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Evaluation of wound healing activity of ethanolic extract and its fraction from *Cucumis melo* var *momordica* Duthie and Fuller

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Abstract

Purpose: The aim of this study was to investigate the wound healing activity of ethanolic extract and its toluene fraction of *Cucumis melo* var *momordica* Duthie and fuller on excision and incision wound models.

Methodology: The study was conducted on excision and incision wound models. Rats were assigned in to four groups untreated control, 5% povidone iodine ointment, ethanolic extract 200mg/kg body weight and fourth group treated with toluene fraction in a dose of 50mg/kg body weight for both the models. One animal from each group was euthanized on 3rd, 6th, 9th, 14th and 21st days after surgical procedures and the wounded areas were analyzed and removed for estimation of hydroxyproline level, microbial count, tensile strength and total protein concentration.

Findings: Excision wound model exhibits marked increase in wound closure on 3rd, 6th, 9th, 14th and 21st days, dwindled microbial load of wound. Incision wound model shows effective strength in wound breaking strength hydroxyproline level and total protein concentration at the surface of wound site when compared with control animals. Dose dependent changes at ($P < 0.0001$) were found significant.

Conclusion: Based on our findings, we presume that the wound healing potency of toluene fraction was greater on wound contraction, inhibition of microbial count, increased hydroxyproline level, total protein concentration and tensile strength supports the potential for use of toluene fraction of *Cucumis melo* ethanolic extract as an adjuvant in the treatment of wound healing.

Keywords: Wound healing, *Cucumis melo* var. *momordica* (Roxb.) Duthie & Fuller, ethanolic extract, toluene fraction, incision wound model, excision wound model

1. Introduction

The process of wound healing is extremely dynamic and complicated; it proceeds in a sequence with cooperation between blood corpuscles, cells of parenchyma, soluble mediators and extracellular matrix. Dermal wound healing starts immediately after injury, it involves different phases of inflammation, proliferation of cells, and maturation followed by remodeling [1, 2] Platelet adhesion followed by platelet aggregation at the wound site first appears then formation of a clot on the surface of the wound materialize which leads to inflammation [3]. In proliferative phase, there is formation of granulation tissue, proliferation and migration of connective tissue cells, and re-epithelialization of the wound surface. Maturation involves extracellular matrix deposition, tissue remodeling, and wound contraction. Dermal wound healing is a complex phenomenon characterized by a sequence of independent and overlapped events described as exudative/inflammatory, proliferative phases [4]. Since herbs are the mines of useful drugs, a herb *Cucumis melo* var. *momordica* belonging to family Cucurbitaceae grows in humid areas near the rivers. Chemically the fruit contain a saponin (stigmasta-7-16-25(26-3-O-β-D-Glucopyranosyl (1→5)-O-β-D-xylofuranoside. Curcumin and leptodermin is also reported in this fruit traditionally, the plant is used in eye infection, ulcers, Bronchitis, kidney troubles and chronic fever [5]. Many pharmacological activities have been mentioned in traditional literatures and it was observed to show a great antiulcer potential when ethanolic fruit extract was investigated on experimental animals [6]. In this work we have made an attempt to explore this plant scientifically for its wound healing activity.

Experimental

Materials and method

Chemical and reagents: Povidone iodine ointment 5% w/w was purchased from Glide chem New Delhi. Ethanol, toluene and petroleum ether were purchased from Sigma Aldrich Bangaluru. All the chemicals and reagents used were of analytical grade.

Collection of plant material: The fruits and plant material was collected from the river side area of District Allahabad, Uttar Pradesh, India and authenticated from Botanical Survey of India, Allahabad by taxonomist Dr. G.P.Sinha with voucher specimen no. BSI/CRC/Tech./2015-16. TR. No.GC 950221. Collected fruits were washed with water, chopped and dried under shade then weighed.

Extraction and fractionation: The dried fruit powder (300gm) was macerated with petroleum ether to remove the fatty substances. Marc was exhaustively extracted with ethanol (95%) by maceration method. Ethanolic extract concentrated on rota vapour at 10,000 rev/min. The concentrated extract was fractionated with chloroform, toluene and ethyl acetate successively. All the fractions were concentrated and kept in air tight containers. Ethanolic extract and fractions were further subjected to preliminary phytochemical screening to find out various secondary metabolites by adopting standard procedure.

Phytochemical screening: Preliminary phytochemical screening of extract and all fractions was performed carried out by standard procedure [7] which revealed the presence of various chemical constituents shown in table-1

Experimental animals: The protocol of the study was approved by the Institutional Animal Ethical Committee of United Institute of Pharmacy, Allahabad, India (Ref.no. UIP/IAEC/2014/April/23). The wistar albino rats were obtained from Central Drug Research Institute Lucknow, India and kept in animal house in standard conditions of temperature (28 ± 2 °C) and relative humidity ($46 \pm 6\%$) with 12-h light-dark cycle and adequate ventilation. They were provided food and water *ad libitum* during the whole period of the experiment.

Skin Irritation Study: Skin irritation study was conducted on three rats. Ketamine hydrochloride (0.2%) injection was used to anaesthetize rats in order to avoid movement of animals for at least two hours after administration following the guidelines of Organization for Economic Co-operation and Development (OECD) for acute dermal irritation and (corrosion) [8].

Back of the animals was shaved free of fur with an electric clipper 24 hours before application of the sample. This was performed with care to avoid skin injury. Methylated spirit was then applied as an antiseptic to the shaved region with the aid of a cotton wool to prevent infections caused by bacteria. A millimetre rule was used to measure an area of 40mm x 30mm on the shaved skin. Control group animal was not given any treatment while ethanolic extract in a dose of 50, 100, 1000 and 2000mg/kg body weight in a simple ointment base (USP) was topically applied on a free surface of the skin. Test materials were removed and the surface of the skin was rinsed with distilled water and 1 hour later the sites were examined for skin irritation. The sites were observed at 24 hours after application of dose and repeated at 48 and 72 hours, 7th and 14th day.

Experimental Design

Excision wound model: The animals were anesthetized by using ketamine 100 mg/kg, and xylazine 16 mg/kg through intramuscular route. An impression was making on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear on the anaesthetized rat. The skin area was shaved prior to the experiment. The skin of impressed area was excised to the full thickness to obtain a wound area of about 500 mm². A cotton swab soaked in normal saline was used to achieve haemostasis at the wound site. All the animals in each group were treated with drugs daily as per the experimental design from 0th day to 21st post wounding day. Wound area is measured on 3rd, 6th, 9th, 14th and 21st day post wounding for determination of wound contraction and percentage wound contraction was calculated. Wound surface microbial load and hydroxyproline level were measured during the study on specified intervals. Data shown in table 2.

Incision Wound model: The animals were divided into four groups of six animals each. Group 1: was treated as control and no drug was applied. Group 2: was treated with 5% povidone iodine ointment (5%). Group 3: was treated with 200mg/kg body weight ethanolic extract and fourth group was treated with 50mg/kg toluene fraction topically. A 5 cm long Para-vertebral incision in longitudinal direction was made through the entire thickness of the skin and cutaneous muscle with the help of a scalpel. After complete homeostasis the wound was stitched by means of interrupted sutures applied 1 cm apart. The sutures were removed on the 8th post wound day and the topical treatment of wounds with different standard, extract and fraction was continued. Tensile strength and protein concentration were measured during the study.

Wound surface microbial load

Cotton swabs were taken from the excision wound sites on day 4, 8, 12, and 16. The collected sample was inoculated in sterile peptone water with the swab sample and incubated for 20 minutes at 37 °C. Test tubes were arranged in a rack and filled each with 9 ml of 0.85% physiological saline. Bacterial suspension of 1ml was pipetted after the 20 minutes of incubation and colony forming units were estimated by the standard method [9].

Bacteria per ml = Number of CFU/Volume plated (ml) x total dilution used

Total dilution = 10^{-3} , Volume plated = 0.1ml

Breaking-Strength Measurements

Wound breaking strength was measured by using a standard tensiometer. After sacrifice of the animal the wound area of 2 cm width was cut in rectangular shape. In this test scar breaking forces was measured by providing a stretch to the treated wound in a tensiometer. Forces leading to rupture of obtained scar are divided into sample size on each sample and standardized as gram.cm⁻² [10]

Estimation of total proteins: tissue homogenate was prepared and centrifuged to obtain supernatant. Biuret method was used to estimate the peptide bonds of protein react with cuprous ions in alkaline medium to form a blue-violet ion complex. Tartrate was added as a stabilizer whilst iodide is used to prevent auto-reduction of the alkaline copper complex. The colour formed is proportional to the protein concentration and is measured at 546 nm [11].

Hydroxyproline content: 1 ml tissue homogenate was taken and hydroxyproline content was measured by the standard procedure using copper sulphate, hydrogen peroxide sodium hydroxide and n-propanol ^[12]

Statistical Analysis: The results were subjected to statistical analysis by using one way ANOVA followed by Tukey Kramer Multiple Comparison Test. Values of $p < 0.0001$

were considered statistically significant.

Results

Phytochemical screening: it was observed that toluene fraction shows more phytochemicals containing greater amount of flavonoids which may be responsible for suppression of microbes so this fraction was selected for performing the wound healing activity.

Table 1: Phytochemical investigation

Test Performed	Name of Test	Ethanollic extract	Toluene fraction
Test for Flavonoids	FeCl ₃ Test	++	++
Test for carbohydrates	Molisch test	+	++
	Benedict test	+	+
Protein	Biuret test	+	+
	Millons Test	+	+
Amino acids	Ninhydrin test	-	-
Glycosides	Legal's test	++	++
	Bromine water test	+	++
Steroids	Salkowski test	++	+
	Lieberman burchard test	++	+
Tannins	Gelatin test	++	+
Phenolic compounds	Lead acetate test	++	+
	Acetic acid	-	-
Fats	Filter paper test	-	-
Alkaloids	Hagers test	-	-
	Murexide test	-	-
	Ammonium renicate test	-	-

Skin irritation test: At the site of application of ethanolic extract of drug no score, redness or inflammation was observed at any dose levels from 50 to 2000 mg/kg. body weight. On the basis of toxicological studies a dose of 200mg/kg body weight for the extract and its one fourth dose 50 mg/kg body weight for toluene fraction were selected for the wound healing activity.

Contraction of wound and duration of epithelialization: Appearance of wound was clean and free of exudates or

secretions throughout the study in all the animals treated with standard drug and toluene fraction. Granulation tissue was evident at the wound site from 3rd day of injury in all groups. The results of wound contraction and epithelialization period after topical administration of the toluene fraction are shown in Table 2. Wound contraction following treatment was significantly greater on Day6, 9 and 14 as compared to the respective control group. The epithelization period was also higher on the 14th day in subjects treated with toluene fraction.

Table 2: wound contraction in excision wound healing model.

Group/treatment	day 3	day 6	day 9	day 14	day 21
Control	5.41 ±0.61	15.45 ±1.06	28.65±1.94	80.93±3.09	95.57±0.77
Povidone iodine 5% oint.	11.99±0.56	36.61±3.05**	82.07±0.47***	99.05±0.37***	99.97±0.01***
Toluene fraction 50mg/kg	8.68±0.53	30.29±1.14**	71.60±0.73***	95.41±0.27***	99.42±0.14***
Extract 200 mg/kg	6.17±0.51	28.15±0.62	66.12±0.32**	84.15±.421*	96.97±0.21**

Values are the mean ± SEM, one way ANOVA, n = 6 *** $p < 0.0001$, ** $p < 0.01$ vs. control.

Wound breaking strength: wound breaking strength in toluene fraction shows significant at $p < 0.0001$ vs. control ethanolic extract also gave significant response which is near to standard but less than toluene fraction.

Table 3: wound breaking strengths in incision wound model.

Group/treatment	Mean tensile strength in gm.cm ⁻²
Control	337.00±5.39
Povidone iodine (5%) ointment	587.33±2.29***
Toluene fraction (50mg/kg)	528.66±2.76***
Extract 200mg/kg	502.21±2.14

Values are the mean ± SEM, one way ANOVA, n = 6 *** $p < 0.0001$ vs. control.

Total protein concentration: on 16th day significant increase in protein concentration was observed significant at $p < 0.0001$, when compared with the control group in toluene fraction and up to a certain extent in ethanolic fraction.

Table 4: Protein concentration

Group/treatment	Percentage protein concentrations after days		
	8 Days	16 Days	24 Days
Control	8.26±0.09	13.23±0.12	16.8±0.11
Standard	18.53±0.12	21.4±0.32**	26.3±1.2***
Toluene fraction	16.21±0.2	19.540±0.16**	23.5±0.18**
Toluene fraction	14.18±0.12	17.21±0.15	19.34±0.17

Values are the mean ± SEM, one way ANOVA, n = 6 *** $p < 0.0001$, ** $p < 0.01$ vs. control.

Microbial count: Estimation of microbial count on the surface of the wounds gives dynamic response. The number of microbes isolated on the wounds was decreasing on days 8, 16 and 24th days. There was significant decrease in the number of microbes in the extract treatments as well as toluene fraction treated wounds. The decrease in the bacterial count on toluene fraction was more significant than ethanolic extract when compared with control group.

Table 5: Microbial count

Group treatment	Microbial count after days		
	8 Days	16 Days	24 Days
Control	91±0.3	79±0.21	50±0.42
Standard	35±0.12	23±0.13***	17±0.28***
Toluene fraction	41±0.5	32±0.32**	22±0.17***
Extract	51±0.04	45±0.43	32±0.19

Values are the mean ± SEM, one way ANOVA, n = 6 *** p<0.0001, **p<0.01 vs. control.

Hydroxyproline content: significant increase in hydroxyproline level was observed in toluene fraction followed by ethanolic extract.

Table 6: Hydroxyproline content

Treatment group	Hydroxyproline level (µg/mg)
Control	28.14± 0.78
Povidone iodine 5% ointment	67.89±0.56
Toluene fraction	63.67±0.88
Ethanolic extract 200mg/kg body weight	55.13±0.31

Values are the mean ± SEM, one way ANOVA, n = 6 *** p<0.0001, **p<0.01 vs. control.

Discussion

Significant promotion in wound contraction was observed in the toluene fraction and ethanolic extract of *Cucumis melo* var *momordica* Duthie and fuller in both the models. Increase in tensile strength, protein concentration hydroxyproline level shown in incision wound model reveals the increase in proliferation of granulation tissue followed by enhanced breakdown of collagen. Suppression of microbial load supports antimicrobial action of the toluene fraction and ethanolic extract. Greater activity of toluene fraction was certainly due to presence of more amount of active flavonoids, glycosides and few more as supported by phytochemical screening.

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