracts were taken for wound healing activity. The effect of petroleum ether, alcohol, and aqueous extract of leaves was evaluated in excision, incision and dead space wound healing models using Albino wistar rats. Among the extracts, petroleum ether extracts showed significant wound healing activity on all three wound models compared to other extracts by calculating the parameters, percentage closure of excision wound, period of epithelization, tensile strength, dry weight granulation tissue, breaking strength of granulation tissue and hydroxyproline content. Histopathological study of the granulation tissue of the petroleum ether extract treated group evidenced increased collagenation when compared to control group of animals. Presence of sterols, flavonoids, tannins in various extracts was also confirmed by preliminary phytochemical investigation, TLC and HPTLC methods.

KEYWORDS: *Hyptis Suaveolens*, wound healing activity, antibacterial activity, petroleum ether extract.

INTRODUCTION

Natural products are a source of synthetic and traditional herbal medicine and are still the primary health care system[1]. The presence of various life sustaining constituents in plants made scientists to investigate these plants for their uses in treating certain infective diseases and management of chronic wounds.

A wound is the result of physical disruption of the skin, one of the major obstacles to the establishment of infections by bacterial pathogens in internal tissues, which leads to loss or breaking of cellular and anatomic or functional continuity of living tissue[2].

Wound healing occurs in three stages: inflammation, proliferation, and remodeling. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction. In angiogenesis, new blood vessels grow from endothelial cells. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix by excreting collagen and fibronectin. Collagen, the major component which strengthens and supports extracellular tissue, contains substantial amounts of hydroxyproline, which has been used as a biochemical marker for tissue collagen[3]. In epithelialization, epithelial cells proliferate and spread across the wound surface. Wound contraction occurs as the myofibroblasts contract. Platelets release growth factors and other cytokines[4]. Chronic wounds are wounds that fail to heal despite adequate and appropriate care. Such wounds are difficult and frustrating to manage[5]. Current methods used to treat chronic wounds include debridement, irrigation, antibiotics, tissue grafts and proteolytic enzymes, which possess major drawbacks and unwanted side effects.

The plant, *Hyptis suaveolens* (L) Poit commonly known as *Wilayati tulsi* belongs to the family Lamiaceae and is an ethnobotanically important medicinal plant. The plant has been considered as an obnoxious weed, distributed throughout the tropics and subtropics. Almost all parts of this plant are being used in traditional medicine to treat

various diseases. The leaves of *H. suaveolens* have been utilized as a stimulant, carminative, sudorific, galactogogue and as a cure for parasitic cutaneous diseases[6]. Crude leaf extract is also used as a relief to colic and stomachache. Leaves and twigs are considered to be antispasmodic and used in antirheumatic and antisuporific baths, an antiinflammatory, antifertility agents[7], and also applied as an antiseptic in burns, wounds, and various skin complaints. The decoction of the roots is highly valued as appetizer and is reported to contain urosolic acid, a natural HIV-integrase inhibitor[8]. Fumes of the dried leaves are also used to repel mosquitoes and control insect pests of stored grains. Though there is no scientific evidence to support the wound healing effect of *Hyptis suaveolens*, tribal men continue to use the plant in the treatment of wound. The objective of this investigation was to ascertain the scientific basis for the use of this plant in the treatment of wound, using different wound models.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Hyptis suaveolens* (L) Poit. (Lamiaceae) were collected in flowering stage during late September from the natural population of Belgaum and authenticated by Dr. Harsha Hegde, Chief Botanist, Indian Council of Medical Research (RMRC), Belgaum branch, shade dried and powdered then passed from 40# mesh size.

Preparation of Various Extracts of *Hyptis suaveolens* (L) Poit.

Around 1 kg fresh shade dried leaves were powdered and extracted by hot percolation method by soxhlet apparatus with five liters of each petroleum ether, solvent ether, chloroform, alcohol, chloroform water and. The percolation process was continued until the extraction process was completed (indicated by fade coloured menstrum). All the extracts finally reduced to dryness at 40^{o}C by Rotovapour (Rotavapour Buchi, Switzerland). The traces of the solvents were removed by keeping the dried extracts in to a desiccator. The concentrated extracts were stored carefully for phytochemical investigation.

Preliminary Phytochemical Investigation

All the extracts were screened for the presence of various secondary metabolites like steroids, alkaloids, carbohydrates, proteins, flavonoids, tannins and glycosides using the standard methods.

Powder Analysis

The powdered crude drug was subjected to determination of pH, extractive value, total ash, water soluble ash, acid insoluble ash and loss on drying as per Indian Pharmacopoeia.

Microorganisms

The test microorganisms used for the antibacterial activity screening were 4 bacteria (2 Gram positive) – *Bacillus subtilis, Staphylococcus aureus*, (2 Gram negative) -*Pseudomonas aeruginosa*, *Escherichia coli*. These organisms were identified and procured from National Chemical Laboratory (NCL), Pune, India.

Antibacterial Activity

The agar diffusion method[9] was used to evaluate the antibacterial activity. Bacteria were cultured overnight at 37 ^{o}C in Mueller Hinton 10 µl Broth (MHB, Oxoid) and used as inoculum. A final inoculum, using 100 µl of suspension containing 10^8 CFV/ml of bacteria spread on Mueller Hinton Agar (MHA) medium.

The disc (6 mm in diameter) was impregnated with 10 µl of 200µl/ml, 150µl/ml, 125 µl/ml, 100 µl/ml, 75 µl/ml and 50 µl/ml of each extracts and for each organism placed on seeded agar. Streptomycin (200µl/ml, 150µl/ml, 125 µl/ml, 100 µl/ml,75 µl/ml and 50 µl/ml) was used as positive control bacteria. The test plates were incubated at 37 ^{o}C for 24h for bacteria depending on the incubation time required for a visible growth.

PHARMACOLOGICAL ACTIVITY

Experimental animals

Albino wistar rats of either sex (150-200g weight) and Swiss albino mice of either sex (18-22g weight) were procured from Venkateshwara Enterprises, Bangalore. The animals were divided into different groups comprising of six animals in each group. They were kept in polypropylene cages at 23±1^{0}C in 12:12h dark: light cycle, with free access to standard pellet feed (Chakan Oil Mill, India) and water *ad libitum*. This project was cleared by Institutional Animal Ethical committee.

Acute Oral Toxicity study

Swiss albino mice of either sex (18-22g weight) and of 90 days were used to determine the LD_{50} of various test extracts. The animals were fasted over night prior to the acute experimental procedure. The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD)[10]. The LD_{50} was found to be more than 5000 mg/ kg b.w. p.o. in acute toxicity testing. The therapeutic dose 500mg/ kg b.w. p.o. (ED_{50}) was calculated as 1/10 th of the lethal dose for the purpose of wound healing investigation.

WOUND MODELS

The animals were starved for 12h prior to wounding. Studies were carried out using ether-anaesthetized rats. The rats were divided into four groups (n = 6). Animals were depilated at the dorsal thoracic region before wounding. The first group served as control similarly second, third and fourth groups received alcoholic, petroleum ether and aqueous extract by oral route at a dose of 500 mg/kg body weight by oral route daily for 10 consecutive days in incision and dead space wound model and for 20 days in the excision wound model.

Excision wound model[11]

An impression was made on the dorsal thoracic region 1cm away from vertebral column and 5cm away from ear using a round seal of 2.5cm diameter on the anaesthetized rat. The skin of impressed area was excised to the full thickness to obtain a wound area of about 500 mm^2 diameters. Hemostasis was achieved by blotting the

wound with cotton swab soaked in normal saline. Contractions, which contribute for wound closure in the first two weeks, were studied by tracing the wound on a transparency paper initially. Then an impression was taken on a millimeter scale graph paper, scar area after complete epithelization and time for complete epithelization in days was evaluated to calculate the degree of wound healing. The parameters were studied were wound closure, epithelization time and scar features. The observation of the percentage wound closure were recorded on 4^{th} , 8^{th}, 12^{th}, 16^{th} and 20^{th} post wounding day and also for epithelization and size and shape of scar area.

Incision wound model

In the incision model[12], the rats were anesthetized by anesthetic ether and two longitudinal paravertebral incisions of 6cm length were made through the skin and cutaneous muscle at a distance of about 1.5cm from the midline on each side of the depilated back. After the incision, the parted skin was sutured 1cm apart using a surgical thread (No. 000) and curved needle (No. 11). The wounds were left undressed. The extracts were given by oral route once a day, till complete healing. The sutures were removed on eighth post-wound day. The skin-breaking strength of the 10-day-old wounds was measured by the method of Lee[13].

Dead Space Wound model

For the dead space wound four groups of six animals each were used. Dead space wound was made by implantation of polypropylene tube (0.5cmX2.5cm), beneath the dorsal Para vertebral skin. On the 10^{th} day the granuloma tissue form on the dead space wound was dissected and tensile strength was determined. The excess tissue was cut into two approximately equal halves. One of the granuloma tissues was dried in an oven at 60° C and the dry weight was noted. The granulation tissue so harvested was subjected to hydroxyproline estimation. Their weights were expressed as mg/100 gms body weight as suggested by Dispaquale and Meli[14].

STATISTICAL ANALYSIS

All the results were analyzed by One-way Analysis of Variance (ANOVA) followed by Dunnett's test. The level of significance was set at $P<0.05$.

Histopathological study

The healing tissues obtained on the 11th day from all four groups of animals of the incision wound model were processed for histological study to determine the pattern of lay-down for collagen. The amount of collagen was quantified using Vangeison stain.

RESULTS AND DISCUSSION

The average percentage yield of various extracts of *Hyptis suaveolens* is shown in table 1. Powder analysis parameters like pH, extractive value, total ash, water soluble ash, acid insoluble ash and loss on drying were determined on the powder of *Hyptis suaveolens.* The results of physicochemical characterization of *Hyptis suaveolens* are presented in table 2. As per the preliminary photochemical investigation, the major chemical constituents such as sterols were present in petroleum ether and solvent ether extract. The other constituents like flavonoids, tannins, glycosides, carbohydrates and proteins were found in ethanolic and chloroform water extract. Leaves of the plant possess alkaloids in chloroform extracts. The results of phytochemical screening are given in table3.

The LD_{50} and ED_{50} values for the various extracts of *Hyptis suaveolens* are summarized in table 4. The therapeutic dose 500mg/ kg b.w. p.o. was calculated as $1/10^{th}$ of the lethal dose for the purpose of wound healing investigation.

Antibacterial activity was done for all the five, pet ether, solvent ether, chloroform, ethanol and chloroform water extracts. During antibacterial study chloroform water, petroleum ether and alcoholic extracts showed maximum zone of inhibition against almost all organisms in cup plate method. So the chloroform, alcohol and petroleum ether extract were taken for wound healing activity. The results of antibacterial activity of various extracts of *Hyptis suaveolens* are shown in table 5.

The results of the excision wound model are given in table 6. In an excision wound model, petroleum ether extract at a dose 500mg/kg BW p.o. of *Hyptis suaveolens* showed significant wound healing activity (% wound contraction on 18^{th} day (97.225± 0.170, $P < 0.05$) compared to control (77.446± 0.406). It also showed complete epithelization (18.09± 0.213days $P < 0.05$) when compared to control (23.34± 0.222). The petroleum ether extract showed a scar area of (9.67± 0.486 mm^2, $P< 0.05$) as compared to control 15.2± 1.550mm^2.

The results of incision wound model are given in table 7. In incision study, the petroleum ether extract showed significant (294.69±5.540, $P< 0.05$) breaking strength when compared to control (143.62±8.084).

The results of dead space wound model are given in table 8. The tensile strengths of the granuloma tissue were determined by the water flow technique of Lee. Petroleum ether extract showed highly significant increase in breaking strength (370.66±5.637, $P<0.05$) when compared to control (156.35±8.803) and petroleum ether also showed significant increase in the dry weight of granulation tissue (74.00±1.29, $P<0.05$) as compared to control(42.33±2.28).Petroleum ether also showed significant increase in the hydroxyproline content (2512 ±21.09, $P<0.05$) as compared to control (1385±9.56).

Histological studies of the tissue obtained from the petroleum ether treated (Figure 1) group showed significant increase in collagen deposition, few macrophages, tissue edema and more fibroblasts. The histological studies of the granulation tissue of the control group of animals showed more aggregation of macrophages with lesser collagen fiber. The wound healing was more significant in petroleum ether treated group of animals.

DISCUSSION

Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound. It mainly depends on the repairing ability of the tissue, type and extent of damage and general state of the health of the tissue. The granulation tissue of the wound is primarily composed of fibroblast, collagen, edema, and small new blood vessels. The undifferentiated mesenchymal cells of the wound margin modulate themselves into fibroblast, which start migrating into the wound gap along with the fibrin strands. The collagen composed of amino acid (hydroxyproline) is the major component of extra cellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of the hydroxyproline could be used as an index for collagen turnover. The data depicted in table 8 showed that the hydroxyproline content of the granulation tissue of the animals treated with petroleum ether extract of *Hyptis suaveolens* was significantly increased when compared to the control and the group of animals treated with alcohol and aqueous extracts of *Hyptis suaveolens* indicating increased collagen turnover. In addition, increase in dry tissue weight also indicated the presence of higher protein content. The preliminary phytochemical analysis of leaf extract of *Hyptis suaveolens* revealed the presence of sterols, alkaloids, flavanoids and tannins. Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity. Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis[15]. Flavonoids[16], triterpenoids[17] are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialisation. *Hyptis suaveolens* also contains tannins[18-20], which are used as antiinflammatory agents and also used topically for treatment of burns. The antioxidant property of the *Hyptis suaveolens* leaves, conferred upon it by the presence of tannin may also be responsible to the prohealing action of the extract. Similar types of wound-healing activity were reported on *Vernonia arborea*[21] and *Pentas lanceolata*[22].

CONCLUSION

Our data demonstrate that the petroleum ether leaf extracts of *Hyptis suaveolens* may be capable of promoting wound-healing activity. However, it needs further evaluation in clinical settings before consideration for the treatment of wounds. Further studies with purified constituents are needed to understand the complete mechanism of wound healing activity of *Hyptis* suaveolens.

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Table 1: The percentage yield of various extracts of *Hyptis suaveolens* L.

Sl. No.	Extracts	Nature of extract	*Colour*	Yield (% w/w)
1.	Petroleum ether	Semisolid	Dark yellow	04.78
2.	Solvent ether	Semisolid	Dark brown	08.52
3.	Chloroform	Semisolid	Dark brown	03.30
4.	Alcohol	Semisolid	Dark brown	5.48
5.	Chloroform water	Semisolid	Dark yellow	15.22

Table 2: Physicochemical Characterization of *Hyptis suaveolens* L.

Sl. No.	Parameters	Result
1.	Total ash (%)	4.00
2.	Acid insoluble ash (%)	1.75
3.	Water soluble ash (%)	1.2
4.	Loss on drying (%)	1.86
5.	Extractive value (%)	9.0
6.	pH	6.4

Table 3: Phytochemical Screening of different extracts of *Hyptis suaveolens* L.

Extracts	Steroids	Alkaloids	Glycosides	Saponin	Flavonoid	Tannin	Carbohydrates
Petroleum Ether	+++	-	-	-	-	-	-
Solvent Ether	+	-	-	-	-	-	-
Chloroform	-	+	-	-	-	-	-
Alcohol	-	-	+	-	++	++	+
Chloroform water	-	-	+	-	+	++	++

+++: high concentration; **++:** medium concentration; +: low concentration; - : constituents not detectable

TABLE 4: Results of Acute oral toxicity studies of various extracts of *Hyptis suaveolens* L.

Sl. No	Extracts	LD_{50} (mg/kg)	ED_{50} (mg/kg)
1.	Petroleum ether	5000	500
2.	Solvent ether	5000	500
3.	Chloroform	5000	500
4.	Alcohol	5000	500
5.	Chloroform water	5000	500

TABLE 5: Results antibacterial activity of various extracts of *Hyptis suaveolens* L.

Sl. No.	Name of the Extract	Zone of Inhibition in mm at conc. of 200 µg/ 0.1 ml			
		Bacillus subtilis	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*
1.	Alcohol	19	17	18	24
2.	Chloroform	14	12	13	17
3.	Chloroform water	17	15	16	21
4.	Solvent ether	14	10	12	18
5.	Petroleum ether	16	13	14	19
6.	Control (DMF)	R	R	R	8
7	Standard	21	19	21	26

Diameter of cup –6mm;Standard drug –Streptomycin; DMF - Dimethyl formamide; R - Resistance

TABLE 6: Effect of various extracts of *Hyptis suaveolens* L. on healing of Excision wound

Group (N)	Dose (oral)	Excision Wound						
		% Wound Contraction						
		4th day	8th day	12th day	16th day	18th day	Mean size of scar area mm^2	Period of epithelization (days)
Control	1 ml of 2% Gum acacia	11.96± 0.326	24.416± 0.254	38.225± 0.716	66.983± 0.273	77.446 ± 0.406	15.2± 1.550	23..34± 0.222
Petroleum ether	500 mg/kg suspended in 2% acacia	17.74± 0.150*	35.751± 0.654*	66.423± 0.827*	83.535± 0.718*	97.225 ± 0.170*	9.67± 0.486*	18.09± 0.213*
Chloroform water	500 mg/kg suspended in 2% acacia	14.80± 0.217	27.335± 0.970	58.89± 0.904	78.685± 0.588	87.815 ± 0.618	11.40± 0.497	20.89± 0.230
Alcohol	500 mg/kg suspended in 2% acacia	16.72± 0.159	33.46± 0.698	64.30± 0.854	81.45± 0.736	94.69± 0.174	10.30± 0.457	19.40± 0.199

* indicates significant difference at P<0.05 when compared to control. Values are Mean ± SEM from 6 animals in each group), Data analyzed by One-way ANOVA followed Dunnett's test.

Table7: Influence of various extracts of *Hyptis suaveolens* L. on healing of Incision wound

Group (n)	Dose (oral)	Wound breaking strength (g)
Control	1 ml of 2% Gum acacia	143.62± 8.084
Petroleum ether	500 mg/kg suspended in 2% acacia	294.69±5.540*
Chloroform water	500 mg/kg suspended in 2% acacia	176.36±6.357
Alcohol	500 mg/kg suspended in 2% acacia	250.31±6.521

* indicates significant difference at P<0.05 when compared to control. Values are Mean ± SEM from 6 animals in each group), Data analyzed by One-way ANOVA followed Dunnett's test.

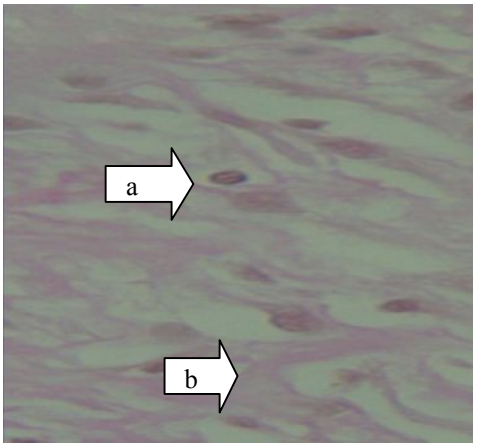
Table 8: influence of various extracts of *Hyptis suaveolens* L. on healing of dead space wound

Group (n)	Dose (oral)	Breaking strength (g)	Granulation tissue dry weight (mg/100g)	Hydroxyproline (µg/100mg)
Control	1 ml of 2% Gum acacia	156.35±8.803	42.33±2.28	1385±9.56
Petroleum ether	500 mg/kg suspended in 2% acacia	370.66±5.637*	74.00±1.29*	2512 ±21.09*
Chloroform water	500 mg/kg suspended in 2% acacia	278.43±0.933	56.00±2.37	1475±11.86
Alcohol	500 mg/kg suspended in 2% acacia	292.34±7.148	61.67±1.87	1920±5.53*

* indicates significant difference at P<0.05 when compared to control. Values are Mean ± SEM from 6 animals in each group), Data analyzed by One-way ANOVA followed Dunnett's test.

Figure1. Histology of the Granulation tissue of *Pet ether* treated animal showing more collagen and less macrophages (Vangeison stain).

a- macrophages, b- collagen



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