ISOLATION AND CHARACTERIZATION OF BOSWELLIC ACID FROM BOSWELLIA SERRATE AND LUPEOL FROM FICUS RACEMOSA FOR STUDY OF SYNERGISTIC WOUND HEALING ACTIVITY ON ALBINO WISTAR RATS.

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Abstract

In the present study boswellic acid was isolated from *Boswellia serrate* and lupeol isolated from *Ficus racemosa* were characterized for their Structure elucidation and purity using different physiochemical parameters, chromatographic and spectroscopic methods including ultra-violet –visible spectroscopy, high performance thin-layer chromatography (HPTLC) and high performance liquid chromatography (HPLC) technique. HPTLC fingerprint patterns of lupeol have been therefore evolved to check the purity of isolated compound from ethanolic extract of sample. The Rf value of standard lupeol 0.3 was matched with the Rf value of isolated compound was about 0.3 was shown in peak. The lupeol is white amorphous solid compound with melting point 213°C which corresponds to the molecular formulae $C_{30}H_{50}O$. The UV λ max value of compound lupeol was 372 nm. HPLC profile of boswellic acid estimates both 11-KBA and A-11-KBA, the active acids among the boswellic acids. The mobile phase with pH 4.0 gives greater stability to the analytical column. The λ_{max} of the TA fraction was analyzed using UV-vis spectrophotometer and compared with standard BAs namely, AKBA, (α+β) BA and Ac (α+β) BA. The calibration curve of U.V. depicts that it obeys Beer's Lambert law where, slope = 0.0025 and R2 = 0.996 at 260 nm. Both the extracts of *Boswellia serrate* and *Ficus racemosa* were combined in different ratios including 1:1 (2%+2%) and 1:2 (2%+4%) to produce gel formulation which showed synergic effect in wound healing in the rats when observed on 16th day post wounding day. This indicates that boswellic acid and lupeol both synergistically possess wound healing property.

Keywords: Boswellic acid, Lupeol, spectroscopy, *Boswellia serrate*, *Ficus racemosa*, wound healing.

Introduction

Chronic wounds are one of the many risks that older people face; they are a silent killer. Chronic wounds that don't heal can affect people of all ages as well as those with a variety of lifestyle conditions, including diabetes, nephropathies, cardiovascular disease, etc. The skin, being the largest organ in the body, is essential for preserving body homeostasis and shielding the body from harmful microorganisms, ultraviolet radiation, toxins, and other substances (Percival et al., 2015). Wounds that are caused by disruptions to the skin's integrity often heal in three months. According to Järbrink et al. (2017), wounds that do not heal within three months are referred to as chronic wounds, and they can occur for a variety of causes. Microbiological infections at the site of the wound are one of the main causes of nonhealing wounds (Leaper et al., 2015). The majority of the medications on the market aim to enhance the body's ability to heal wounds, but they are unable to successfully prevent microorganisms from colonizing. (1)

The human skin serves as the body's semi-permeable barrier. Skin integrity is essential for communication, metabolism, feeling, protecting the body, and maintaining body temperature. Skin conditions are rather prevalent, particularly wounds. A wound is a disruption in the tissue's continuity. The wound is regarded as healed when the injured tissue returns to its pre-injury state.

The wound healing process is a very intricate and dynamic process that encompasses several cell types, chemical mediators, and extracellular matrix.3. Even though wound healing occurs naturally, it cannot be quick, efficient, or aesthetically pleasing on its own. Inflammation is the first step in tissue repair, which is then followed by the development of granular tissue, which produces new connective tissues and closes epithelial wounds, and scar remodeling, which produces a physical barrier that is functional. (2)

Gum resin called B. serrata extract is made from the gum tree known as the frankincense tree, B. serrata Roxb., which is native to Punjab and India (Family: Burseraceae; Genus: Boswellia). Its traditional uses include treating rheumatoid arthritis and cardiovascular conditions with a herbal medicine. Boswellic acids are a class of pentacyclic triterpenes that are the main active ingredients in boswellia. Compounds such as 11-keto-β-boswellic acid (KBA), acetyl-11-keto-β-boswellic acid (AKBA), and β-boswellic acid (BBA) have been discovered to inhibit 5-lipoxygenase, microsomal prostaglandin E synthase-1, or the serine protease cathepsin G. Boswellic acids (BAs) have been shown to have very potent anti-inflammatory qualities. Specifically, 3-acetyl-11-keto-boswellic acid (AKBA) and 11-keto-boswellic acid (KBA) both have anti-inflammatory effects and block 5-lipoxygenase. (4)

Material & Method

Plant Material Collection and Authentication: Gum resin of Boswellia serrata and stem bark of Ficus. racemosa (F. racemosa) were purchased from local herbal store, Ghaziabad, Uttar Pradesh India in the month of March 2023. The plant material was authenticated by Botanist Dr. K.C Bhatt, Principal Scientist at [ICAR-(National Bureau of Plant Genetic

Resources)] (NBPGR), New Delhi, A herbarium was prepared (voucher specimen no. AC-05/2024) and deposited at the Department of Institute.

Chemicals and Reagents:

All the analytical grade chemicals and reagents were used for the study. The chemicals were procured from Sigma chemicals Co., USA and Qualigens fine chemicals, Mumbai, India. Organic solvents like ethanol, methanol (high-pressure liquid chromatography grade, Merck), Triron-X, 5% Tween, formalin, eosin, paraffin, chloroform, ethyl acetate, sulphuric acid, potassium permanganate and trichloroacetic acid were used.

1) Preparation of Test Extracts:

a) **Extraction of boswellic acid from *Boswellia serrate*:** The fraction containing BAs will be prepared by extracting *B. serrata* gum resin successively with ethanol (95%) in a percolator and evaporated under reduced pressure on a thin film evaporator at 40ºC to obtain a thick brown residue. Further, the extraction and isolation process will be carried out using different chemical ratio and crude mixture will be obtained. Then after, the crude mixture will again be subjected to dissolution process for finally obtaining the creamish powder of boswellic acids (5).

b) **Extraction of lupeol from *Ficus racemosa*:** The F. racemosa plant was procured locally in accordance with established protocols. The bark was chopped and let to air dry for ten days. The process of fine grinding produced the powder. The powder was heated three times in a day, using 200 g of powder and 500 ml of distilled water in a water bath set at 750C for five to ten minutes. Wattman's filter paper was used to filter the contents of the beaker, and the filtrate was then collected. The leftover powder on the filter paper was placed back into the beaker after an hour, and this time, 200 ml of distilled water was added. The mixture was again boiled in the same manner for 24 hours, and the process was repeated. The filtrate was then collected in another beaker. The powder on the paper was removed again after an hour, and the identical process was carried out. Thus, 200 grams of powder were mixed with 900 milliliters of distilled water. Two hundred grams more of powder underwent the same process. In other words, 400 grams of powder and 1800 milliliters of distilled water were combined. Four milliliters of chloroform were added to the big beaker containing the entire filtrate as a preservative. At 1000C, the beaker was placed on the water bath. Consequently, after three days, a large amount of aqueous extract was obtained as the water evaporated. By weighing the beaker containing the extract (222 gms) and the one without (182 gms), the weight of the aqueous extract was determined. Thus, it weighed forty grams. Consequently, 40 grams (10%) of aqueous extract were taken from 400 grams of dried powder. (6)

I. Isolation and characterization of phytochemicals

1) Isolation and characterization of boswellic acids: Boswellic acids were isolated by the method reported by Simla Basur. 50 gm gum resin of *B. serrata* will be extracted with methanol. After filtration, the extract will be concentrated to a viscous solution using various required chemicals. The resulting dichloromethane extract will be dried over anhydrous sodium sulphate and the solvent will be evaporated to obtain a white

powder of mixture of boswellic acids. Boswellic acid mixture will then be characterized by comparing its melting point, Fourier Transform Infrared (FTIR) spectrum and Differential Scanning Calorimetry (DSC) thermogram with those reported in the literature (7).

2) **Isolation and characterization of lupeol:** By using column chromatography, fractionates of methanolic extract were produced. The separation that was obtained by TLC was used to determine the solvent system for column chromatography. Silica gel G was used as the stationary phase in the preparation of TLC plates. Following a thorough cleaning and distilled water rinse, the plates were coated. After being exposed to sunlight for a while, the plates were heated to 120 degrees Celsius for 60 minutes. Filter paper was used to line the standard chamber, and 30 minutes were spent saturating the chamber with 100 milliliters of solvent. After testing a number of solvent systems, petroleum ether (60–80o) was ultimately chosen as the solvent system for column chromatography based on the degree of separation attained. The column was packed using a wet packing technique with an adsorbent of 300 g of silica gel (# 60-120). Hexane was used to make a slurry, which was then put into the column. After combining them with a small amount of silica gel, 13 g of sticky extract was placed to the top of the column. Column chromatography was performed using the gradient elution technique. At 60–80 degrees Celsius, petroleum ether was used to elute the column, and seven fractions, each containing 100 milliliters, were collected . To find the presence of a single component, TLC was used to concentrate the obtained fractions. Following that, five fractions were collected from the column after it was eluted in a 50:50 ratio using petroleum ether and chloroform. TLC was done after fractions were concentrated. After that, the solvent system was adjusted to a chloroform:methanol (1:1) ratio, fractions were collected, the solvent system's methanol concentration was progressively raised, and a column was run with the methanol:chloroform ratios of (60:40), (70-30), (80:20), and (90:10), respectively. Five 100 ml fractions of methanol:chloroform were collected in a 90:10 ratio, and after TLC analysis, a solitary spot was found in the third fraction. This portion was given the letter A. When a chemical test was run on the third portion that was collected, a positive result for steroids was revealed. After the final two fractions, 4th and 5th, were collected, they were combined and weighed. The result was 700 mg. (8).

PHARMACOGNOSTICAL EVALUATION

Macroscopical Characterization: Macroscopical examination was conducted to identify the key morphological features of Boswellia Serrata. Various parts of the plant were observed, including the branch with leaves (Fig 1), trunk with exfoliating bark, and the exudation of resin following tapping). Additionally, the crude gum-resin was collected and analyzed for its physical characteristics.

Pharmacognostical Evaluation

Organoleptic Characters: The organoleptic properties of the Shallaki oleo-gum-resin were characterized based on its consistency, nature of the powder, color, taste, and odor. These

observations are summarized in Table 2. The oleo-gum-resin was found to have a coarse, hygroscopic powder form, with a whiteyellow color. Its taste was sub-acrid, bitter, pungent, and sweet, while the odor was sweet, balsamic, and aromatic.[9, 10]

Phytochemical constituent's determination

The following phytochemicals such as tannins, alkaloids, flavonoids, saponins, steroids, terpenoids, carbohydrate, and proteins were determined by the methods described by Mikail and Venkitachalapathi kalaiselvi (11) (12)

Solubility Tests

The chemical properties of the oleo-gum-resin of Boswellia serrate and extract of Ficus serrate were evaluated using various solubility tests. The resin demonstrated solubility in water and alcohol, insolubility in acids, and solubility in glycerin and concentrated HCl, which produced a reddish or brown color.[13]

Physico-Chemical Parameters

The physico-chemical properties of the oleo-gum-resin were assessed, including loss on drying, total ash value, water-soluble extract, volatile oil content, and pH value. The results indicated a loss on drying of 20.74% w/w, a total ash value of 6.00% w/w, and a water-soluble extract content of 24.20% w/w. The volatile oil content was measured at 2.40% w/w, and the pH was determined to be 6.09.[14, 15]

CHARATERIZATION OF BOSWELLIC ACID AND LUPEOL

Identification Tests

Identification tests such as Solubility, Ultraviolet (UV) spectroscopy, Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and High-performance liquid chromatography (HPLC) were conducted.

Solubility

Solubility of *Boswellia serrata* and *Ficus racemosa* was checked by using different solvent like water, methanol, hexane, dichloromethane, dimethyl formamide and acetonitrile.

U.V. Spectroscopy Method: Preparation of Standard Plot of Boswellia serrata Exudates in 6.8 Phosphate Buffer Solution

For making the standard curve of Boswellia serrata extract serial dilution were made. Initially a solution of the concentration of 1000 mcg/ml was made by weighing 10 mg of drug using digital balance (Shimadzu, AU X 220) and dissolving in 10 ml of 6.8 phosphate buffer solutions. From this stock solution different concentration ranging from 25-100 mcg/ml were made in using 6.8 phosphate buffer solution as blank at 260 nm for detection of boswellic acid. A graph was plotted between the concentration (X-axis) and absorbance (Y – axis).

UV Spectroscopy Method: Preparation of Standard Plot of *Ficus Racemosa* extract

Weight accurately 0.1gm of powders FRS stem extract. It dissolves in 20ml of ethanol and kept it for 15 minutes in sonicator, and makeup to volume 100ml. then subjected to UV from wavelength between 900-200nm. Record the UV spectra.

High Performance Liquid Chromatography of boswellic acid

'H and 13C NMR spectra were recorded at 400 and 100 MHz, respectively, on an AMX-400 FT NMR spectrometer and EIMS on Jeol D-300 spectrometer. Melt- ing points were recorded on a V. Scientific melting point apparatus (MP-1) and are uncorrected. UV spectra were recorded on a PDA detector of Shimadzu HPLC; IR spectra on a Perkin-Elmer spectrum BX FTIR. The preparative HPLC (Shimadzu) conditions are: Supelcosil PLC-18, 250 × 21.2 mm, 12 µ, mobile phase 0.1% v/v phosphoric acid in water: acetonitrile (10:90), flow rate 20 mL/min and UV detector at Amax 210 and 248 nm.

The HPLC (Shimadzu) system for estimation of boswellic acids equipped with Alltima C18,5 µ (250 mm × 4.6 mm) column, LC-10AT pumps, SCL-10A system controller, SIL-10A autoinjector, SPD-M10 AVP photo diode array detector set at a wavelength of 210 and 248 nm for detection and class M10A software were used. A Millipore Swinnex type filter (pore size = 0.45 µm) was used for filtration.

Isolation of boswellic acids: 20 g of commercially available extract of boswellic acids (85%) (B.No. L 001008) was subjected to column chromatography over silica gel (ACME 100-200 mesh) using mixtures of hexane and ethyl acetate in increasing polarity. Selected fractions were combined based on TLC and analytical HPLC into four fractions. Fractions 1 and 2, on recrystallization from a mixture of hexane-ethyl acetate gave 11-keto-ß-boswellic acid 1 (1.3 g) and 3-O-acetyl-11-keto-ß-boswellic acid 2 (810 mg) respectively. Preparative HPLC of fraction 3 gave a-boswellic acid 3 (150 mg) and ß-boswellic acid 4 (1.3 g) and fraction 4 yielded 3-O-acetyl-a-boswellic acid 5 (300 mg) and 3-O-acetyl-ß-boswellic acid 6 (1.15 g), respectively (6).

Chromatographic conditions

The mobile phase consisted of acetonitrile–water (90:10, %v/v) adjusted to pH 4 with glacial acetic acid. Samples were analyzed using the following parameters: flow rate, 2.0 mL/min; injection volume, 20 µL; run time 18 min; temperature, 27 ± 2ºC; detection wavelength, 260 nm.

Preparation of stock solution of standard BSE

Accurately weighed 50 mg standard BSE powder were dissolved in 25 mL of methanol to get a BSE stock solution (2 mg/mL; corresponding to 116.6 µg/mL of KBA and 65 µg/mL of A-11-KBA).

Identification of boswellic acids

Standard BSE powder (100 mg) was hydrolyzed with aqueous sodium hydroxide solution (20 % w/v, 25 mL) by heating on a steam bath for 2 h. The content was cooled to room temperature, neutralized with hydrochloric acid, and extracted with chloroform (10 mL × 3). The chloroform extract was dried with anhydrous sodium sulphate and evaporated to dryness. The obtained solid (5 mg) was dissolved in methanol (100 mL) and analyzed by the proposed method.

Calibration curve of standard 11-KBA and A-11-KBA

Aliquots (1, 2, 3, 4, and 5 mL) of the standard solution of BSE were diluted up to 10 mL with methanol (corresponding to 11.66, 23.32, 34.98, 46.64, and 58.30 µg/mL of KBA and 6.50, 13.00, 19.50, 26.00, and 32.50 µg/mL of A-11-KBA). Twenty microliters of solution from each flask were injected manually into the chromatographic system. The calibration curve was prepared by plotting peak areas of 11-KBA and A-11- KBA against respective concentrations. Validation of the proposed method Validation of the proposed method was carried out in terms of linearity, accuracy, precision, limit of detection, and limit of quantitation as per ICH guidelines. The linear responses for 11-KBA and A-11-KBA in the range 11.66–58.30 µg/mL of 11-KBA and 6.50–32.50 µg/mL of A-11- KBA were assessed in terms of slope, intercept, and correlation coefficient values. The accuracy was determined by standard addition method. To a fixed amount of pre-analyzed sample of BSE, increasing amount of standard BSE solution was added in all the levels of calibration curve. The recovery of 11-KBA and A-11-KBA was calculated at each level (n = 3). The inter-day precision (RSD) was determined by analyzing a standard solution of BSE over the entire calibration range for five days. The intra-day precision (RSD) was determined by analyzing a standard solution of BSE over the entire calibration range for five times on the same day. For determinations of limit of detection, the concentrations of standard lower than that of the lowest point of calibration curve were injected to the HPLC and responses were measured.

Purification of isolated compounds of *Ficus Racemosa* by HPTLC and High performance liquid chromatography

Preparative High-performance Thin-layer chromatography (HPTLC) of *Ficus Racemosa*: The isolated pure compound was dissolved in appropriate solvents. 5 µl of isolated compounds (lupeol) were applied to silica gel plates, Merck (Germany) 20×20 cm, 0.25 mm in thickness. Plates were developed using the solvent system nHexane: Ethyl acetate (80:20v\v) for lupeol. The separated zones were visualized with freshly prepared Libermann Burchard reagent and heated at 100°C for 10 minutes. Chromatograms were then examined under daylight within 10 minutes (17).

High-performance liquid chromatography (HPLC) of *Ficus Racemosa*

The analytical HPLC system (Shimadzu) was equipped with a diode array detector, a 20µl loop, 200 x 4.6 mm C18 column, methanol (HPLC grade, 0.2mm filtered) used as a mobile phase. The isolated Lupeol compounds were separated using a mobile phase of methanol:

water (75:25 v/v) and isopropyl alcohol and water (80:20) as mobile phase and detected by UV at wave length of 210 nm and flow rate of 1.0 ml/min, column temperature 30 °C. Injection volume was 40 µl and detection was carried out at 346 nm (98). The content of lupeol was calculated using the formula:

Content of lupeol = Sample area x Standard weight x Purity/Standard area x Sample weight.

4.7 FORMULATION OF GEL

4.7.1 Method of Preparation

The ointment bases were combined and gradually heated while stirring until a homogeneous mixture formed. To cool, stirred it. 1 gm of appropriate extract was be combined with 10 g of ointment base inside this formulation (10 percent). The mixture was next be thoroughly mixed until a homogeneous base is formed. Bases (water miscible bases) used in the formulation of *Boswellia serrate* and *Ficus racemosa.*

Table1: Bases (water miscible bases) used in the formulation of *Boswellia serrate* and *Ficus racemosa.*

S.no	**Ingredients**	**Formula (official)**	**Formula (Working)**
1	Emulsifying wax	30 gm	3 gm
2	White soft paraffin	50 gm	5 gm
3	Liquid paraffin	20 gm	2 gm

Table 2: Bases (water miscible bases) used in the formulation of *Boswellia serrate* and *Ficus racemosa.*

S.no	**Ingredients**	**F1**	**F2**
1	Carbopol-934	1.5g	1.5g
2	Polyethylene	15g	15g
3	Triethanol Amine	1.5g	1.5g
4	Sodium Sulphite	0.1g	0.1g
5	Plant extract	2%+2% (1:1)	2%+4% (1:2)
6	Water up to (ml)	50	50

Evaluation of ointment formulation:

a. Color and Odour:

Visual inspection was used to assess color and odor.

b. pH: The pH of all the formulations was determined by using digital pH meter. 1.5gm of gel was accurately weighed and dispersed in 15ml of distilled water and stored for two hours. The measurement of pH of each formulation was carried out in triplicate and the average values are represented in Table. The pH of dispersions was measured using pH meter.

c. Spreadability Test: Spreadabilty can be expressed as the extent of area to which the gel readily spreads on application to skin or affects part. It is used to describe the region across

whereby the gel spreads easily when applied to skin or afflicted area. It was measured in terms of time it takes 2 slides to slide off the gel and position between the slides below a specific load. The less time it takes to separate two slides, the greater the spreadability. The equation was used to calculate spreadability.

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

d. Extrudability: For such an investigation, a straightforward methodology was used. After the ointments were placed in the bottle, the compositions were placed in collapsible tubes. The weight in grams necessary to extrude a 0.5 cm ribbon of ointment in 10 seconds was used to evaluate the extrudability of the various ointment compositions.

The extrudability was calculated using the following formula: Extrudability = Applied weigh to extrude gel from tube (gm) / Area (cm^2).

e. Viscosity: Brookfield viscometer was used to determine the viscosities of gels. The measurement was made over the whole range of speed settings from 10rpm to 100rpm with 30 seconds between two successive speeds and then in a descending order

f. Stability Study: A physical stability of herb ointment was performed at 80°C for 50 minutes, and the stability was maintained with one month. During first month, those formulations were tested for color, odor, texture, traces of gritty particles, and skin irritation.

ANIMALS EXPERIMENTAL STUDY: EXPERIMENTAL PROTOCOL

The pharmacological investigation will be carried out by using excision, incision and burn wound heal model in Swiss albino rats. Wistar albino rat weighing 150-220 gm will use. The animal will be housed a 23 degree C with 12:12 hr light and dark cycle. They have free access to food and water ad libitum. The animals will be acclimatized for a period of 7 days before the study. Animals will be procured from CDRI. All the experimental study will be conducted in accordance with CPCSEA and IAEC guideline.

Formulation of Ointment: Bases used in the formulation of boswellic acid and lupeol

Grouping: Animals was divided into four groups of six animals each as Group I, Group II, Group III, Group IV.

Table 3: Grouping of animals

S.NO	Group	Treatment	Animals
1	I	Ointment base (control)	6
2	II	10% Povidone Iodine (standard)	6
3	III	Gel Formulation 1	6
4	IV	Gel Formulation 2	6

Type: Water miscible base.
Method of Preparation: Mixed the ingredients, heated gently with stirring until homogenous mixture forms (table 1). Stirred to cool. In this preparation 1gm of suitable extract is mixed with 10gms of ointment base (10%).Then it is stirred well until homogenous base is obtained.

Pharmacological screening : Incision Wound Model
Excision Wound Model
Burn wound heal Model
Animal - Wister Albino rats (150-200gms).

Procedure:

1. Excision Wound Model - The rats were anesthetized by administering ketamine (0.5 ml/kg b. w. i.p.). A full thickness of the excision wound of circular area (approx. 500 mm2) and 2 mm depth was made on the shaved back of the rats 30 min later the administration of ketamine injection. The wounding day was considered as day 0 .The wounds were treated with topical application of the ointment as described above till the wounds were completely healed. The wounds were monitored and the area of wound was measured on 4, 6, 8, 10, 12, 14, 16 post-wounding days and the mean % wound closure is report.. The period of epithelization was calculated as the number of days required for falling of the dead tissue remnants without any residual raw wound. Wound healing rate:
% of wound closure = wound area on day 0 − wound area on day n/wound area on day ×100 (18)

2. Incision wound model: The rats were anesthetized by administering ketamine (0.5 ml/kg b. w. i.p.). Incision wounds of about 6 cm in length and 2 mm in depth were made with sterile scalpel on the shaved back of the rats 30 min later the administration of ketamine injection. The parted skin was kept together and stitched with black silk at 0.5 cm intervals. Surgical thread (no. 000) and a curved needdle (no. 9) were used for stitching. The continuous thread on both wound edges were tightened for good closure of the wounds. The wounds of animals in the different groups were treated with topical application of the Ointments as described above, for the period of 10 days. The wounding day was considered as day 0.When wounds were cured thoroughly, the sutures were removed on the 8th post-wounding day and the tensile strength of the skin that is the weight in grams required to break open the wound/skin was measured by tensiometer on the 10th day report. Incision wound on the day 0.A completely healed incision wound after 8 day treatment. Tensile strength was calculated using the following formula (19)

Tensile strength =breaking strength (g)/cross-sectional area of skin (mm2

PARAMETERS FOR EVALUATION

1. Wound contraction: Planimetric tracking of gradual changes in wound area was used to monitor this. After the wounding day, the wounds would be drawn on a translucent on a different day. While tracing, the animal would be confined in the appropriate position. After that, the tracings would be copied on a 1 mm^2 graph sheet. Wound areas would be taken this

from, as well as the percentage of wound contraction would be computed using the size of a wound (100 mm^2) as the starting point. Wounded area was calculated throughout the model by drawing the wound with a transparent sheet on a millimeter-based graph on days 0, 1, 4, 8, 12, and 16 for all groups.

Percentage of Wound closure = $\frac{\text{Wound area on day 0} - \text{Wound area on day n}}{\text{Wound area on day 0}} \times 100$

2. Epithelialization period: It was monitored by noting the number of days required for eschar to fall away, leaving no raw wound away.

The no. of days necessary for tissue remains of wound to fall off with no remaining raw wound has been estimated as epithelialization time

2. Estimation of Antioxidant and biochemical parameters.

The granulation tissue was removed in order to examine pro-healing biochemical markers such as hydroxyproline (Woessner Jr., 1961) [70]and total protein concentration (Lowry et al., 1951) [71]. In 0.15M KCl with 5mM EDTA, a 10% homogenate of granulation tissue was produced. After homogenization, materials were sonicated (10 bursts of 5 s each at 5 s intervals) and an aliquot was taken for reduced glutathione quantification (GSH). Triton X-100 was added at 0.1 percent (v/v) to the residual homogenate. The samples were then incubated at 4°C for 2.5 hours before being centrifuged at 4226 g. The supernatant has been used to calculate superoxide dismutase(Marklund & Marklund, 1974) and catalase (Aebi, 1984).

A. Hydroxyproline

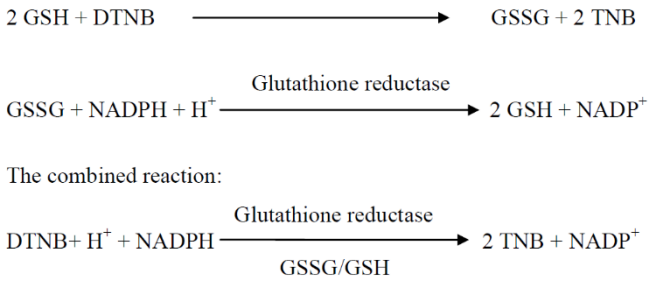
2 ml of isopropanol and 1 ml of oxidant sample was added to a 50 ml screwcapped tube holding 1 ml standard solution, as well as the solution was well mixed. After four minutes, 13 ml of Ehrlich's reagent solution were poured, the top was tightened, as well as the solution was well stirred. After being heated in a water bath at 60ºC for 25 minutes, the tubes were cooled in tap water for 2 to 3 minutes. The absorbances of samples and standards were read against the reagent blank at 558 m in 1 cm cells within 2—3 hours, and the hydroxyproline values were estimated using a standard curve generated from three to four working standards. After usage, the cell was rinsed with equal parts 95 percent ethanol and 3 N HCl.after use and rinsed with 95% ethanol and dried before use in order to avoid deposition of solid reagent that could affect absorbance readings.

B. Glutathione assay

GSH has many features, including reductive converting of hydroperoxides to respective alcohols via electron donation, xenobiotic metabolism catalysed by glutathione S-transferases (GSH acts as a co-substrate), ion channels, antioxidant, co-factor over several metabolic enzymes, radioprotectant, and facilitating protein folding and degradation.

DTNB (5,5 â dithiobis-2-nitrobenzoic acid) is used to evaluate the reaction of reduced Glutathione (GSH) to a yellow-coloured product that absorbs at 412 nm. In the setting of

GSH, the thiol-general reagent (DTNB) interacts with the GS-TNB, the 5-thionitrobenzoic-acid (TNB), and the chromophore. Finally, GS-TNB is decreased by generating a second TMB molecule and reforming GSH, which is enhanced further by the usage of glutathione reductase and -nicotinamide adenine dinucleotide phosphate (NADPH). Any GSH oxidised (GSSG) that was present in or produced as a result of the GSH GS-TNB mixed disulphide reaction is rapidly reduced to GSH.



Procedure

To measure the GSH level, the tissue homogenate in 0.1 M phosphate buffer (pH 7.4) was taken. Equal volumes of 20% trichloroacetic acid (containing 1 mM EDTA) and homogenate were mixed to precipitate the tissue proteins. The mixture was centrifuged for 10 min at 200 rpm and the supernatant (200 µl) was then transferred to a new set of test tubes. Simultaneously Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) was prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution and was added (1.8 ml) to the test tube. Then all volume of the test tubes was made up to 2 ml. The solutions were measured at 412 nm against blank after completion of the total reaction. Absorbance values were evaluated with a standard, obtained from standard curve of known GSH.

4.10 Histological Processing

On day 0, 1, 4, 8, 12, and 16 post wounding, the wound samples were preserved using buffered formaldehyde solution at 3.7 percent (pH 7.4) and analysed using normal regular light microscopy tissue techniques. The treated tissues have been inserted in paraffin, Gomori's trichrome (GT) was applied, and serial sections 5.0 mm thick have been placed on microscope slide, rehydrated, dewaxed, and stained individually with (GT) and picrosirius red (PR) for Collagenesis assessment, including one slide dedicated to every staining across all post wounding days.

RESULTS

Pharmacognostical Evaluation : The mean value was calculated and the result recorded in the table is under given below 5.1, 5.2 and 5.3. Each test was performed three times and calculate the value.

Ash Value

The total ash value of the plant *Ficus Racemosa and Boswellia serrata* was found to be 12.5 % and 3.5% respectively (w/w). The acid insoluble ash value of *Ficus Racemosa and Boswellia serrata* was found to be 0.8% (w/w) and 1.9 respectively, and water-soluble ash value of *Ficus Racemosa and Boswellia serrata* was found to be 3.3 and 9.5 % (w/w) respectively as given in table 4

Table 4: Ash value of powdered of. *Ficus Racemosa and Boswellia serrata*

Name of the Plant	**Ash value (%w/w)**		
	Total Ash	**Acid insoluble ash**	**Water soluble ash**
Boswellia serrata	3.5	1.9	9.5
Ficus Racemosa	12.5	0.8	3.3

Extractive value

The water- soluble extractive value of the herbal plant *Ficus Racemosa and Boswellia serrata* was found to be 15.9 % (w/w) and 26.5 % (w/w) and alcohol soluble extractive value of both plant was found to be 5.1 and 35.9 % (w/w) respectively as given in table 5

Table 5: Extractive value of powdered of *Ficus Racemosa and Boswellia serrata*.

Name of the Plant	**Extractive value (%w/w)**	
	Water soluble Extract	**Alcohol soluble extract**
Boswellia serrata	26.5	35.9
Ficus Racemosa	15.9	5.1

Moisture Content

The moisture content of *Ficus Racemosa and Boswellia serrata* was determined and found to be 2.41 % and 6.9 % as reported in table 6

Table 6: Moisture content of powdered seeds of *Ficus Racemosa and Boswellia serrata*

Name of the plant	**Wt. of powder plant part (gm)**	**% of moisture content**
Boswellia serrata	390	6.9
Ficus Racemosa	498	2.41 % w/w

5.2 Qualitative Chemical Examination Extract

The extract obtained were subjected to qualitative chemical to detect the chemical constituents present in them as shown in table 7.

Phytochemical Analysis

Table 7:Phytochemical analysis of *Boswellia serrate and Ficus racemosa bark* extract

Type of extract	***Ficus racemosa***	***Boswellia serrate***
Alkaloids	+++	---
Carbohydrates	+++	+++
Glycosides	+++	---
Oil and Fats	---	---
Steroids	+++	+++
Tannins and	+++	---
Flavonoids	+++	+++
Proteins	---	---
Amino Acids	---	---
Phenolic compounds	+++	+++

(+) - Presence of phytochemical compounds; (−) - Absence of phytochemical compounds

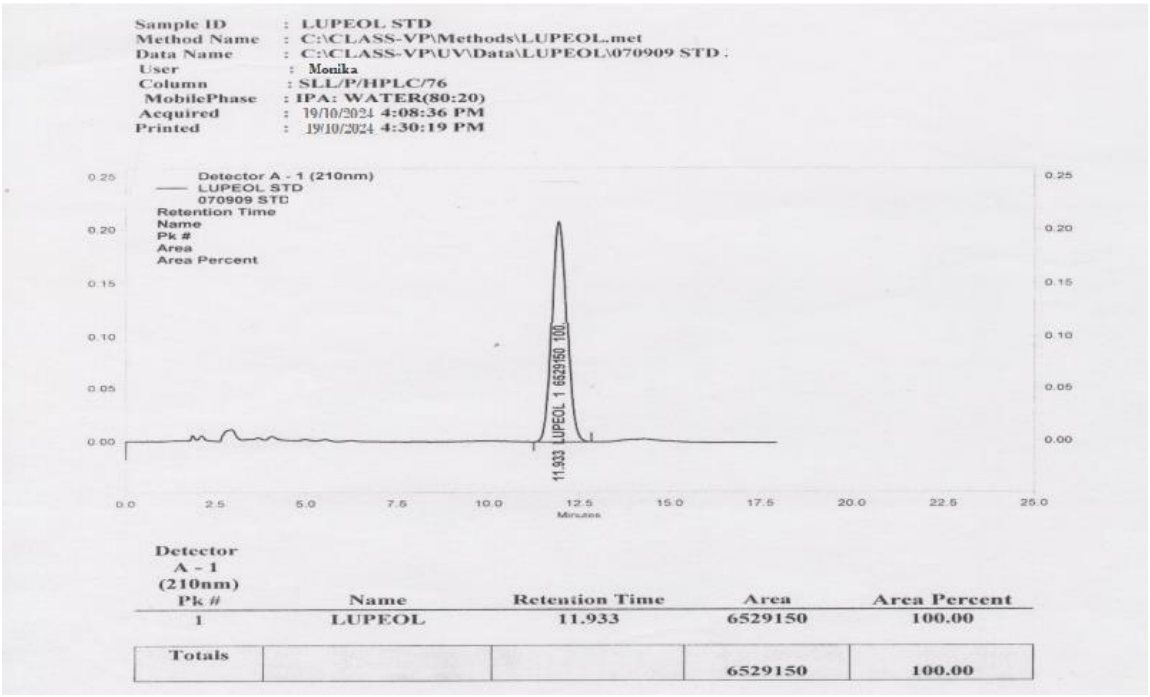
The presence of phytochemical in the leaves of *Adhatoda vasica* including phenols, tannins, alkaloids, sterols,flavonoids, and reducing sugar in ethanolhave been shown in the Table 5.1.

EVALUATION OF OINTMENT FORMULATION

Table 8: Evaluation parameters of ointment

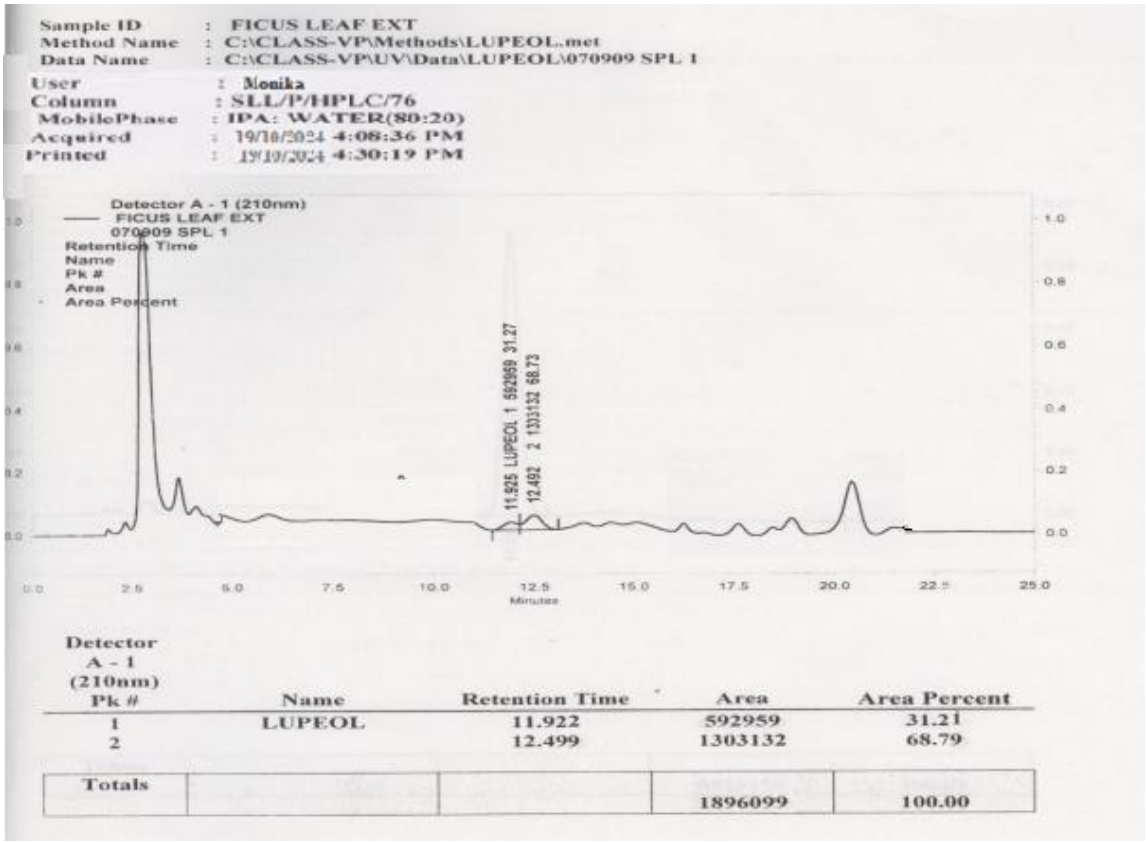
S.No	**Parameters**	**Observation & Result**
1.	Nature	Semisolid
2.	Colour	Brown
3.	Odour	Characteristic
4.	Consistency	Smooth
5.	Trace of gritty particles	Absent
6.	Skin irritation	Non-irritant
7.	pH	6.78
8.	Spreadability	6.83 g.cm/min
9.	Extrudability	0.5g
10.	Viscosity	5177pa/s

Purification of isolated compound by HPTLC and HPLC: HPTLC fingerprint patterns have been therefore evolved to check the purity of isolated compound from ethanolic extract of sample. The Rf value of standard lupeol 0.3 was matched with the Rf value of isolated compound was about 0.3 was shown in peak below figure 1, 2



Detector A - 1 (210nm) Pk #	Name	Retention Time	Area	Area Percent
1	LUPEOL	11.933	6529150	100.00
Totals			6529150	100.00

Fig 1. : HPLC graph of Standard Lupeol



Detector A - 1 (210nm) Pk #	Name	Retention Time	Area	Area Percent
1	LUPEOL	11.922	592959	31.21
2		12.499	1303132	68.79
Totals			1896099	100.00

Fig 2.: HPLC quantification of Lupeol from Ficus racemosa extract

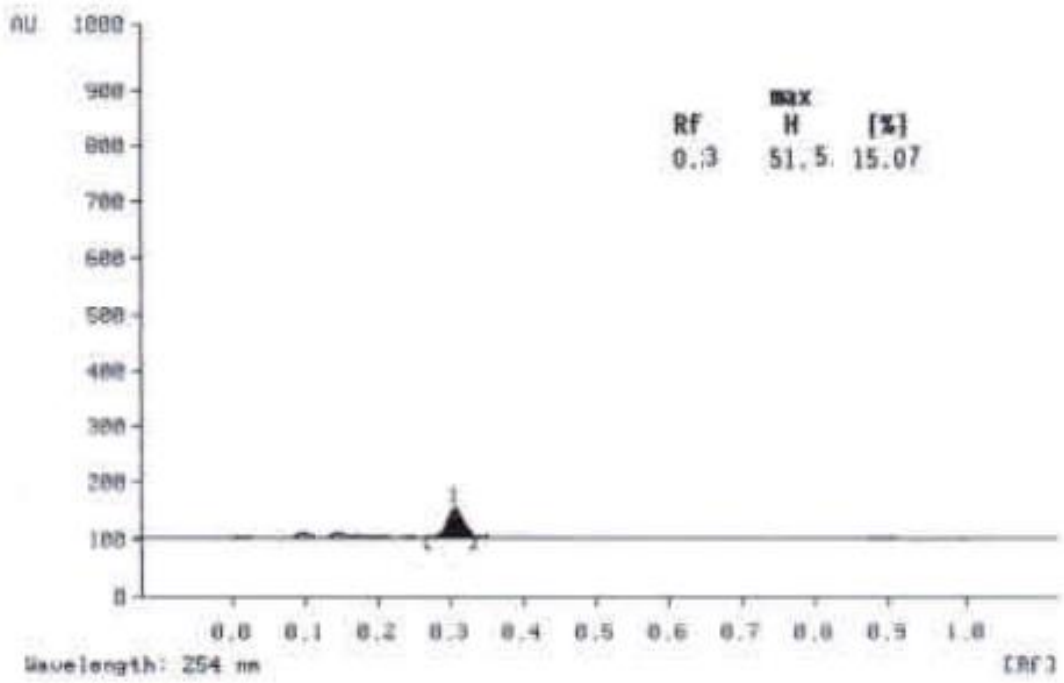


Fig. 3. HPTLC finger print profile of lupeol (5µl)

Structural Elucidation of isolated compounds: UV spectroscopy: The lupeol is white amorphous solid compound with melting point 213°C which corresponds to the molecular formulae $C_{30}H_{50}O$. The UV λ max value of compound lupeol was 372 nm (fig 4).

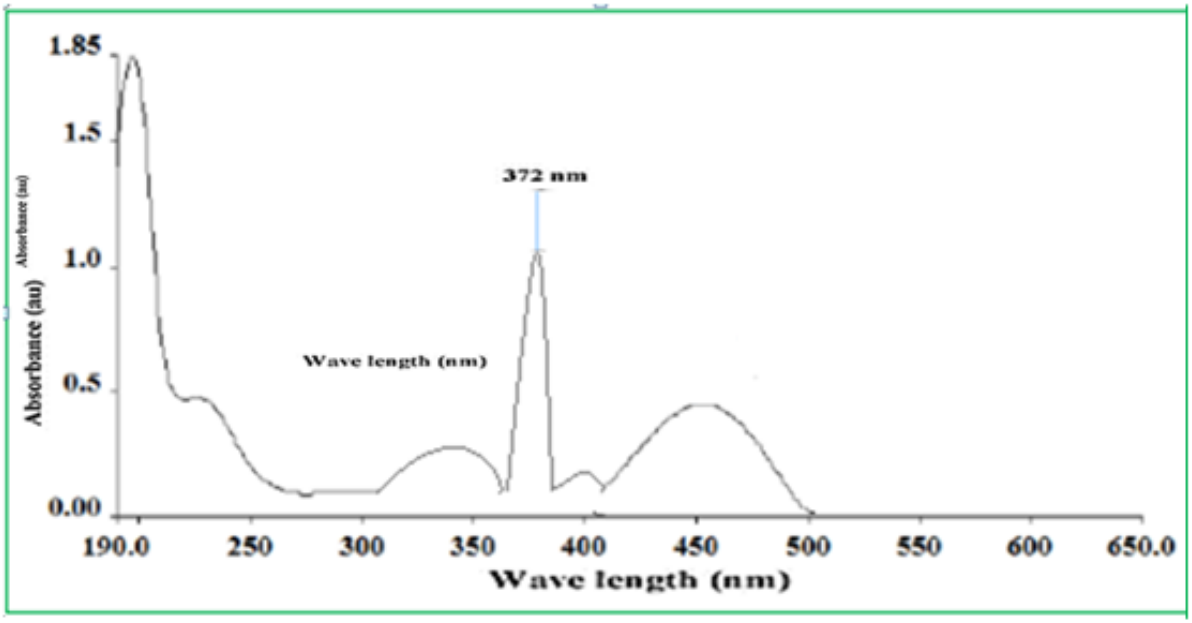


Figure 4: Displays the UV λmax value of lupeol which was found to be 372 nm. The melting point was found to be 215-216ºC. Properties computed from the structure of lupeol show that it has a molecular weight of 426.7174 (g/mol).

High Performance Liquid chromatography profile of Boswellic acid: It estimates both 11-KBA and A-11-KBA, the active acids among the boswellic acids. The mobile phase with pH 4.0 gives greater stability to the analytical column. The real advantage of the method is its low retention time: 4.30 and 7.11 min for 11- KBA and A-11-KBA, respectively. It reduces total run time for HPLC, leads to low solvent consumption, and makes the method more economical. The HPLC spectra of the TA fraction and standard BAs are shown in Figure 5,6.

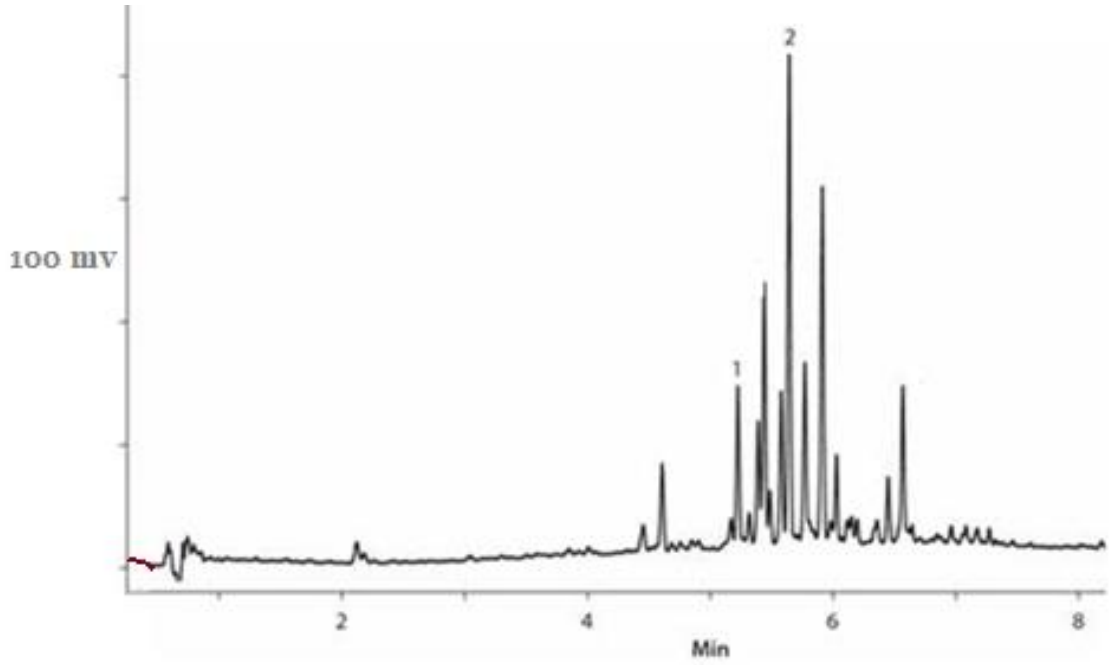


Figure 5: isolated 11-KBA (1) and A-11-KBA (2) of BSE extract

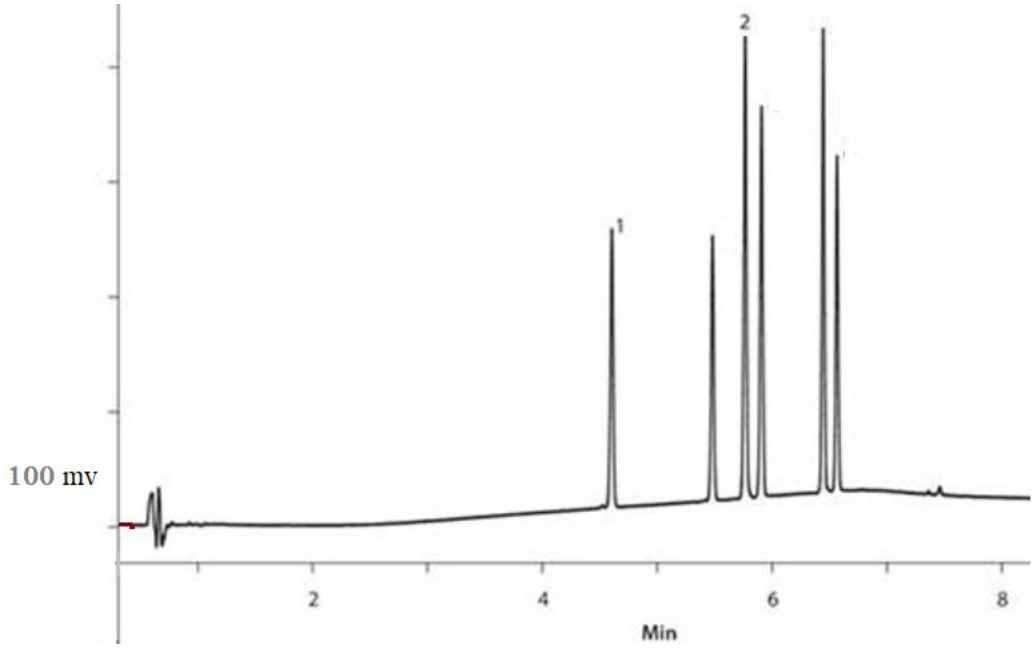


Figure 6: Analytical Standards at 50 µg/mL of 11-KBA (1) and A-11-KBA (2)

UV-vis analysis: It is an important tool for preliminary qualitative analysis as well as quantitative determination of drug molecules. The λ_{max} of the TA fraction was analyzed using UV-vis spectrophotometer and compared with standard BAs namely, AKBA, (α+β) BA and Ac (α+β) BA. The UV-vis spectra of the TA fraction and standard BAs are shown in Figure 7along with the structures of BAs shown in Figure 7.

Ultraviolet Spectroscopy: The calibration curve of U.V. depicts that it obeys Beer's Lambert law where, slope = 0.0025 and R2 = 0.996 at 260 nm

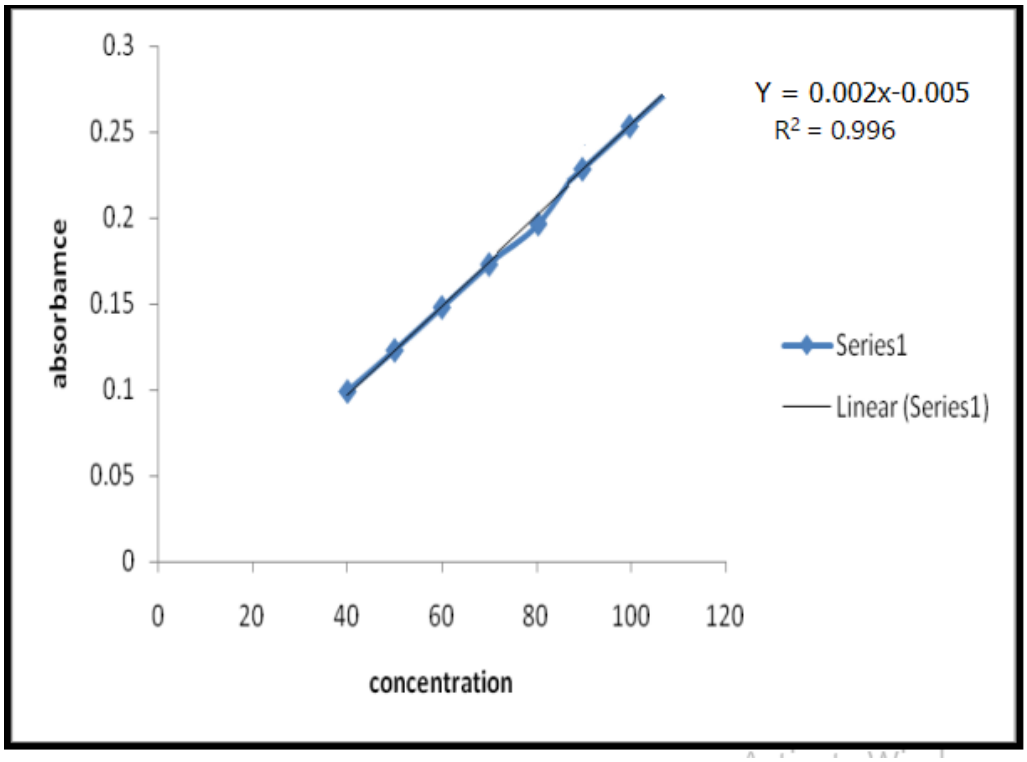


Figure 7: Calibration curve of boswellic acid through U.V.

Table 9 - Effect of formulation 1 amd 2 including boswellia serrate and ficus racemosa linn. Extracts on closure of wound area on post wounding days

S.no	Groups	**% Contraction**			
1		**4th Day**	**8th Day**	**12th Day**	**16th Day**
	Control	64.32±3.2 (16.9%)	60.54±2.3 (24.15%)	52.37±2.9 (29.7%)	56.25±2.1 (33.5%)
2	Standard	53.39±3.4 (33.6%)	25.53±2.88 (68.9%)	2.31±2.6 (95%)	2.59±2.3 (98%)
3	Formulation 1	69.39±2.7 (14.8%)	35.90±2.5 (56.5%)	15.25±3.4 (79.7%)	4.03±2.2 (93%)
4	Formulation 2	68.90±2.4 (13.3%)	35.54±1.2 (55.9%)	14.07±2.4 (80.7%)	3.88±3.5 (95.1%)

TABLE 10: EFFECT OF FICUS RACEMOSA LINN LEAF EXTRACTS ON TENSILE STRENGTH OF RESUTURED INCISION WOUND ON 10th POST WOUNDING DAY

Group	Treatment	Tensile strength (Grams ± S.E.M)
I	**Ointment base (Control)**	**411 ±5.07**
II	**Standard**	**741 ±8.27**
III	**Formulation 1**	**708±10.1**
IV	**Formulation 2**	**714 ±9.2**

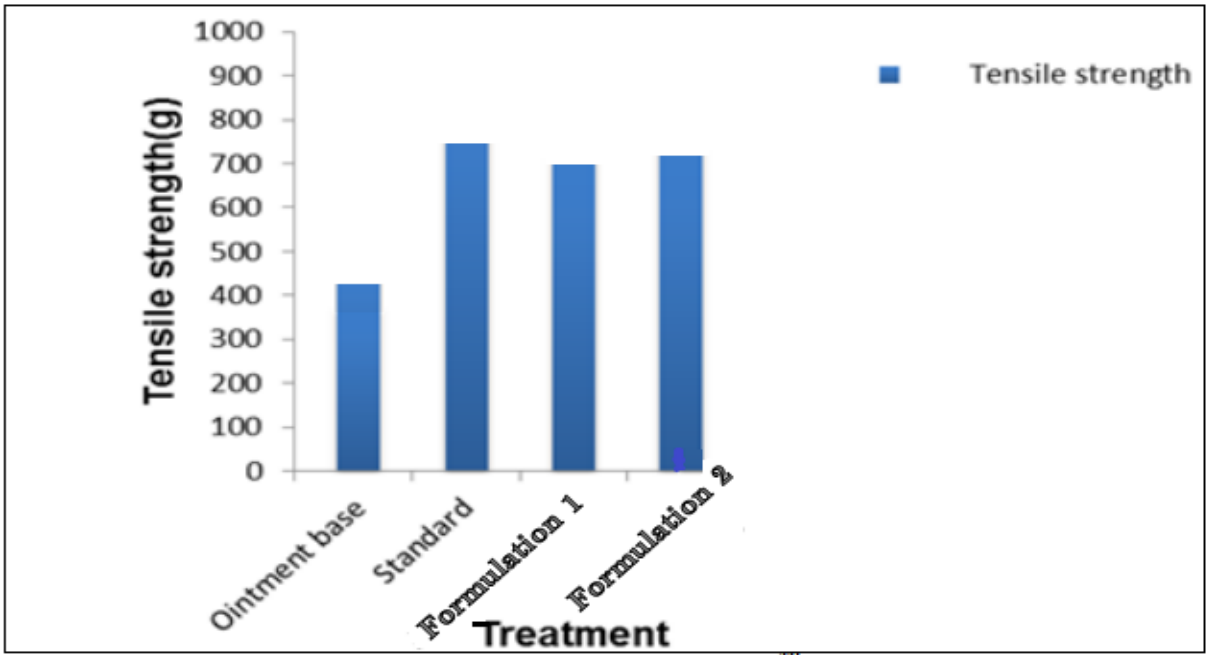


Figure 8 : Tensile strength of Incision wound on 16th Day post wounding day

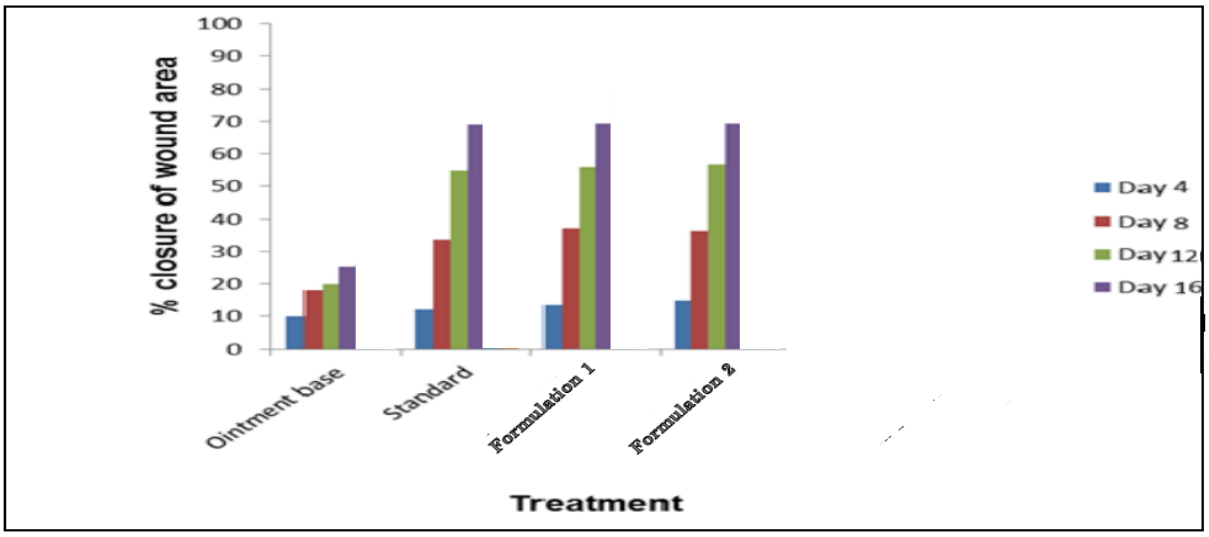
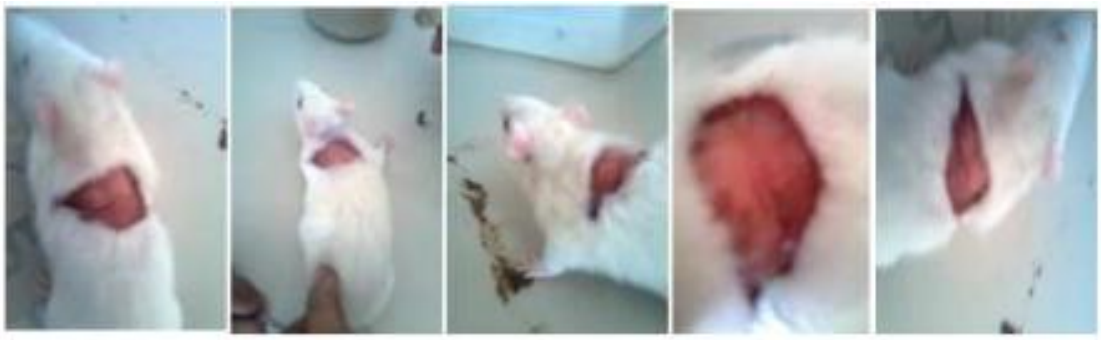
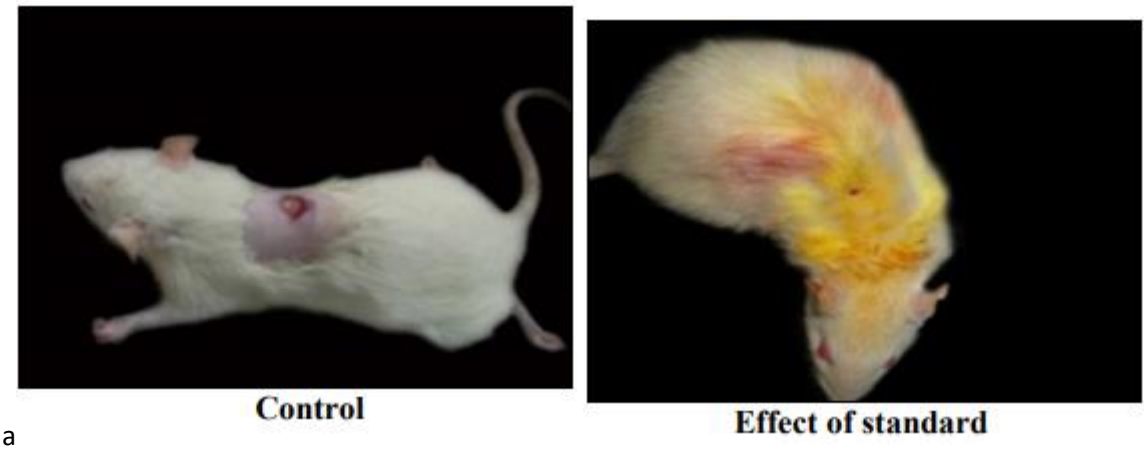


Figure 9: Percentage closure of excision wound area

Figure 10: Control, Standard, Formulation 1, Formulation 2 groups respectively on 0 day of excision wound model





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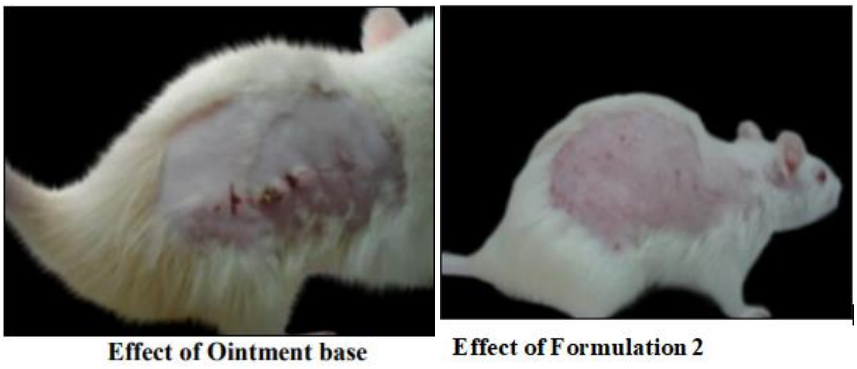

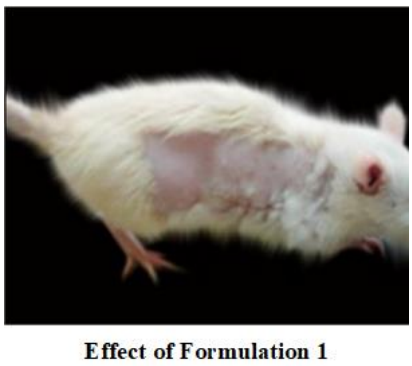

Figure 11: Control, Standard, Formulation 1, Formulation 2 groups respectively on 16th day of excision wound model

Wound contraction on different days is shown in Fig 10 and 11 and in Table 1. The wound contraction rate was measured as the percentage reduction in wound size on day 4, 8, 10 and 12. The wound contraction percentage was determined from the measurements for the first time on the 4th day after the application of oleo-gum resin of Boswellia serrate along with Ficus racemosa and carried out at 4-day intervals for the duration of 16 days. On 16 th day, all treated animal groups exhibited significant increase in the percentage of wound contraction as compared to control and standard groups. The percentage of wound contraction in formulation 1 (93%) and formulation 2 (95%) showed good wound healing activity as compared to that of control (33.4%) and standard groups (98%) which indicates complete healing of wounds.

Significant progress in the percentage of wound contraction was observed in the treated excision wounds compared with the untreated control. On days 4, 8, 10, and 12, the formulation 2 had significantly increased the percentage of wound contraction compared to formulation 1 and control. Results were comparable to that of the standard.

Biochemical Estimation in the Incision Model: Biochemical estimations like hydroxyproline and protein content in the incision model further supports the wound healing activity.

Estimation of Hydroxyproline: Hydroxyproline is one of the biochemical parameter implicated in the wound healing process. Hydroxyproline stimulates collagen formation. Therefore, estimation of hydroxyproline is yet another evidence for the wound healing effect of the extracts.

Hydroxyproline is estimated in the granulation tissue of the wound as per the literature reported procedure. Hydroxyproline was higher in animals treated with alcohol extract, enriched diet and curcumin comparable to chloroform and ethyl acetate extracts. Results are shown in Table 4, Fig. 5.

A representative calibration graph of absorbance Vs. concentration resulted in the regression

equation. (Y=mx).

$Y = 0.005x$, $R^2 = 0.994$

Where, Y = Absorbance, m = slope = 0.005, x = Concentration.

The calibration curve was found to be linear from 5 - 15µg/ml with coefficient of 0.994

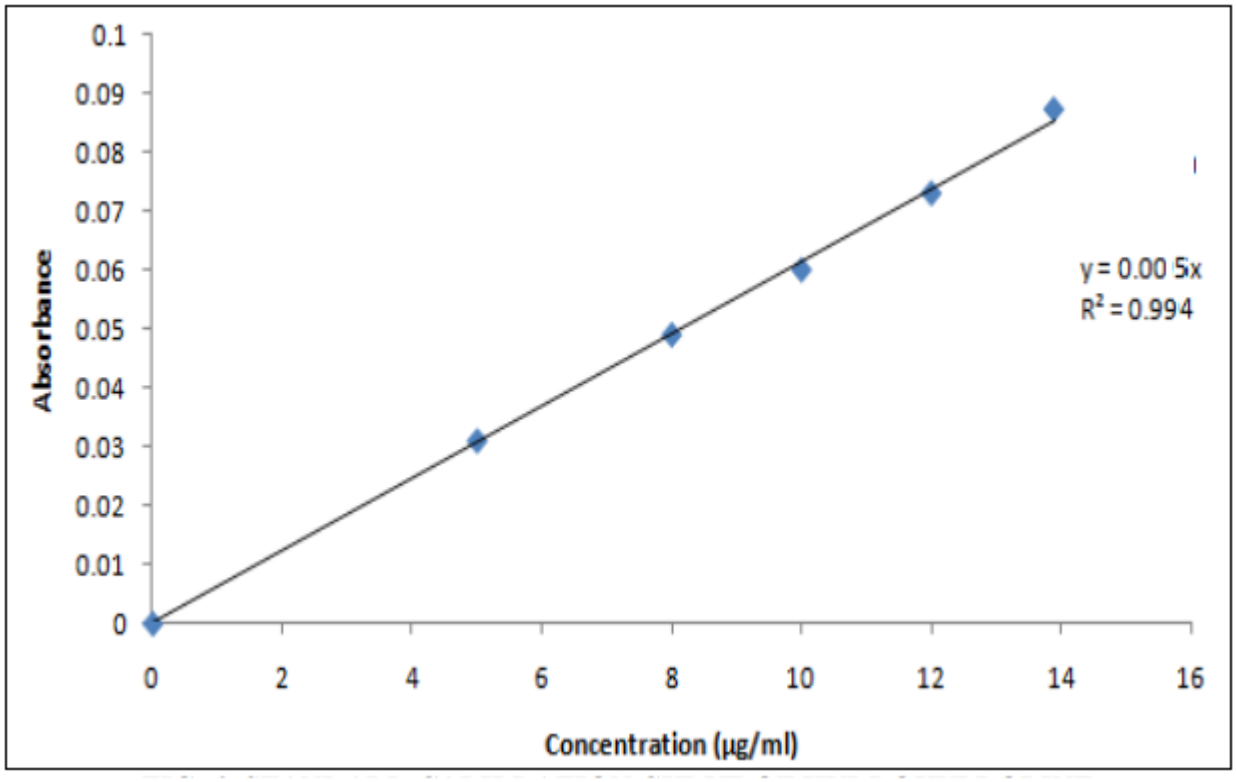

Table 12: ESTIMATION OF HYDROXYPROLINE CONTENT IN GRANULATION TISSUE

Group	Hydroxyproline in µg/50mg tissue
Ointment base	3.72 µg/ml
standard	14.01 µg/ml
Formulation 1	12.99 µg/ml
Formulation 2	13.89 µg/ml

Estimation of Total Protein: Total protein is another biochemical parameter implicated in the wound healing process. There is an increase in protein content, which was predominantly due to enhanced collagen synthesis. Total protein content was more in animals treated with alcoholic extract, enriched diet and standard compared to formulation 1 and formulation 2. Results are shown in Table 13

TABLE 13: EFFECT OF FICUS RACEMOSA and BOSWELLIA SERRATA EXTRACTS ON TOTAL PROTEIN

Group	Hydroxyproline in µg/50mg tissue
Ointment base	16.99
standard	49.01
Formulation 1	50.20
Formulation 2	48.68

DISCUSSION AND CONCLUSION:

The goal of this study was to develop a rapid and specific isocratic HPLC method for the estimation of boswellic acids (11- KBA and A-11-KBA) from their market formulations. UV-vis spectral analysis is performed for FRL and BSE shows strong absorption maxima at 372 nm. Wounds are physical injuries that result in an opening or break of the skin. Proper healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin. It is a product of the integrated response of several cell types to injury. Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wound closure and progressing to the repair and remodeling of damaged tissue. In spite of tremendous advances in the chemical drug industry, the availability of substances capable of stimulating the process of wound repair is still limited (20). Moreover, the management of chronic wounds is another major problem due to the high cost of therapy and the presence of side effects (21). Wound healing, a complex sequence of events, is initiated by the stimulus of injury to the tissues. A positive stimulus may result from the release of some factors by wounding of tissues. This sequence of physiologic events occurs by a process of connective tissue repair. These events involve four phases (22):

(i) Coagulation, which prevents blood loss.
(ii) Inflammation and debridement of wound.
(iii) Epithelial repair, including proliferation, mobilization, migration and differentiation.
(iv) Tissue remodeling and collagen deposition.

In the excision wound healing model the alcohol extract of F. racemosa and Boswellia serrata oleo-gum resin and enriched diet showed a significant increase in percentage wound closure by enhanced epithelialization. This enhanced epithelialization may be due to the effect of F. racemosa and Boswellia serrata oleo-gum resin extracts on enhanced collagen synthesis. In incision wound, the increase in tensile strength of treated wounds may be due to the increase in collagen concentration and stabilization of the fibers. A healing tissue synthesizes collagen, which is a constituent of the growing cell. Increase in blood vessels and the role of antioxidants were experimentally proved. The higher tensile strength indicates better healing of wounds. Thus it supports the wound healing activity of Ficus racemosa and Boswellia serrata oleo-gum resin. From the observations, it was evident that Ficus racemosa and Boswellia serrata oleo-gum resin possesses a definite potential healing action. The tensile strength of the incision wounds was increased in alcohol extract and enriched diet groups. The wound healing activity of extracts is further evidenced by the estimation of hydroxyproline, and total protein content which was found to be at higher levels in alcoholic extract and enriched diet treated animals compared to control.

Any agent which accelerates the above processes is a promoter of wound healing. The application of medicinal concoctions from plants to treat skin lesions, in particular, burns and wounds, has had a long tradition. Plants with wound healing activity have been reported and experimentally studied on various animal models to reveal the most active promising compounds (23). Results obtained in the present study suggest that treatment

of rat excision wounds with Boswellia serrata oleo-gum resin and Ficus racemosa has accelerated the wound healing process. Treated excision wounds showed an increased rate of wound contraction, leading to faster healing as confirmed by the increased healed area when compared to the control group. Tensile strength was measured to confirm the wound healing activity of Boswellia serrata oleo-gum resin and Ficus racemosa (figure 8). The increase in tensile strength of treated wounds may be due to increase in collagen concentration and stabilization of the fibers (24, 25). The results obtained in this study were similar to the influence of Aloe vera on collagen characteristics in healing dermal wounds in rats. It was observed that Aloe vera increased the collagen content of the skin ultimately and contributed to wound strength (26, 27). The results suggest that treatment with Boswellia serrata oleo-gum resin and Ficus racemosa may have a beneficial influence on the various phases of wound healing like fibroplasias, collagen synthesis, and wound contraction, resulting in faster healing. In conclusion, the observations and results obtained in this study indicated that the Boswellia serrata oleo-gum resin and Ficus racemosa significantly stimulated wound contraction. The breaking strength of the treated excision wounds increased in the treated groups compared with the control and standard groups. These findings could justify, role of this plant material in the management of wound healing. Further experiments are needed to test the effect of this plant in treatment of chronic wounds.

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