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Chemical Composition and Wound healing activity of Volatile oil of Leaves of Azadirachta *indica* A.juss

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Abstract : The volatile oil of *Azadirachta indica* was extracted by hydro distillation method and analyzed chemically by GC-MS. Analysis of volatile oil of *Azadirachta indica* leaves was determined. Thirty two compounds were identified which represent 84.98% of total oil. Intramuscular administration of the volatile oil to albino rats resulted in LD_{50} value 0.428ml/kg. Wound healing potential of volatile oil of leaves of *Azadirachta indica* for treatment of wounds in rats was studied on excision and incision wound models. Various parameters of incision wound, viz epithelization period, scar area, tensile strength and hydroxyproline measurements along with wound contraction were used to evaluate the effect of *Azadirachta indica* on wound healing. Intramuscular administration of the oil to anaesthetized rats (0.2143ml/kg body wt) decreases the surface area of the wound and increasing the tensile strength. Dexamethasone was used as a positive control. Complete epithelization was observed within 15 days with *Azadirachta indica* volatile oil. Measurements of the healed area and the hydroxyproline level were in good agreement.

***Keywords*:** *Azadirachta indica*, volatile oil, GC/MS, wound healing

INTRODUCTION

Neem (Azadirachta indica A juss), a Meliaceae family tree, is a hardy evergreen tree commonly found in all parts of India. *Azadirachta indica* leaves are eaten as vegetable, and twigs are used as toothbrushes. It is a nature's pharmacy *(Vietmeyer, 1992)*. Today, researchers opinion that Neem could be called "a wonder tree" and eventually expect it to benefit everyone on the planet. The medicinal properties of Neem have been known since time immemorial. The earliest ayurvedic literature refers to the benefits of all parts of this majestic tree - fruit, leaf, bark, flower and root *(Schmutterer, 2002; Subapriya and Nagini, 2005)* Neem elaborates a vast array of biologically active compounds which are chemically diverse and structurally complex (*Vietmeyer, 1992; Siddiqui et. al., 1992; Garg et. al., 1998; Ramesh and Balasubramanian, 1999; Koul et. al., 2003; Koul 2004; Kaur et. al., 2004).* Every part of this plant is used as herb. Neem oil contains active ingredients that directly work with the wound healing process. Because of this, neem directly affects the wound healing process and helps the skin retain its suppleness as the wound heals. Clinical studies have also inhibits inflammation as effectively as cortisone acetate, this effect further accelerates wound healing. The present study is therefore an attempt to assess the efficacy of the volatile oil of *Azadirachta indica* using different parameters of wound healing in rats. Also, since the plant has been used traditionally in the treatment of skin diseases.

MATERIALS AND METHODS

Plant Material

The leaves of *A.indica* leaves were collected from the Himalaya Drug Company, Sahranpur road, Dehradun.

Preparation and analysis of oil

The Neem leaves were washed thoroughly with water to remove dust particles etc. The dry powdered leaves of *Azadirachta indica* were subjected to hydrodistillation.The hydro-distillation process using Clevenger apparatus *(Clevenger,J.H,1928)* of sigma chemical company for 5 h. in accordance with the British pharmacopoeia. The yield of volatile oil was 0.13% v/w. the GC/MS analysis was performed by using Perkin –Elmer Clarus-500 equipped with a flame ionization detector. The GC column was (30m X 0.25µm) fused silica capillary column. The GC conditions were as follows: injector and detector temperature, 250^0c; oven temperature programmed to rise from 100^0c to 230^0c at 10^0c per minute. Injection volume:

2µl. helium the carried gas at 1ml/min; ion source temperature 300°c; ionization voltage 70 eV.

Source of Animals: The albino rats of weighing between (150±10)gms. were used for the present study. They were kept in the departmental animal house under controlled condition of temperature 23±2^{0}C, containing humidity of 50±5% , light and dark cycles of 10 and 14 hours respectively. The animals were housed individually in polypropylene cages sterile paddy husk (procured locally) as beeding throughout the experiment and has free access to sterile food and water *ad libitum.*

Determination of LD50 value for the volatile oil of neem leaves

According to the method of *Weill (1952)* for determination of the dose of LD_{50}, exploratory trial were performed in five groups each (n=5). They were administered single dose of volatile oil of the Neem leaves with increasing doses of 0.2, 0.4, 0.8, 1.6, 3.2 ml/kg body weight respectively. The dose up to 0.4286ml/kg b.wt was well tolerated without producing any sign of toxicity when administered intramuscularly. 50% of the maximum tolerated dose i.e. 0.2143ml/kg b.wt. of volatile oil was selected for the study.

Group Classification

Group I (Normal Control Group): Rats of this group were given sesame oil at a dose level of 2.5ml /kg body weight (i.m).

Group II (Normal group treated with volatile oil): The rats included in this group were gavages with volatile oil (0.22ml/ kg i.m) daily to each rat.

Group V (Dexamethasone group):

Received dexamethasone, 0.17 mg/kg i.m

Wound Models

The studies were carried out using ether anesthetized rats and their back was shaved,
in two different wound models, at dose levels of 0.2143ml/kg body wt.

Excision wound model

The rats were inflicted with excision wound model as described by *Umachigi et.al. (2007).*An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear using a round seal of 2.5 cm diameter. The skin of impressed area was excised to the full thickness to obtain a wound area of about 500 mm^2 diameter. Homeostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The percentage of wound healing (Indian Pharmacopoeia, 1996) was calculated of original wound size (500 mm^2) for each animal of group on predetermined days i.e., 4, 8, 12, , 16 days post-wounding for final analysis of results. Falling of scar leaving no raw wound behind was taken as end point of complete epithelization and the days required for this was taken as period of epithelization.

Incision Wound Model

Two paravertebral-long incisions were made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the rat as described by *Tara et.al. (2006)*. All the groups were treated in the same manner as mentioned in the case of the excision wound model. No ligature was used for stitching. After the incision was made, the parted skin was kept together and stitched with black silk at 0.5 cm intervals. Surgical thread and a curved needle (No. 11) were used for stitching. The continuous thread on both wound edges were tightened for good closure of the wounds. The wound was left undressed; volatile oil and Dexamethasone were injected intramuscularly once a day for 9 days. When wounds were thoroughly cured, the sutures were removed on the 9th day and tensile strength was measured with a tensiometer *(Sharma et.al.2003)*

RESULTS:

GC/MS Analysis of Neem oil

The chemical composition of essential oil isolated from leaves by hydro distillation was analyzed by GC/MS. Analysis of volatiles oil was determined that thirty two compounds were identified, which represented 84.98 % of total oil. The oil contains ketones, terpenes and phenolic esters. The monoterpene(Limonene, 7.17%; α-pinene,3.14%; 3-phenyl-1,2 butanol, 3.70%; Terpinen-4-ol, 5.29%) Sesquiterpenes (b-caryophyllene, 12.73%; Logifolene, 1.18%; germacrene B, 5.41 %) and diterpenes ware also identified. Differences in oil composition were observed between the reported literatures given by (*Helmy, Wafaa A. et. al. 2007)*

Table 1: GC/MS Analysis of Volatiles Constituents of *Azadirachta indica* A. juss Leaves

Identified peak No.	RT	Area %	Name
1	6.31	1.15	Hexanal
2	7.02	3.14	α-Pinene
3	7.14	0.41	Ethyl butyrate

4	**8.35**	**0.53**	**2-Methyl-2,3-pentanediol**
5	**9.16**	**0.85**	**1-Pentanol-5-cyclopropylidene**
6	**9.91**	**1.07**	**β-Pinene**
7	**10.32**	**0.59**	**Myrcene**
8	**10.72**	**0.08**	**2, 4--Heptadienal**
9	**11.04**	**7.17**	**Limonene**
10	**12.39**	**0.08**	**2-Pinen-4-one**
11	**12.82**	**1.81**	**Isopropyl benzyl alcohol**
12	**14.67**	**3.74**	***trans*-Pinocarveol**
13	**14.76**	**6.31**	**n-Undecane**
14	**15.00**	**3.02**	***cis*-Verbenol**
15	**15.89**	**0.06**	**2-Undecanone**
16	**16.34**	**3.26**	**Terpinen-4-ol**
17	**19.93**	**6.62**	**Bornyl acetate**
18	**24.81**	**3.7**	**(2R, 3R)-3 Phenyl-1,2-butandiol**
19	**25.82**	**0.13**	**Ethyl propyl disulfide**
20	**27.33**	**0.46**	**d-Elemene**
21	**27.61**	**1.62**	**a-Cubebene**
22	**27.88**	**4.7**	***n*-Tetradecane**
23	**28.29**	**1.18**	**Longifolene**
24	**28.83**	**12.73**	**b-Caryophyllene**
25	**28.35**	**1.66**	**Eudesma-4(14), 11-diene**
26	**30.12**	**0.41**	**Humulene-oxide**
27	**30.7**	**0.97**	**Bornyl isovalerate**
28	**31.11**	**1.8**	**g-Muurolene**
29	**31.71**	**5.41**	**Germacrene B**
30	**31.99**	**5.02**	**Spathulenol**
31	**33.48**	**1.33**	**Muurola-4(5), 5-diene**
32	**33.97**	**1.97**	**b-Eudesmol**

Table 2: Effect of Volatile oil on excision wounds

Wound Model : Excision						
Percentage of wound contraction						Epithelization period (days)
Treatment group	**Dose/Route**	**4thday**	**8th day**	**12th day**	**16th day**	
Group I (Control)	**2ml / i.m**	**22.07 ±1.09**	**41.27 ±1.82**	**59.56 ±1.91**	**69.75 ±1.56**	**21.33 ±0.44**
Group II (Volatile oil)	**0.22ml/kg / i.m**	**27.06 ±1.31**	**52.72 ±1.86**	**72.58 ±1.16**	**83.93 ±1.38**	**15.86 ±0.33**
Group III (Dexamethasone)	**0.17 mg/ kg / i.m**	**35.66 ±1.21**	**62.98 ±1.64**	**82.45 ±1.34**	**95.26 ±1.56**	**13.19 ±0.37**

Values are mean ± SEM for four rats

Table 3: Effect of Volatile oil on incision wounds

Wound Model : Incision wound					
Treatment group	**Dose/Route**	**Epithelization period (days)**	**Tensile strength (g)**	**Scar area (mm^2)**	**Hydroxyproline (mg/100mg tissue)**
GroupI (Control)	**2ml / i.m**	**25.3 ±1.28**	**281.5 ±21.6**	**47.2 ±3.4**	**6.04 ±0.4**
Group II (Volatile oil)	**0.22ml/kg / i.m**	**19.43 ±1.50**	**385.5 ±21.9**	**39.6 ±3.2**	**8.49 ±.82**
Group III (Dexamethasone)	**0.17 mg/ kg / i.m**	**13.6 ±1.26**	**418.5 ±21.7**	**30.5 ±3.8**	**9.97 ±0.34**

Values are mean ± SEM for four rats

DISCUSSION

The chemical composition of essential oil isolated from leaves was analyzed by GC/MS. Analysis of volatiles of leaves was determined that thirty two compounds were identified, which represented 84.98 % of total oil. The oil contains ketones, terpenes and phenolic esters.

The results showed that upon administration of volatile oil, the wound contraction and epithelization were faster when compared to control. As shown in Table-2, wound contraction progressed faster when the volatile oil was administered in rats compared to untreated wounds. Table-2 shows the results for excision wounds. The percentage of wound contraction for simple wounds was 22.07±1.09, 41.27±1.82, 59.56±1.91, 69.75±1.56 as measured on the 4^{th}, 8^{th}, 12^{th} and 16^{th} day respectively in the control group. Wound contraction rate significantly increased in volatile oil treated group (83.93 ± 1.38) as compared to control group (69.75 ± 1.56) on 16^{th} day. The epithelization period for volatile oil is (15.86 ± 0.33) which is very less as compared to control group (21.33 ± 0.44). In the dexamethasone treated rats the wound were completely healed.

There was a significant increase in the tensile strength and hydroxyproline content compared to the control group and comparable to the Dexamethasone group (Table-3).

The observation and results obtained in this study indicate that the volatile oil of *Azadirachta indica* significantly stimulated wounds.

A.indica is a source of terpenoids, which play an important role in wound healing *(Hawkins and Ehrlich, 2006)*.

Terpenoid strengthen the skin, increase the concentration of antioxidants in wounds, and restore inflamed tissues by increasing blood supply. The volatile oil was found effective due to the synergetic effect of thirty two components of volatile. This paper may be combined judiciously in the development of a globally acceptable wound healing agent, which if validated properly and proven scientifically can act as substitute or may even replace the modern wound healing agents.

CONCLUSION

The use of *Azadirachta indica* in Indian traditional systems of medicine for various skin diseases has been justified 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 volatile oil in wound healing are very clearly defined, wound healing potential of *Azadirachta indica* may be partly due to the potent 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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