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FORMULATION AND EVALUATION OF TOPICAL DOSAGE FORM OF *EUPATORIUM ODORATUM* LINN. AND THEIR WOUND HEALING ACTIVITY

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ABSTRACT

Topical application of ointments and gels prepared from the methanol extract of *Eupatorium odoratum* L (Family-Asteraceae) were formulated and evaluated for its efficacy and safety. Ointments and gels were prepared at different concentrations i.e. 5%, 7.5% and 10%(w/w) by fusion method using different excipients. These topical formulations were tested for pH, viscosity, spreadability, drug contents uniformity, *in vitro* diffusion. The stability study was carried out at 4, 25 and 37^0C. The drug content uniformity of ointments and gels were found with in the range of 98.18% to 98.96% and 97.23% to 98.66% respectively. The formulations of O-III and G-III showed maximum drug release of 78% and 90% over a period of 8h. All the formulations were evaluated for its acute skin irritancy, wound healing activity in Swiss Albino rats. These formulations did not produce any skin irritation for about a week when applied over the skin. Comparative studies showed that the viscosity of the formulations increases, spreadability decreases and vice versa. From the stability studies, ointments and gels showed no changes in pH, viscosity, spreadability, extrudability, drug contents, consistency, and phase separation after keeping at different temperatures for 90 days. All the formulations and the normal methanol extracts of *Eupatorium odoratum* showed significant ($P< 0.001$) wound healing activity by excision wound model and comparable with that of the reference standards and control bases. The measurement of the wound areas were taken on 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th} and 18^{th} days and the percentages of wound closures were calculated.

KEYWORDS

Eupatorium odoratum leaves, methanol extract, ointments, gels, *in vitro* evaluation, wound healing.

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INTRODUCTION

Eupatorium odoratum L. Syn: *Eupatorium conyzoides* Vahl. (Family-Asteraceae) is a Christmas bush, also known as bitter bush, Siam weed, baby tea, Santa María, is a scrambling shrub. It may reach 1m or more as a free standing shrub and 4m or more when climbing into trees or shrubs. Stems reach 2cm in diameter. The plants are maintained by a system of abundant, yellowish, fine lateral roots. Multiple sprouts arise from the root crown and lower stems. The individual branches are long with relatively few branches. The opposite, three-nerved leaves are deltoid to ovate-lanciolate, usually with a dentate margin and a long pointed tip. The leaves are aromatic when crushed. The inflorescences are corymbs of cylindrical heads located on the terminals of lateral branches. There are 15 to 25 tubular florets per head, white, lavender, pink, or blue in color. The seeds are a brownish gray to black achene that is 4mm long with a pale brown pappus 5 or 6 mm long [1, 2]. E. *odoratum* L. has acquired a reputation as a medicinal herb for a variety of ailments including malaria, fever, and the aqueous leaf extract of the plant is used as antiseptic for wound dressing. The decoction of the leaf was used as a cough remedy and the stem decoction can be used in pulmonary hemorrhage [3]. The phytochemical investigation revealed the presence of α-pinene, β- pinene, myreene, limonene, β- caryophyllene [4], d-eupatene, eupatol, lupenol, β-sitosterol [5], β-amyrin, intermedine, pinderine, tamarixetin, salvigenin, odoratin, rhamnetin[6,7,8]. The literature reveals that the *E. odoratum* leaves are used orally against wounds, inflammation in traditional system. Hence, an effort has been made to establish the scientific validity to investigate the possible wound healing activity of different formulations such as ointments and gels made from the dried methnol extract of *Eupatorium odoratum* leaves in animal models.

MATERIALS AND METHODS

Plant material and extraction

The leaves of *Eupatorium odoratum* Linn. w*as* collected freshly during the month of February from Mohuda in Ganjam Distrit, Orissa, India, identified and authenticated by Prof. S. K. Dash, Head, PG Department of Biosciences, College of Pharmaceutical Sciences, Mohuda comparing with the voucher specimen (EO-1) present in the herbarium, has been kept in the laboratory for future references. The leaves were dried under shade and pulverized into powder by a mechanical grinder. The powder was then passed through 40-mesh sieve and stored in a closed vessel. The dried, powdered leaves of *Eupatorium odoratum*. (1kg) was extracted successively with of petroleum ether ($60–80^0$C), chloroform, and methanol in soxhlet apparatus. A dark greenish black colored petroleum ether extract (yield 4.8% with respected to dry powdered plant material) was obtained. The marc, after proper drying, was extracted with chloroform to produce a dark greenish semisolid (yield 3.4% with respected to dry powdered plant material); again the marc was dried and extracted with (95.5%) methanol by the same procedure to yield a dark greenish semisolid (yield 3.7% with respected to dry powdered plant material). All the extractions procedures were carried out until the solvent systems becomes a colorless. All the extracts were collected and concentrated by

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evaporating the solvent completely and was selected for all experimental procedure. The chemical constituents of the extract was identified by qualitative analysis and confirmed by the thin layer chromatography (i.e. hR_f values).

Preliminary phytochemical analysis

The methanol extract of *Eupatorium odoratum* was subjected to preliminary phytochemical screening for detection of major chemical groups. In each case test 10% w/v solution of the extract in methanol was used and unless otherwise mentioned in individual test [9]. Results of different chemical tests on the methanol extract of *Eupatorium odoratum* showed the presence of phytoconstituents viz., steroids, triterpene, alkaloids and flavonoids.

Chemicals used

Petroleum Ether, Liquid paraffin, Chloroform, Hard paraffine, Glycerin, Triethanolamine, Sodium hydroxide (purchased from Merck specialties pvt. Ltd Mumbai, India), Petrolatum, White soft paraffine, PEG 6000 (Polyoxyethylene glycol), Petroleum jelly, White Wax, Methylparaben (purchased from Himedia Laboratories Pvt. Ltd Mumbai, India), Cholesterol (purchased from sd fine-CHEM. Ltd Mumbai, India), Cetostearyl alcohol, Disodium edetate, Sodium borate, Stearyl alcohol, Propylene glycol, Sodium lauryl sulphate, Carbomer 934P, Sodium alginate (purchased from Loba Chemie Pvt. Ltd Mumbai, India).

Animals

Swiss Albino rats of either sex weighing 150-200g obtained from M/s Ghosh & Ghosh Enterprises., Kolkata, India, were housed in standard polypropylene cages at room temperature of 30 ± 2 ^{0}C and 60-65% relative humidity and had free access to food and water *ad libitum*. The animals were fed with a commercial diet (Hindustan Lever Ltd., Bangalore).

Formulations

The dried methanol extracts of *Eupatorium odoratum* was taken for the preparation of ointments and gels. Six different formulations were prepared using an ointment base and gel base according to the formula [10, 11] given in the Table-1. Appropriate standard methods of fusion were adopted, where the solid fats were melted and mixed, and trituration was followed for preparation of the ointments and gels [12,13]. The methanol extract of *Eupatorium odoratum* was incorporated in the bases to get three different concentrations (5%, 7.5% and 10%). All preparations were packed in wide-mouthed plastic jars with screw-capped lid. The following tests were carried out on all the preparations.

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Table-1.

Physicochemical evaluations of different formulation of ointments and gels

Formulations	Ingredients	Concentration (%, m/m)	Drug content (%) Mean ± SD	pH	Viscosity (cps) Mean ± SD	Spreadability (sec)
O-I	Extract	5	98.26±0.27	6.68	16.000±050	23
	Cetostearyl alcohol	10				
	PEG6000	5				
	Petroleum jelly	75				
	Liquid paraffin	10				
	Methylparaben	0.18				
O-II	Extract	7.5	98.18±0.56	6.54	16.050±150	23
	Cetostearyl alcohol	10				
	PEG6000	5				
	Petroleum jelly	75				
	Liquid paraffin	10				
	Methylparaben	0.18				
O-III	Extract	10	98.96±0.28	6.46	16.100±100	25
	Cetostearyl alcohol	10				
	PEG6000	5				
	Petroleum jelly	75				
	Liquid paraffin	10				
	Methylparaben	0.18				
G-I	Extract	5	97.23±0.42	6.58	13.600±050	18
	Carbomer934P	0.5				
	Glycerin	10				
	Triethanolamine	0.5				
	Water	89				
	Methylparaben	0.18				
G-II	Extract	7.5	98.28±0.31	6.74	13.650±075	18
	Carbomer934P	0.5				
	Glycerin	10				
	Triethanolamine	0.5				
	Water	89				
	Methylparaben	0.18				
G-III	Extract	10	98.66±0.22	5.98	13.700±100	20
	Carbomer934P	0.5				
	Glycerin	10				
	Triethanolamine	0.5				
	Water	89				
	Methylparaben	0.18				

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Drug content

Each formulation (1gm) was accurately weighed and transferred to 100ml volumetric flask to which about 70ml of methanol was added. After shaking, the volume was made up to 100ml with methanol. The content was filtered through a suitable filter paper. 1ml filtrate was taken and suitable diluted and the drug content (extract) was estimated by using UV/Visible spectrophotometer, (SL-159-Shimadzu-1700, SI-164 Double Beam) at 271nm. Results given in Table-1 are the average of triplicate values. Drug contents values are expressed as Mean ± Standard deviation.

Drug release

The *in vitro* diffusion studies of the ointments and gels were performed by using dialysis membrane (Sigma Inc. MO, USA; dry, unwashed, pre-cut and open ended; fiat width: 35 mm; inflated diameter, 21mm; Length: 30mm). The membrane soaked in phosphate buffer pH 7.4 for 6-8 h was clamped carefully to one end of the hollow glass tube of dialysis cell (2.3 cm diameter, 4.16 cm^2 area). 100ml of phosphate buffer was taken in a beaker, which was used as receptor compartment for the study. 1gm of each formulations both the ointments and gels were spreaded uniformly on the membrane. The donor compartment was kept in contact with the receptor compartment and the temperature was maintained at 37±0.5 ^{0}C. The solutions on the receptor side were stirred by externally driven Teflon-coated magnetic bars. At pre-determined time intervals, 5ml of solution from the receptor compartment was piprtted out and immediately replaced with 5ml fresh phosphate buffer solution. The drug concentration of the receptor fluid was determined spectrophotometrically at 271nm against appropriate blank [14,15,16]. This experiment was carried out in triplicate and the results were extrapolated in the Fig-1

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Figure.1.

In vitro diffusion profile of gels (G-I to G-III) and ointments (O-I to O-III) (mean ± SD, n = 3)

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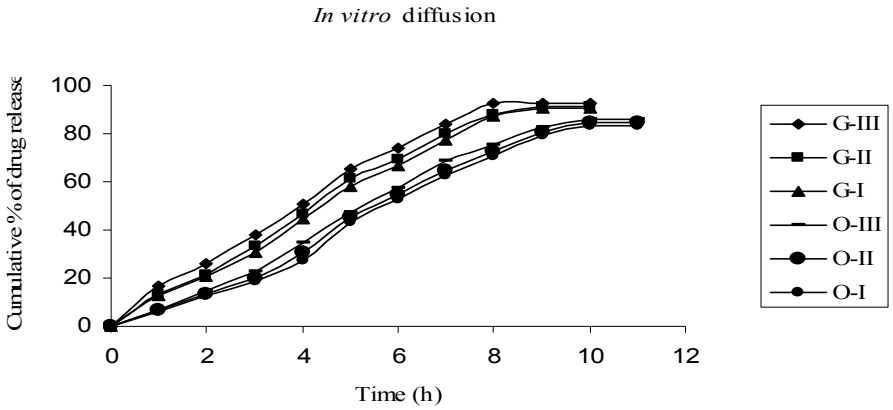

Physical evaluations

Preliminary evaluation of formulations at different concentrations was carried out as follows:-

pH [17,18]: The pH of various formulations was determined by using Digital pH meter (Digital pH meter 335, Systronics, Noroda, Ahmedabad). One gram of ointment was dissolved in 100ml of distilled water and stored for two hours. The measurement of pH of each formulation was done in triplicate and average values were depicted in Table-1.

Viscosity [19]**:** The measurement of viscosity of prepared ointments was carried out with Brookfield Viscometer (model LV-DV-II, Helipath-spindle type S-96). The values of each formulation were done in triplicate and average values were depicted in Table-1. The viscosity values are expressed as Mean ± Standard deviation.

Spreadability [20,21]**:** Spreadability is a term expressed to denote the extent of area to which the ointment and gel readily spreads on application to skin or affected part. A special apparatus has been designed by Multimer to study the spreadability of formulations. The spreadability is expressed in terms of times in seconds taken by two slides to slip off from ointment and placed in between the slides under the direction of certain load. Lesser the time taken for separation of two slides, resultant the better spreadability. Spreadability was calculated by using the formula. (S = M.L/T). Where S = spreadability, M = Weight tied to upper slide, L = Length of glass slides and T = Time taken to separate the slides completely from each other. In this present experiment, M = 250 gm, L = 3.8 cm and T was recorded in the Table-1.

Acute skin irritation study [22,23]

The primary skin irritation test was performed on albino rats and weighing about 150-200gm. The animals were maintained on standard animal feed and had free access to water *ad libitum*. The animals were kept under standard laboratory condition. The total mass was divided into four batches, each batch containing seven animals. Two batches of each were

used for control and test. Dorsal hairs at the back of the rats were clipped off one day prior to the commencement of the study. Animals showing normal skin texture were housed individually in cages with copography meshes to avoid contact with the bedding. 50mg of the each formulation of different concentrations were applied over one square centimeter area of intact and abraded skin to different animals. Aqueous solution of 0.8% formalin was applied as standard irritant. The animals were observed for seven days for any signs of oedema and erythema.

Extrudability [24]

A simple method was adopted for this study. The formulations were filled in the collapsible tubes after the ointments were set in the container. The extrudability of the different ointment formulations was determined in terms of weight in grams required to extrude a 0.5cm ribbon of ointment in 10 second.

Stability studies [25,26,27]

The stability studies were carried out in all formulations at different temperature conditions (4°, 25° and 37°C) for 3 months. All the evaluation parameters i.e. pH, viscosity, spreadability, extrudability, drug contents, consistency and phase separation studied at different time intervals i.e. 15th, 30th, 60th and 90th days.

Evaluation of wound healing activity (Excision method)

The rats were inflicted with excision wounds as described by Morton and Malone [28]. The rats were anaesthetized with ether solution prior to creation of the wounds. The dorsal thoracic region of the animal was shaved with electric clipper and the area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 500mm^2 was created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound left open to the atmosphere [29,30]. The animals were divided into eleven groups of six each. The animals of Group I were applied with ointment base and considered as the control I, Group II received gel base and considered as control II, Group III served as reference standard I (Neosporin; Neomycin and Polymyxin B Sulfates and Bacitracin Zinc M/S Glaxo Smith Kline Pharmaceuticals Limited, Mumbai), Group IV served as reference standard II (Betadine; Povidone-Iodine IP 5% w/w, M/S Win-Medicare Pvt. Ltd, New Delhi). The animals of Group V, VI and VII were treated with ointment 5% (O-I), 7.5% (O-II) and 10% (O-III) respectively. The animals of Group VIII, IX and X were treated with gels 5% (G-I), 7.5% (G-II) and 10% (G-III) respectively. The animals of Group XI were treated with pure extract with 1% caboxy methyl cellulose base (E-I). The ointment was topically applied once in a day, starting from the day of the operation, till completion of epithelialisation. The measurement of the wound areas of the excision wound model were taken on 3rd, 6th, 9th, 12th, 15th and 18th days**.** Thereafter on alternate days until healing were complete; the percentage of wound closure was calculated. All the protocols were reviewed and permitted by the Animals Ethical Committee (Reference Code: 1170/ac/08/CPCSEA), College of Pharmaceutical Sciences, Mohuda, Berhampur, Orissa.

Statistical analysis

The experimental results were expressed as the Mean ± Standard error of mean (SEM) and the statistical significance was evaluated by One-way analysis of variance (ANOVA) followed by Dunnett's t-test [31].

RESULTS AND DISCUSSION

The various physicochemical parameters utilized to evaluate the prepared ointment formulations are shown in Table-1. From the results, it was clearly evident that all the ointments and gels formulations showed good homogeneity and extrudability. *In vitro* release of ointment and gels prepared from *Eupatorium odoratum*. were found to be slow and extended over the longer period of time. All the ointments and gels showed only slight difference in release profile at the particular time period. The O-III and G-III showed maximum release of 73% and 90% over a period of 8 h. Based on the results, the O-III and G-III were selected for further study. The pH of the formulations from O-I to O-III and G-I to G-III were in between 6.46 to 6.86 and 5.98 to 6.58 respectively, which lie in the normal pH range of the human skin. The drug content uniformity of the ointments and gels were found with in the range of 98.18% to 98.96 % and 97.23% to 98.66% respectively. All the formulations did not produce any skin irritation, i.e., erythema and edema for about a week when applied over the skin. The rheological behaviors of the different formulations of ointments and gels were studied with Rotational Brookfield Viscometer. The results indicated that the torque and shear stress increases where as viscosity decreases. A comparative study of viscosity and spreadability showed that the viscosity of the formulations increases, spreadability decreases and vice versa. From the stability studies, Ointments and gels at the concentrations of 5%, 7. 5% and 10%, showed no changes in pH, viscosity, spreadability, extrudability, drug contents, consistency and phase separation after keeping at different temperatures for 90days. All the formulations and the normal methanol extracts of *Eupatorium odoratum* showed significant promotion of wound-healing activity with statistically significant (*P<0.001) in all the seven groups of animal which were depicted in the Table-2.

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Table-2:

Topical application of ointments and gels from extract of *Eupatorium odoratum* on wound healing activity in rats. [% of wound healing = (1-t/c) x 100]

Group	Post wounding days						
	0 day	3rd days	6th days	9th days	12th days	15th days	18th days
Control I	506.24±0.96* (0.00)	480.42±1.02* (5.10)	398.22±1.12* (21.36)	3525.42±0.88* (30.34)	270.46±0.88* (46.57)	188.63±0.62* (62.73)	86.52±0.52* (82.90)
Control II	508.26±1.17* (0.00)	408.72±0.86* (5.41)	404.62±1.28* (20.39)	363.42±0.72* (28.49)	282.36±0.50* (44.44)	202.32±0.42* (60.19)	97.20±0.60* (80.87)
Standard -I	508.42±1.62* (0.00)	406.32±1.18* (20.08)	323.56±1.58* (36.35)	250.80±0.58* (50.67)	135.42±0.50* (73.36)	9.42±0.22* (98.14)	0 (100)
Standard-II	510.38±1.02* (0.00)	414.72±1.32* (18.74)	332.32±1.22* (34.88)	260.42±0.64* (48.97)	144.32±0.56* (71.72)	19.54±0.42* (96.17)	0 (100)
O-I	508.24±1.05* (0.00)	457.62±0.88* (9.95)	369.42±0.86* (27.31)	302.14±0.52* (40.56)	184.36±0.59* (63.72)	82.22±0.55* (83.82)	18.20±0.42* (96.42)
O-II	510.06±1.06* (0.00)	458.42±0.88* (10.12)	350.48±1.37* (31.28)	290.12±0.42* (43.12)	166.32±0.56* (67.39)	64.22±0.52* (87.41)	8.14±0.33* (98.40)
O-III	506.03±0.92* (0.00)	440.24±1.32* (13.00)	329.52±1.20* (34.88)	266.28±0.84* (47.38)	140.42±0.46* (72.25)	18.50±0.42* (96.34)	0 (100)
G-I	508.06±1.02* (0.00)	466.57±1.22* (8.13)	382.52±0.93* (24.71)	313.42±0.43* (38.31)	197.72±0.93* (61.08)	96.55±0.53* (80.99)	22.52±0.42* (95.57)
G-II	506.12±0.92* (0.00)	46.32±0.64* (9.05)	370.52±0.42* (26.79)	302.22±0.48* (40.29)	184.26±0.39* (63.59)	88.22±0.58* (83.75)	15.52±0.56* (96.93)
G-III	508.12±0.88* (0.00)	455.24±0.74* (10.40)	352.48±0.94* (30.63)	288.40±0.66* (43.24)	168.56±0.57* (66.83)	72.52±0.52* (85.72)	9.36±0.31* (98.16)
E-I	506.22±1.12* (0.00)	454.82±0.76* (10.15)	360.40±0.52* (28.80)	296.56±0.52* (41.42)	178.54±0.46* (64.73)	77.42±0.46* (84.71)	17.42±0.43* (96.56)

Results are expressed mean ± SEM of six readings; Significance evaluated by One-way analysis of variance (ANOVA) followed by Dennett's t-test versus control group,*P < 0.001, (n = 6). Figures in parentheses indicate the percentage of wound contraction. The ointment formulation of different concentrations comparable with the control I,standard-I and standard-II, similarly the gel extracts of different concentrations also comparable with the control II, standard-I and standard-II

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The mean percentage closure of wound area was calculated on the 3rd, 6th, 9th, 12th, 15th and finally 18th days. Post wounding days also shown in Table-II. The wound-healing activity was found to be comparable with that of the reference standards and control bases. The percentages closure of excision wound area in animals treated with O-I to O-III and G-I to G-III were found to be 96.42%, 98.40%, 100% and 95.57%, 96.93%, 98.16% respectively. The animals treated with normal methanol extract with 1% caboxy methyl cellulose base were found to be 96.56%. Both the ointments and gels formulations containing 10% methanol extract of *Eupatorium odoratum* showed significant wound healing activity and comparable with that of the commercial products of Neosporin and Betadine. The mechanical properties of pharmaceutical formulations from the methanol extract of *Eupatorium odoratum*. are important tests that often form part of a manufacturer's own specification which are quantifiable by the viscosity, spreadability, extrudability, consistency of the formulations. The viscosity provides a measure of strength, spreadability expressed to denote the extent of area to which the prepared formulations readily spreads on application to skin or affected part, extrudability is a measure of removal the orifice of the container, consistency is a measure of flow when stress is applied. Both the ointments and gels prepared by fusion method using different excipients on the other hand showed acceptable viscosity, spreadability and extrudability, values at the concentrations employed, indicating the suitability of method for the production of pharmaceutical formulations from the methanol extract of *Eupatorium odoratum*. Thus, the mechanical properties were affected by the type and concentration of excipient employed. The results indicate that all the formulations were generally stable under tropical storage conditions. Thus, the methods of preparation of the *Eupatorium odoratum* formulations need to be carefully selected to ensure the production of ointments and gels with adequate viscosity, spreadability, extrudability and at the same time release the active compound(s) for biological action. Furthermore, the type and concentration of excipients employed in the formulations of *Eupatorium odoratum*. formulations need to be carefully chosen to enable the production of suitable formulations of ointments and gels.

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contraction is a process that occurs throughout the healing process, commencing in the fibroblastic stage where by the area of the wound undergoes shrinkage. It has 3 phases; inflammatory, proliferative and maturational and is dependent upon the type and extent of damage, the general state of the host's health and the ability of the tissue to repair. The inflammatory phase is characterized by hemostasis and inflammation, followed by epithelization, angiogenesis and collagen deposition in the proliferative phase. In the maturational phase, the final phase of wound healing the wound undergoes contraction resulting in a smaller amount of apparent scar tissue. 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 vessel. 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 wound healing activity of two pharmaceutical formulations both the ointments and gels containing methanol extracts of *Eupatorium odoratum*. was evaluated for its wound

healing potentials in excision wound model in rats. Both the formulations responded significantly in this wound models tested. The results were also comparable to that of the standard drugs Neosporin and Betadine used as standard drugs for comparison in this present investigation. The results were also comparable in terms of wound contracting ability, epithelization period at the wound area. In the present investigation, preliminary phytochemical analysis of methanol leaf extract revealed the presence of steroids, triterpene, alkaloids and flavonoids. The wound-healing property of *Eupatorium odoratum* may be attributed to the phytoconstituents present in the plant and the quicker process of wound healing could be a function of either the individual or the additive effects of the phytoconstituents. However, further phytochemical studies are needed to isolate the active compound(s) responsible for these pharmacological activities. Further studies with purified constituents are needed to understand the complete mechanism of wound healing activity of *Eupatorium odoratum*. Electron microscopic examination will yield the effect of the extract on angiogenesis, epithelialisation or collagen deposition. The data of this study indicated that the leaf extract of *Eupatorium odoratum* possess better wound healing activity and it can be used to treat different types of wounds in human beings too. Thus, this investigation confirms the use of the ointments and gels containing *Eupatorium odoratum* extract as a wound-healing agent as known from folklore medicine.

CONCLUSIONS

Topical route of application has a great potential as an effective and safe way to administer in the form of ointments and gels prepared from the extract of herbal plant of *Eupatorium odoratum* for local wound healing activity. Preliminary tests of skin irritation in rats may indicate negligible systemic absorption and side effects. Further experiments are to be conducted in other animal models for wound healing activity. Based on the results of these tests, trails may be performed on human beings.

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