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ORIGINAL ARTICLE

Excision and incision wound healing activity of apigenin (4',5,7-trihydroxyflavone) containing extracts of *Carissa carandas* Linn. fruits

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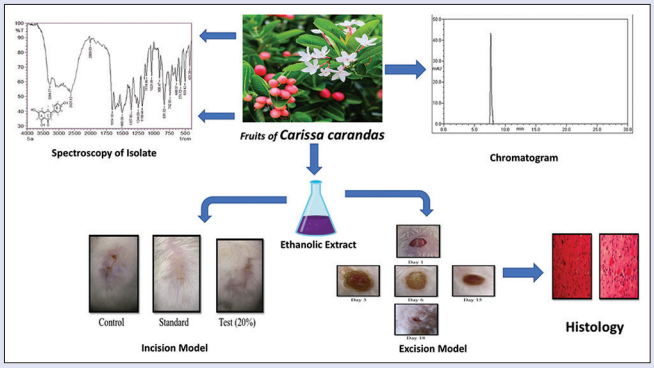
ABSTRACT

Background: *Carissa carandas* Linn. (family Apocynaceae) is a climbing shrub, with a height of 10 or 15 ft (3–5 m). It is found in Jharkhand and other states of India. It is commonly known as "Karunda" or "Jasmin flower Carissa." The major requirement for its growth is full exposure to the sun. *C. carandas* blooms and fruits throughout the year. **Objectives:** Gel of fruit extracts was formulated to determine its wound healing ability by applying it topically on wounds in Wistar rats. **Materials and Methods:** To study the wound healing activities, excision and incision studies, histopathology, and phytochemical studies were done. Ethanolic fruit extract of *C. carandas* was applied topically as 20% w/w gel. Excision and incision wound healing models were employed for determining wound contraction and tensile strength percentage. **Results:** The ethanolic fruit extracts of *C. carandas* (EFCC) 10% and 20% w/w showed effective activity compared to the simple ointment treated and untreated groups. EFCC 20% w/w had a better effect compared to EFCC 10% w/w. There was an increase in the rate of contraction of wounds on 18th post-wounding day (3.04 ± 0.55***) and increase in tensile strength (388.31 ± 0.59***). Tissue pathology of revitalized skin of EFCC revealed increased production of collagen, mononuclear cells, and fibroblasts. Apigenin (4',5,7-trihydroxyflavone) was also isolated from the extract. **Conclusion:** The EFCC ointment (20% w/w) showed a significant increase in wound healing activity, validating its use by the tribal people.

Key words: Apigenin, *Carissa carandas*, excision, histopathology, incision, wound healing

SUMMARY

- Since time immemorial, in India, medications based on herbal origin have been the basis of treatment, cure, and prevention of various diseases. This makes use of a large number of plant species that serve the purpose. One such plant is *C. carandas*, belonging to the family Apocynaceae. The fruits of *C. carandas* had been used by the tribal people for the healing of wounds. Hence, the present study was aimed to explore the wound healing potential of fruit extracts of *C. carandas* using incision and excision wound models through histopathology and phytochemical studies. The dried fruits of the plant were extracted using ethanol as a solvent. Acute toxicity studies of selected fruit extracts showed neither a visible sign of toxicity (no LD_{50}) nor mortality. The excision model revealed that 20% gel of ethanolic fruit extract ointments showed significant wound healing activity by decreasing the period of epithelization and increasing the formation of granulation tissue, synthesis of collagen, and rate of wound contraction, compared to the control group. The topical gel thus formulated was nonirritant upon application to the skin. The incision wound study of EFCC revealed an increase in tensile strength, that is, topical application of 20% gel showed a significant increase in the breaking strength (388.38 ± 3.76). Histopathology study of the ethanolic extract (20% gel) revealed aggregation of macrophages, enhanced migration of fibroblast cells, formation of new blood vessels, and enhanced collagen deposition (prerequisite for the initial phase of wound healing), indicating nearly complete healing of the wound.
- Ten grams of fruits and root extracts was subjected to column chromatography. Silica gel adsorbed extracts were poured into the column. A yellow, amorphous powder was obtained and it showed positive test for flavonoids. It was identified as apigenin through spectral analysis.



Abbreviations used: *m/z*: mass/charge; N: normal; m. p.: melting point; UV: ultraviolet

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INTRODUCTION

The herbal products or herbal medicines are useful for their therapeutic or medicinal values. They may be obtained from leaves, flowers, fruits, barks, seeds, and roots or other parts of the plant.[1] The active principles are diverse and may be unknown. Therefore, identification of bioactive materials is required after assessing the pharmacological activity of the plant extracts.[2]

A wound can be defined as a rupture in the tissue resulting from violence, accidental injury, or trauma. It is said to be healed if there is

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restoration of the wounded or inflamed tissue to normal condition.[3] Wound healing is a significant biological process that involves repair, growth, and regeneration of tissues in a phased manner involving a series of well-organized biochemical and cellular events.[4,5] In other words, wound is a breakdown of the continuity of epithelial integrity of the skin without damaging underlying connective tissues.[6]

Wound healing can be defined as a phenomenon that includes a highly organized cellular, humoral, and molecular mechanism that results in the restoration of anatomic continuity and function. The present study has been designed for the formulation and evaluation of the wound healing activity of ethanolic fruit extracts of *Carissa carandas* Linn. (Apocynaceae) (EFCC), which has been widely used in indigenous systems for this purpose, and to confirm whether this plant has a good potency or not.

MATERIALS AND METHODS

Plant materials

The dried powdered fruit of *C. carandas* (500 g) was subjected to hot extraction using a Soxhlet apparatus with ethanol as the solvent. After extraction, the solvent was evaporated with the help of a rotary evaporator. EFCC were collected, and yield values of the extracts were calculated.

Experimental animals

Male Wistar rats weighing around 150–200 g were used for the study. The animals were housed in standard environmental conditions of temperature (23°C ± 1°C), humidity (45%–55%), and a 12-h light and 12-h dark cycle. Rats were fed with a standard rodent diet and tap water *ad libitum*.

Acute toxicity study

The acute toxicities of the fruit extracts of *C. carandas* were determined as per the Organization for Economic Co-operation and Development (OECD) Guidelines 423.[7,8] The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of Arya College of Pharmacy, Jaipur, Rajasthan, India (1013/PO/c/06/CPCSEA).

A single dosage of 2000 mg/kg, per os (orally), suspended in 0.5% w/v Carboxy methyl cellulose (CMC) was used in the acute oral toxicity investigation of extracts. It was administered to the rats which had been made to fast for the previous 24 h. At 0, 30, 60, 120, 180, and 240 min, as well as once a day over the next 14 days, the toxicity signs and symptoms, as well as any anomalies linked with the administration of EFCC were noted. At the conclusion of the investigation, the number of rats that survived was counted.

Acute dermal toxicity testing of the tested drugs was carried out on the shaved backs of the rats using ointments containing EFCC (10% and 20%). Following that, the animals were monitored for any signs of dermal toxicity using the methodology outlined in OCED guideline no. 402. (Acute dermal toxicity 1987).

Wound model

The two models given below were adopted to perform wound healing.

Excision wound healing model

An excision wound model was developed to study wound contraction rate and epithelization. Vapors of diethyl ether were used as anesthesia. Animals were shaved by the right side and 300-mm^2 and 2-mm depth excision wound was created. The entire wound was then left open.[9] EFCC was applied at a dose of 10% and 20% ointments daily for 18 days.

The Wistar rat models whose wounds were left undressed to the open environment were used to monitor wound contraction and epithelization time, as shown in Table 1. The reference standard drug (Soframycin), simple ointment, and EFCC ointment 10% w/w and 20% w/w were applied every day to the specific areas healed and total areas were measured 1, 3, 6, 9, 12, 15 and 18 for all groups. On every alternate day, wound margin was traced using a graph paper for measurement of progressive changes in wound area using the changes in permanent marker observed in Figures 1–4. Percentage reduction in the size of the original wound was used to express wound contraction.

$$\% \text{ Wound contraction} = \frac{\text{healed area}}{\text{total area}} \times 100$$

Healing was continued for 18 days with the above-mentioned treatments [Figure 5]. Period of epithelization was given by the number of days needed for falling of the scar without any residual raw wound.

Incision wound healing model

For the development of model, the animal was placed in its natural position on the operation table and anesthetized using light ether. Using a scalpel blade, one paravertebral straight incision of 0.5 cm was made on either side of the vertebral column. Animals were kept in separate cages by cleaning their wound using cotton swabs soaked in 70% alcohol. EFCC ointments 10% w/w and 20% w/w were applied for 8 days [Figure 6]. The sutures were removed after 8 days. On the 10th day, the tensile strength was measured by continuous constant water supply technique.[10-12]

Measurement of tensile strength

Tensile strength is a measure of resistance to breaking under tension. It shows how much the repaired tissue can resist to breaking under tension. It may indicate the quality of the repaired tissue. Usually, wound healing agents promote a gain in tensile strength of the tissues. The sutures were removed on the 8th day and the rats were again anesthetized. Tensile strength of the wound was measured by determining the mean of tensile strength on the paravertebral incisions, and the results are shown in Table 2. A comparison was made between test and control groups for tensile strength.[13]

% tensile strength (TS) standard = (TS) standard – (TS) control/(TS) control × 100

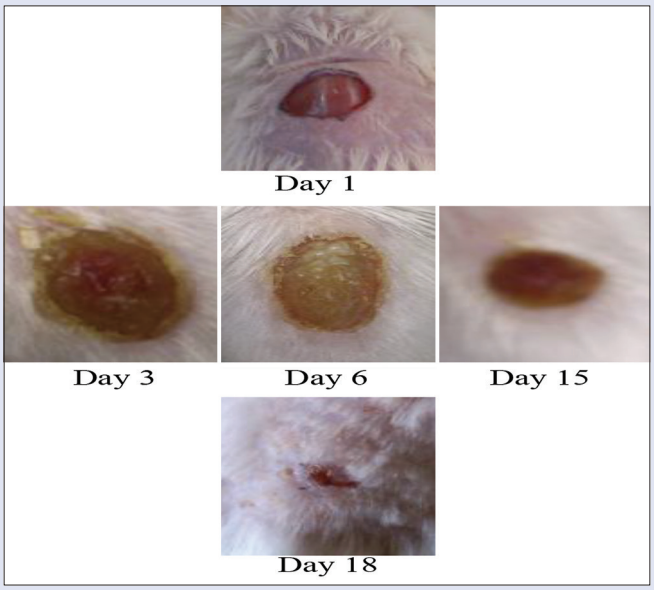

Figure 1: Control group. Excision wound model on day 1–day 18 with different test groups

Table 1: Effect of topical application of ethanolic fruit extracts of *Carissa carandas* ointments (10% and 20% w/w) on excision wounds

Post-wounding days	Wounding area (mm²) (mean±SE) and % of wound contraction			
	Group I (simple ointment)	Group II (standard)	Group III (10% w/w ethanolic fruit extracts)	Group IV (20% w/w ethanolic fruit extracts)
0	312.5±1.87	302.5±1.18	307±1.73	310.83±1.08
3	272.25±1.66	243.66±1.63**	262.33±1.08	249.33±1.40
6	207.16±1.08	153.25±0.93***	186.83±1.50**	158.75±0.93**
9	156.75±1.21	88.58±1.53***	104±1.30**	101.33±1.08**
12	116.66±1.43	37.91±1.59***	52.25±1.33***	32.16±1.08***
15	73.83±1.36	21.16±1.25***	32.33±1.40***	16.08±1.02***
18	40.91±1.82	6.83±0.98***	19.16±1.29***	3.04±0.55***

ANOVA=analysis of variance, SE=standard error, SEM=standard error of mean. Values are mean±SEM (n=6); *P<0.05 and **P<0.01 versus control; one-way ANOVA, Bonferroni's multiple comparison test

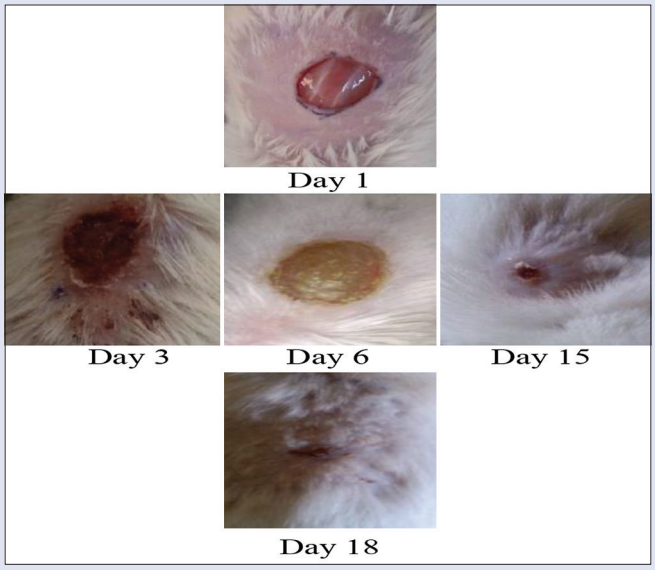

Figure 2: Standard group. Excision wound model on day 1–day 18 with different test groups

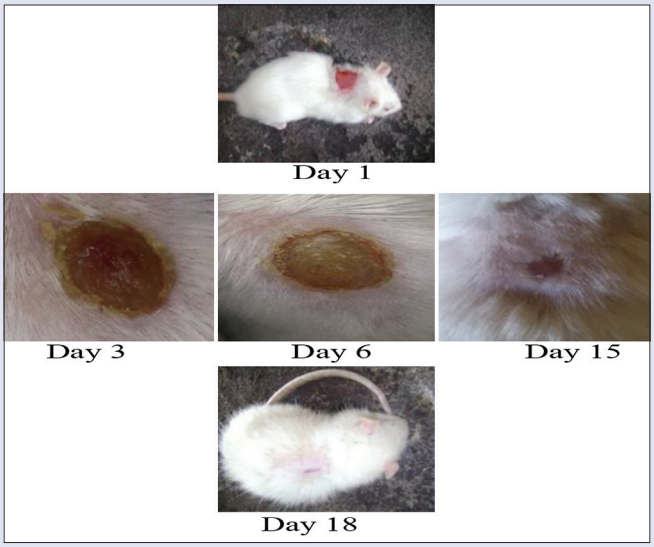

Figure 3: Excision test group (10% ointment). Excision wound model on day 1–day 18 with different test groups

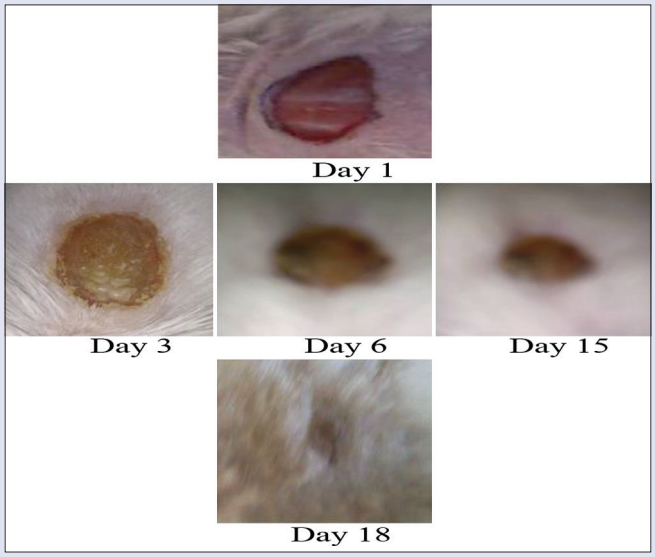

Figure 4: Excision test group (20% ointment). Excision wound model on day 1–day 18 with different test groups

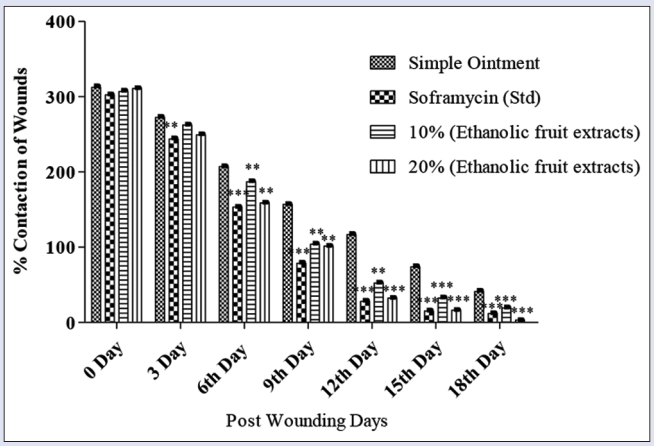

Figure 5: Effect of tropical application of ethanolic fruit extracts of *Carissa carandas* ointments (10% and 20% w/w) on excision wounds

% tensile strength (TS) (10% w/w extract) = (10% w/w extract) – (TS) control/(TS) control × 100

In the incision wound study, EFCC promoted an increase in tensile strength, which was evident from the results shown in Table 2 and the graphical representation in Figure 7. The mean tensile strength (g/mm^2) for the untreated control group was 312.19 ± 2.79; it was 367.16 ± 2.12 (10%) and 388.38 ± 3.76 (20%) for EFCC-treated Wistar rats, and for Soframycin ointment-treated wound, it was 401.58 ± 3.12.

Histopathological studies

On the 10th day of the experiment, the cross-sectional skin specimens were collected from each group of animals for histopathological studies. Neutral buffered formalin solution (10% formaldehyde in phosphate-buffered saline) was used for tissue fixation overnight. Then, tissues were kept for 2 h in 70% isopropyl alcohol (IPA), following which they were kept in ascending strengths (80%, 90%, and 100%) of IPA for 1 h. Alcohol added should be multiple of 15 to the size of tissue. Then, for the occurrence of turbidity, xylene should be added. In case of turbidity, it was again dipped in acetone for half an hour and this process was repeated for dehydration. After dehydration, the tissue was bathed in paraffin wax (m. p. = 56°C) at 58°C–60°C for 1 h. Then, poured it into L-block along to cool and become hard. Very thin sections (2–8 or 5–10 μm) of tissue were prepared using a microtome. Tissues were kept for incubation by mounting on slides using Mayer's albumin solution in the oven at 60°C for 2 h. The paraffin slides section was kept with its holder.

Paraffin was removed using xylene for 30 min and excess xylene was removed. For rehydration of tissues, 100%, 90%, and 80% IPA was used for 2–3 min each and transferred to water for 3 min. The excess water was removed and the tissue was placed in hematoxylin stain followed by tap water for 1–2 min in each. Tissue sections slides was plunged into 1N HCl followed by Scott's water for 1 min each and then dipped in eosin stain for 30 s. The tissue was kept for 2 min in each of 70% alcohol, 90% alcohol, and pure alcohol subsequently. Finally, the section was put on a slide with one drop of gum Dibutylphthalate Polystyrene Xylene (DPX) and covered with coverslip.[14,15] The slide was observed under the microscope with suitable magnification, and this is shown in Figure 8 for the excision wound model and Figure 9 for the incision wound model.

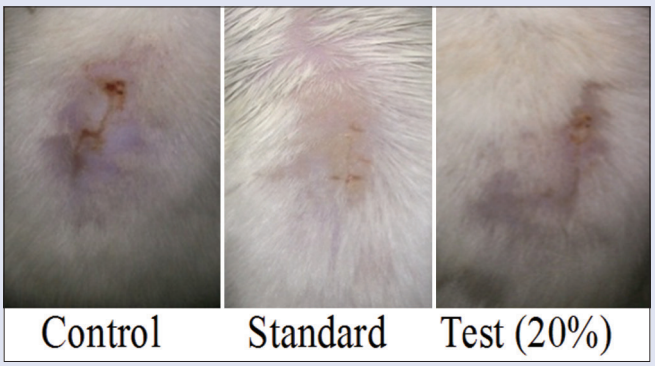

Figure 6: Incision wound model in Wister rat after 08 days

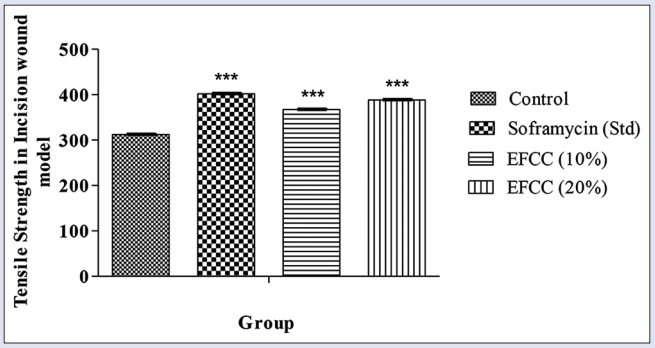

Figure 7: Effect of topical application of ethanolic fruit extracts of *Carissa carandas* ointments (10% and 20% w/w) on tensile strength in incision wound model

Phytochemical analysis

Isolation of phytoconstituents by using column chromatographic technique

Ten grams of fruits and root extracts was subjected to column chromatography. Silica gel adsorbed extracts were poured into the column. Gradient elution techniques were carried out for the isolation of phytoconstituents from the extracts. Solvents of increasing polarity were used for elution in column chromatography. Optimization of Thin Layer Chromatography (TLC) by using solvent system C_6H_6:CH_3OH was done with three separate bands has R_f 0.23, 0.44, 0.51 for fruits extracts and Rf 0.19, 0.32, 0.47 for root extracts of plant resolute. C_6H_6:CH_3OH solvent system with gradient enhancement of polarity was used for isolation of phytoconstituents from the column.

Table 2: Effect of topical application of ethanolic fruit extracts of *Carissa carandas* ointments (10% and 20% w/w) on tensile strength in an incision wound model

Group	Treatment	Tensile strength (g/mm^2)
I	Untreated group	312.20±0.63
II	Soframycin cream treated group	401.68±0.42***
III	Ethanolic fruit extracts of *C. carandas* (10%)	367.16±0.65***
IV	Ethanolic fruit extracts of *C. carandas* (20%)	388.31±0.59***

ANOVA=analysis of variance, SEM=standard error of mean. Valves are mean±SEM; n=6 animals in each group. *P<0.05 and **P<0.001 when compared to control; one-way ANOVA, Bonferroni's multiple comparison test

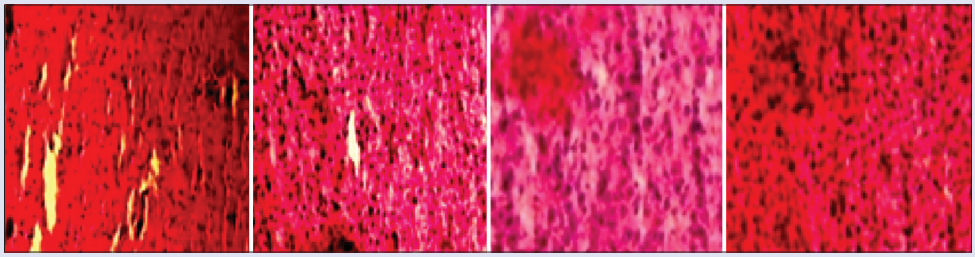

Figure 8: Hematoxylin and eosin-stained sections of granulation tissue of the excision wound model show less aggregation of macrophages (M) and formation of new blood vessels and enhanced collagen (C) deposition, indicating nearly complete healing of wound on the 10th post-wounding day

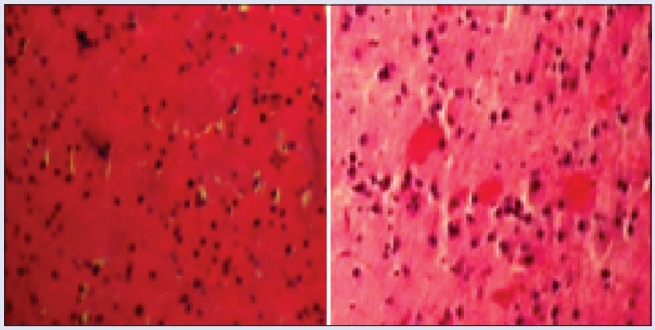

Figure 9: Hematoxylin and eosin-stained sections of granulation tissue of the incision wound model show less aggregation of macrophages (M) and formation of new blood vessels and enhanced collagen (C) deposition, indicating nearly complete healing of wound on the 10th post-wounding day. (Test I 10%, Test II 20%)

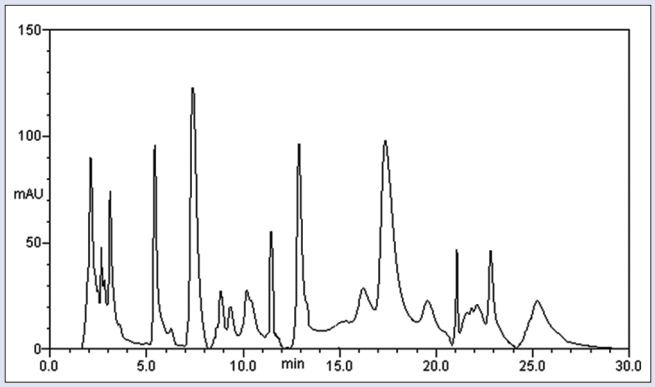

Figure 11: High-performance liquid chromatogram of fruit extracts

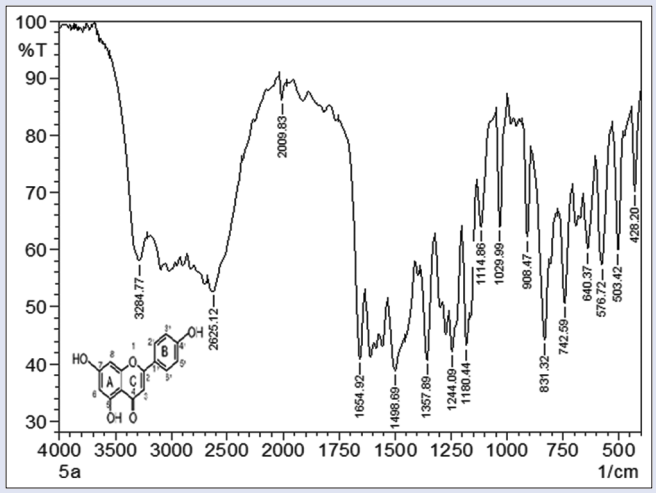

Figure 13: IR spectrum of compound A. IR = infrared

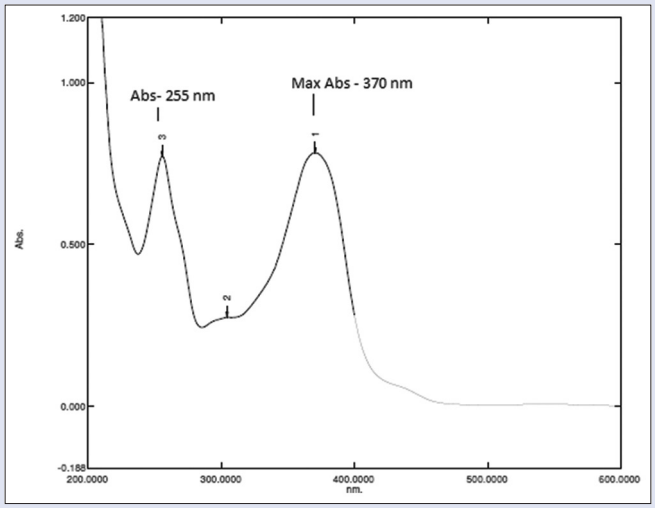

Figure 10: UV spectroscopy of compound A. UV = ultraviolet

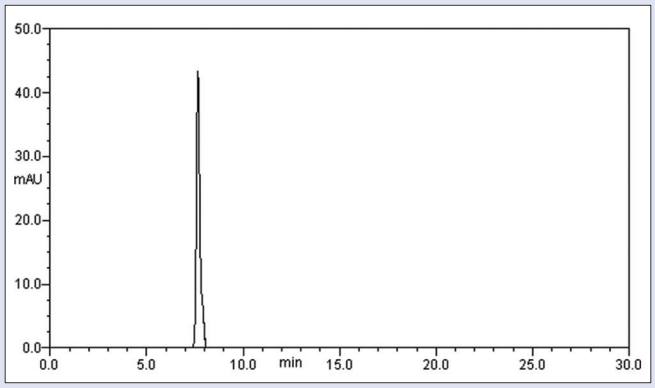

Figure 12: High-performance liquid chromatogram of compound A

Solvent system was 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, and 50% of different polarity of solvent system (C_6H_6:CH_3OH) used for both elution. A total of 32 fractions of eluents were collected from each extract. 18–30 of fruit and 20–28 of root (fraction of eluent having the same R_f value) collected from column and 40%–50% polarity of the same solvent system was used for collection of these 12 and eight fractions. The same R_f value fractions were collected. Purification of the collective fraction was done by repeated crystallization using the mixture $CHCl_3$:CH_3OH. The powder mass of each fraction obtained after crystallization was subjected to physico-chemical and spectral analysis.

Physico-chemical characters of compound A

Yellow, amorphous compound, M. P. 345°C–347.5°C. λ_{max} in MeOH: 255 and 370 nm [Figure 10]. Compound A was giving positive tests for flavonoids. The high-performance liquid chromatogram of fruit extracts and isolated compound A was observed clearly [Figures 11 and 12]. The presence of a prominent broad peak at 3284.77 cm^{-1} in the Fourier Transform Infrared spectroscopy (FTIR) spectra [Figure 13] confirms the presence of –OH group. A peak at 2625.12 shows aromatic ring substations and aromatic C–H and CH_2 stretching. A peak at 1654.92 confirms the presence of C = O group. C–O and C–O–C stretching was visible at 1180.44–1029.99 cm^{-1}.

^{1}H nuclear magnetic resonance (NMR) confirmed the presence of 10 hydrogen atoms [Figure 14]. Correlation spectroscopy (COSY) of compound A is shown in Figure 15. ^{13}C NMR confirmed the presence of 15 carbon atoms [Figure 16]. The peaks at δ 163.9, 104.5, 182.59, 161.07, 98.89, 165.07, 93.86, 157.99, 102.41, 121.89, 129.2, 115.8, 157.7, and 129.2 suggest the presence of C–O group along an aromatic ring. A peak at δ 115.8 confirms the presence of C = O group. Molecular weight was found to be 270.6. The mass spectra of compound A [Figure 17] showed the $[M-H]^+$ ion at *m/z* 270.6, which was attributed to the aglycone

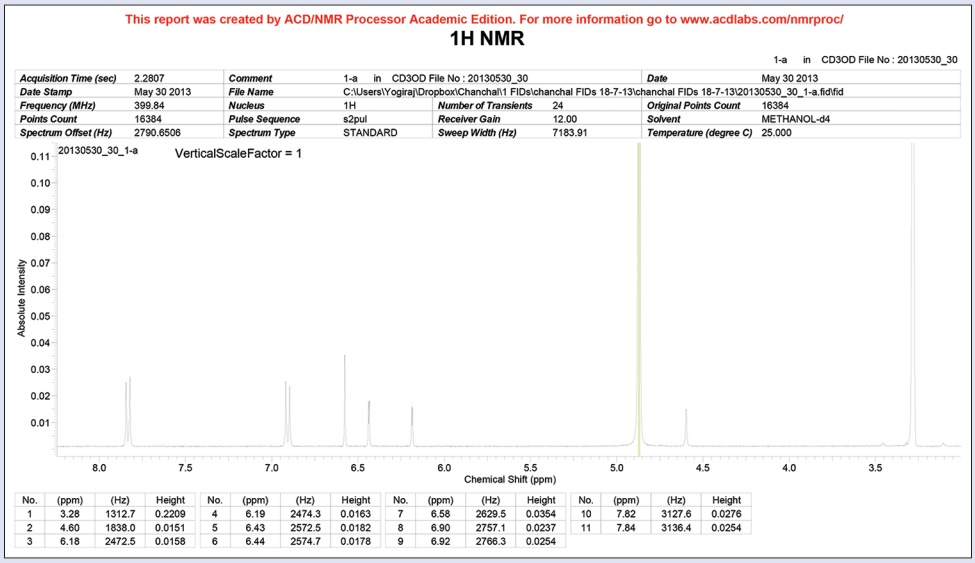

Figure 14: ^{1}H NMR spectrum of compound A. NMR = nuclear magnetic resonance

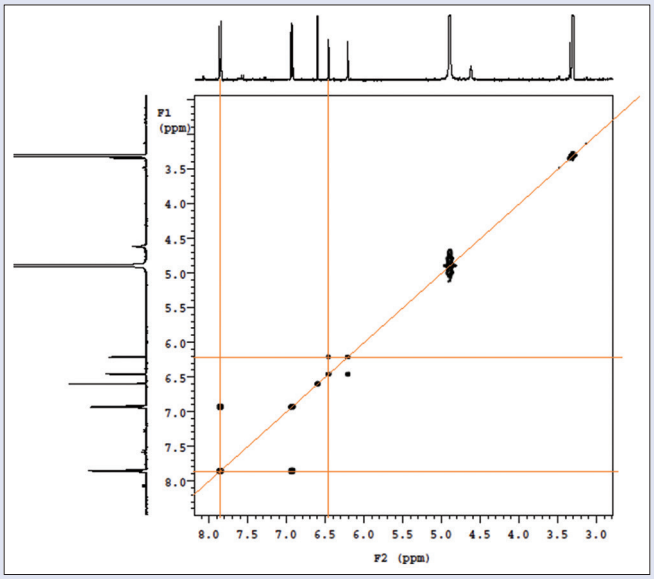

Figure 15: COSY of compound A. C6, C8- 6.2, 6.4; C6′, C5′- 7.84, 6.90; C3′, C2′- 6.92, 7.82

apigenin. So, with all the above information and elemental analysis in the background, the molecular formula was deduced as $C_{15}H_{10}O_5$.

Based upon the above observations and supported by the literature (^{1}H NMR, ^{13}C NMR, mass, and UV data) compound A was identified as apigenin (4′,5,7-trihydroxyflavone) [Figure 18]. The spectrum for the structure was compared by using different reports, which further supported the proposed structure of this compound.[16,17]

RESULTS AND DISCUSSION

A study performed for acute toxicity revealed no sign of toxicity or mortality, and the compound was found to be safe up to 2000 mg/kg as there was no LD_{50}. Hence, we consider all the extracts to be safe and non-toxic.

Data from the excision model revealed that 20% gel of ethanolic fruit extract ointment showed significant wound healing activity by decreasing the period of epithelization and increasing the formation of granulation tissue, synthesis of collagen, and wound contraction compared to the control group. The herbal gel with pH 6.8–7.2 appeared reddish-brown in color and gave a smooth feel when applied, with less variant spreadability. The values of spreadability indicated that the gel is easily spreadable by a small amount of shear. Spreadability of formulated gels (10% and 20%) was 10 and 8 g cm/s, respectively, and showed good homogeneity with the absence of lumps. Physical evaluation was done at the time of gel formulation. The topical gel thus formulated was nonirritant upon application on to the skin. Hence, two different *in vivo* models (excision wound model and incision wound model) had been chosen to assess the effect of EFCC ointments on wound healing.

In the incision wound study, EFCC promoted an increase in tensile strength, which was evident from the results. The mean tensile strength for untreated control group was 312.19 ± 2.79; it was 367.16 ± 2.12 (10%) and 388.38 ± 3.76 (20%) for EFCC-treated Wistar rats, and for Soframycin ointment-treated wound, it was 401.58 ± 3.12. In the incision repair model, the breaking strength of the wounds was measured after topical application of herbal 20% gel. In this study, it was observed that topical application of 20% gel showed significant increase in breaking strength.

The wound healing process is explained by histological examination. Topical application of 20% gel to rats revealed that it was as effective as the standard drug. The control group did not show any sign of healing. Sections of granulation tissue of rats treated with 20% gel showed less aggregation of macrophages, enhanced migration of fibroblast cells, formation of new blood vessels, and enhanced collagen deposition, indicating nearly complete healing of wound in the 8th post-wounding day. It can be concluded that the EFCC ointments (20% w/w) showed more effective activity compared to the simple ointment and untreated group (**$P < 0.001$). An increase in the rate of contraction of open wounds and increase in the tensile strength, as observed in the treated wounds and according to the histopathological slides of incised and excised wound models, attributed to the enhanced production of collagen and its reorganization, which is a prerequisite in the initial phase of wound healing.

CONCLUSION

There are many plants used in the treatment of wounds in the tribal area; through literature survey, it has been found that *C. carandas*

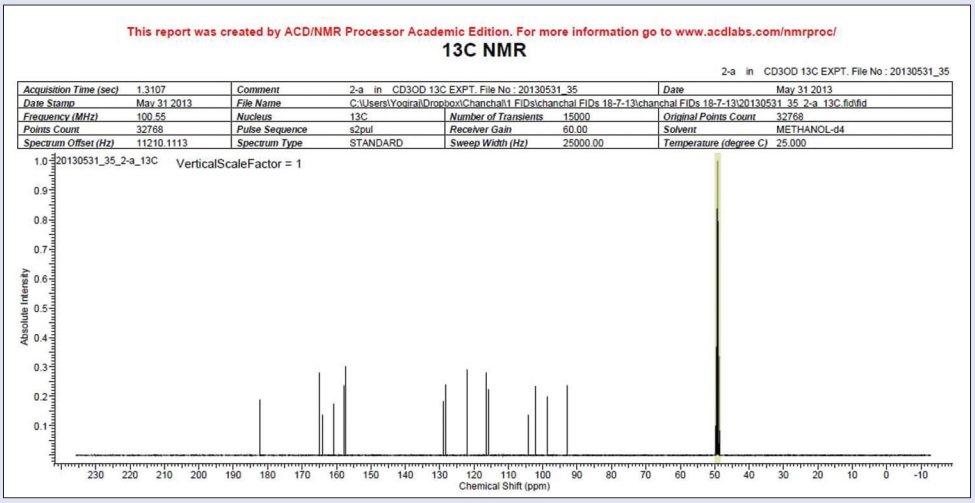

Figure 16: ^{13}C NMR spectrum of compound A. NMR = nuclear magnetic resonance

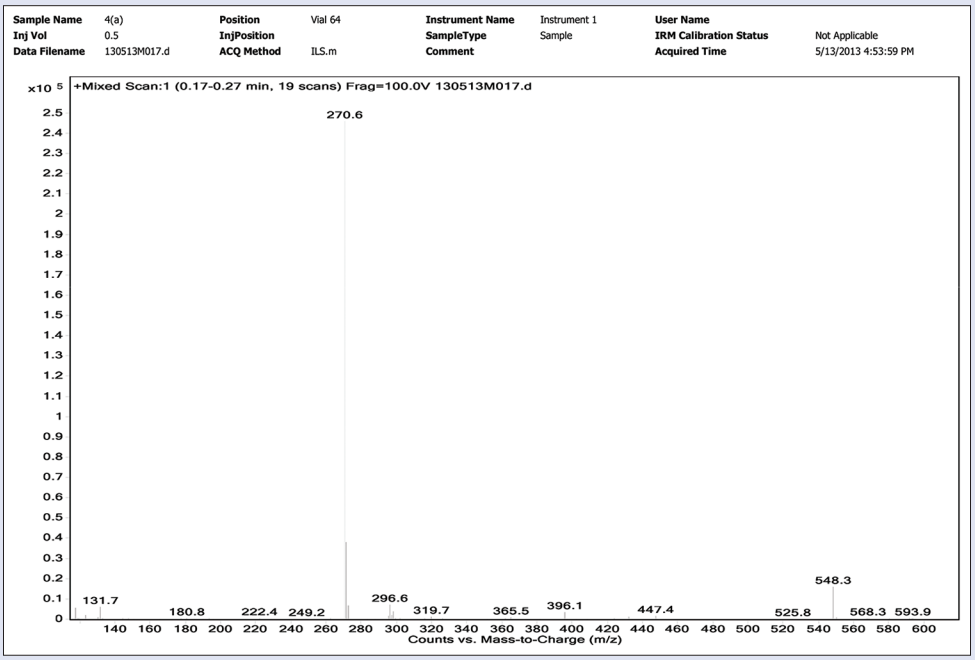

Figure 17: Mass spectrum of compound A

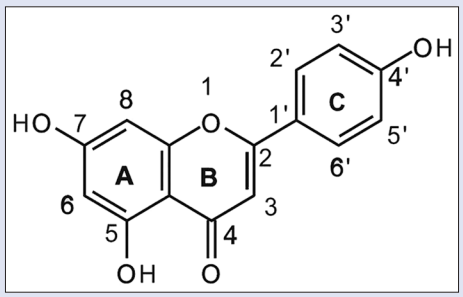

Figure 18: Structure of compound A: apigenin (4′,5,7-trihydroxyflavone). (IUPAC name: 5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran -4-one)

is one of the plants used in the treatment of wounds. From the results, we conclude that EFCC ointments (20% w/w) showed more effective activity compared to the simple ointment and untreated group (**$P < 0.001$). An increase in the rate of contraction of open wounds and increase in the tensile strength, as observed in the treated wounds and according to the histopathological slides of incised and excised wound models, attributed to the enhanced production of collagen and its reorganization, which is a prerequisite in the initial phase of wound healing.

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Conflicts of interest

There are no conflicts of interest.

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