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# Isolation of Effective Wound Healing Compounds from Chloroform Fraction of Ethanol Extract of *Ehretia Laevis* Roxb.(KhanduChakka/AjanVruksha)

**Research Article**

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## Abstract

Phytochemical analysis plays a very important role in identifying effective components of herbs. For phytochemical analysis, ethanol extractions of *Ehtretia Laevis Roxb*. leaves were carried out using the Soxhlet extraction method. Fractionation of collected ethanol extract was carried out using n-Hexane, n-Butanol, chloroform and water as a solvent.Wound healing action of n-Hexane, chloroform, n-Butanol, and water extracts were carried out. It was observed that chloroform fraction was effective for wound healing in animal models and hence chloroform fraction of ethanol extract of *Ehretia Laevis Roxb*. was taken for further phytochemical analysis. Column chromatography of chloroform fraction was carried out using silica gel. 5 Fractions were isolated by using Column Chromatography. Fraction -2 was in sufficient quantity and hence taken for wound healing study. Fraction -2- Dragendorff's test- positive for alkaloids. The rats were marked and divided them into 4 groups of 6 animals each. Isolated fraction-2-2%***w/w*** treated group wound healing was 77.75±0.312 %. The Povidone iodine closest the wound by 96.63±0.205% which is close to the group applied by 4% w/w ointments of isolated fraction-2 i.e. 94.10±0.146 %. Fraction -2- was characterized for $^{1}$H NMR, $^{13}$CNMR and LCMS. On comparing experimental data of fraction-2 , it is observed that the experimental data match with literature data of α-amyrin and β-amyrin. From this, we can conclude that the isolated fraction-2 is the mixture of α-amyrin and β-amyrin responsible for wound healing. We can conclude that mixture of α-amyrin and β-amyrin may have wound healing properties.

**Keywords:** α-amyrin, β-amyrin, Wound Healing*, Ehretia Laevis Roxb., Khandu Chakka, AjanVruksha.*

### Introduction

India is an emerging global leader in traditional and herbal medicine. Since ancient times many civilizations have been using different types of herbs for various ailments. Curiosity is always there to know the exact mechanism of action by various compounds present in herbs.

Phytochemical analysis plays a very important role in identifying effective components of herbs. Many active components have been isolated recently by doing chemical analysis and pre -clinical studies. Such types of studies play a very important role proving the mechanism of action of herbal drugs and establishing evidence. *Ehretia Laveis Roxb*. herb not much known to world identified for study having very good capacity of wound healing and pain relief. Herbs have many chemical compounds responsible for wound healing. (1) Herbs are always advisable to avoid the side effects of modern medicines.

*Ehretia Laevis Roxb*.is a rare species from the boraginaceae family. This herb is also known as AjanVruksha, a very spiritual plant of Nath Sampraday from the holy place of Alandi, Maharashtra, India as Saint Gnyaneshwar Maharaj took sanjeevan samadhi by this plant. It's trade name is Khandu Chakka.( 2)This study was taken to find active compounds, responsible for wound healing by chemical analysis of herbs.

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## Material and Methods

### Compound isolation

For phytochemical analysis, ethanol extraction of *Ehtretia Laevis Roxb*. leaves were carried out using the Soxhlet extraction method.

Fractionation of collected ethanol extract was carried out using n-Hexane, n-Butanol, chloroform and water as a solvent with the help of a separating funnel. Wound healing action of n-Hexane, chloroform, n-butanol, and water extracts were carried out. It was observed that chloroform fraction was effective for wound healing in animal models and hence chloroform

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fraction of ethanol extract of *Ehretia Laevis Roxb*. was taken for further phytochemical analysis.( 3)

**Column chromatography**

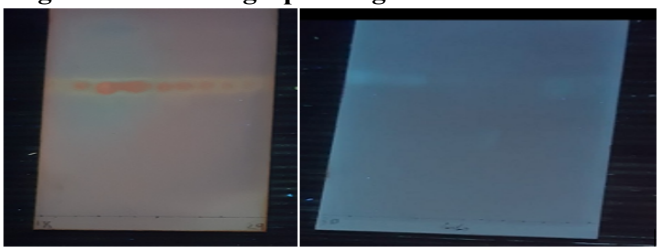
Column chromatography of chloroform fraction was carried out using silica gel(4).The column was first run with pure n-Hexane for 48 hours, then column was run with selected mobile phase n-Hexane & ethyl acetate in proportion 95mL:5mL and 20mL of elute collected at each time. Then the proportion of the mobile phase changed to 90:10 and again 20mL of elute was collected. The process continued and five fractions were collected.

**Figure 1: Photographs of Column Chromatography**

**Thin Layer Chromatography (TLC)**

TLC of each collected elute was carried out using toluene and ethyl acetate in proportion 8:2. From TLC, it was observed that elute 3 and elute 4 showed the same RF values, so they mixed and labeled as fraction-1. Elute 18 to 29 showed the same RF values, so they mixed and labeled as fraction -2. Elute 30-35 show the same RF value, so they were mixed and labeled as fraction-3.Elute 50 was labeled as fraction-4. Elute 59 was labeled as fraction-5.

**Figure 2: TLC Fingerprinting of Various Fractions**



**Fourier-transform infrared spectroscopy (FT-IR)**

Then infrared spectroscopy study of the collected fraction-2 was carried out by using an Infrared spectrometer and pellet press machine of TSI Technosearch and with the help of IT solution software.

**High performance thin layer chromatography (HPTLC)**

Then HPTLC of crude ethanol extract, chloroform fraction, isolated fraction-1, fraction-2, and fraction-3 were carried out by using mobile phase toluene: ethyl acetate in the proportion of 24: 6 with the help of high- performance thin layer chromatography of camag and by using wincat software. (5-6)

**Figure 3: IR Spectrum of Fraction-2**

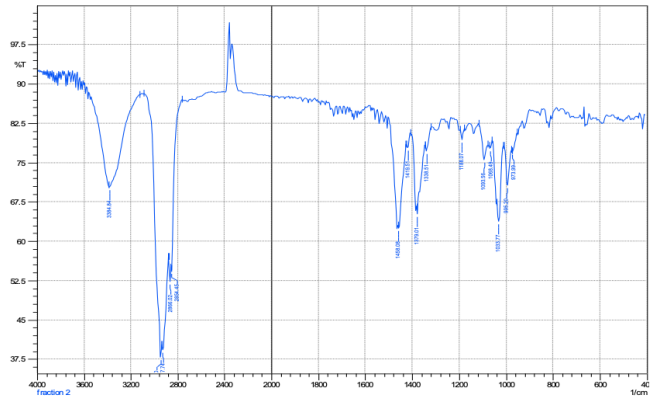
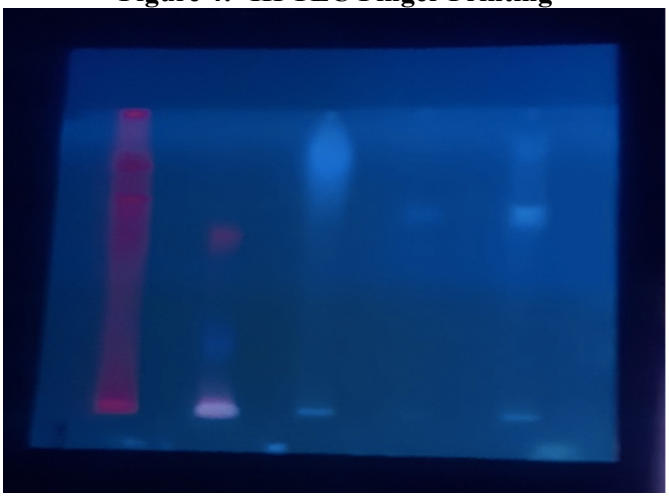


**Figure 4:- HPTLC Finger Printing**



The animal study was conducted as per approval of IAEC (IPER/IAEC/2018-19/03).

**Animal Study** (3)

**Preparation of Ointment:**

Fraction -2 was in sufficient quantity and hence taken for wound healing study and preparation of ointment. A paste was created using wax of white bee's (2%), hard paraffin (3%), $CH_3(CH_2)_nOH$(5%), and white soft paraffin(90%), mixed with isolated fraction-2, and stored for further research work.

The twenty four experimental animals were marked and divided in to four groups equally.

- Group- I (Control):-0.5 g(simple ointment)
- Group- II:- (Standard): 0.5 g (5% w/w povidone iodine ointment.)
- Group- III:-Isolated fraction -2 –(2% w/w ointment 0.5 g)
- Group- IV: -Isolated fraction -2 –(4% w/w ointment 0.5 g)

**Excision Wound Model**

The rats were procured from animal house of Institute of Pharmaceutical Education and Research Wardha(MS) India. Rats were kept in ventilated cages and on standard pallet diet. Rats were slightly anesthetized with the help of diethyl ether. Dorsal hairs were removed from dorsal thoracic region of the rats using hair removing cream purchased from the market i.e Veet manufactured by Reckitt Benckiser. An area of

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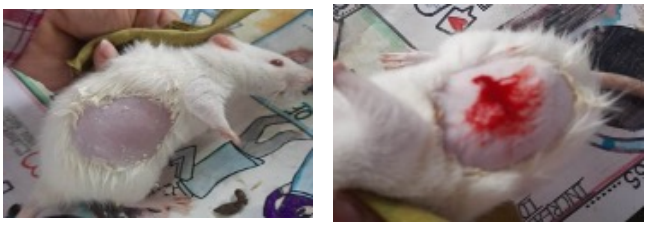
500 mm$^2$ was marked on the shaved area with as indelible ink and rubber seal. The marked area was washed with normal saline and cut throughout the marked area through the skin to create a circular excised wound by sterile blade. The wounded rats were kept individually in separate cages with the wounds left undressed. The prepared ointment of fraction and reference drug Povidon Iodine, were applied topically once daily till the wound was completely healed. All wounds were marked on a transparent paper on fourth, eighth, twelfth and sixteenth days.

% wound closure = (Wound area on day "zero"- n (area of the wound on days) / (area of the wound on day "zero") × 100

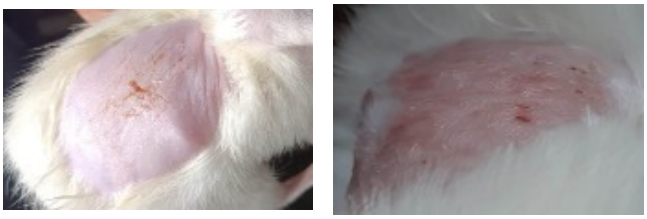
Where, n = number of days (fourth, eighth, twelfth and sixteenth days).

**Figure 5: Wound Healing Photographs**

**Before treatment**



**After treatment**



**Statistical analysis:**

The Statistical calculations were done using a one-way analysis of variance (ANOVA) test followed by Dunnett's comparison test. The results are presented as mean ±SEM and $p< 0.05$ is considered significant.

**Preliminary phytochemical screening**

As fraction -2 was efficacious in wound healing, hence taken for further phytochemical screening. Preliminary phytochemical screening of fraction- 2 was done for identification of the type of compound present in the fraction-2.(7-11)

**LCMS, $^{13}$CNMR, $^{1}$HNMR study of isolated fraction 2 of *Ehretia laevis* Roxb. Leaves:**

As fraction 2 showed better wound healing property it was selected for further analytical study i.e., LCMS, $^{13}$CNMR, $^{1}$HNMR . For this study the sample was sent to SAIF Punjab University, Punjab. At first permission for the work was taken from the authorities of SAIF lab by mail. After filling their online form and following their all terms and conditions, 50mg of sample was measured and poured into glass vial with all precautions and sent to SAIF along with hard copy of the filled form.

## Results & Discussion

From column chromatography we have isolated five fractions from chloroform fraction of ethanol extract of *Ehretia Laevis Roxb.* by using TLC. From the preliminary study, it is observed that alkaloids are present in fraction 2.

Percentages of reduction of wounds of various groups until the 16$^{th}$ day were calculated and presented in Table 1 & Figure 6. Control groups presented with the lowest rate of wound closure (60.69±0.373%). The highest rate of wound closure was observed in groups treated with 4% w/w ointments of isolated fraction-2. Isolated fraction-2-2% treated group was 77.75±0.312 %. The povidine iodine closes the wound by the rate of 96.63±0.205% and this is similar to the animals treated with 4% w/w ointments of isolated fraction-2. Wound closer is mentioned in percentage of wound closer in Table 1.

**Table 1: % of wound closure**

| Treatments | Fourth day | Eight day | Twelfth day | Sixteenth day |
|---|---|---|---|---|
| Group I(Control) | 14.60±0.200 | 30.88±0.421 | 45.35±0.0272 | 60.69±0.373 |
| Group II(Std) | 33.94±0.264 | 60.22±0.212 | 87.83±0.237 | 96.63±0.205 |
| Group III(***fraction-2. 2% w/w)*** | 22.98±0.312 | 45.36±0.510 | 58.40±0.476 | 77.75±0.312 |
| Group IV(***fraction-2. 4% w/w)*** | 32.42±0.496 | 56.35±0.270 | 75.91±0.705 | 94.10±0.146 |

**Figure 6:- % of wound closure**

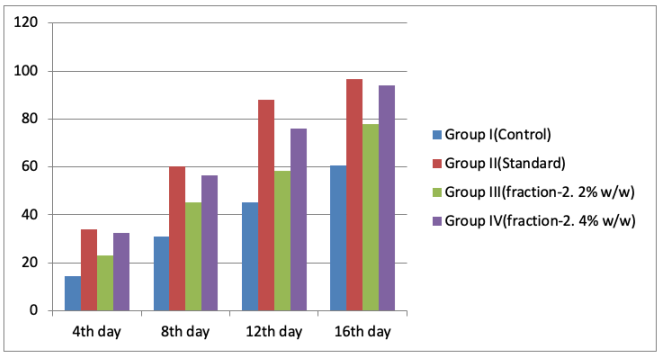



**Table 2: Preliminary phytochemical screening of fraction-2**

| Sr no. | Compound | Test | Observation | Result |
|---|---|---|---|---|
| 1 | Carbohydrate | Fehling's test | Brick red color precipitate not found | Negative |
| | | Barfoed's test | Red color precipitate not found | Negative |
| 2 | Proteins | Biuret test | Violet colour not appeared | Negative |

| | | | | |
|---|---|---|---|---|
| 3 | Amino acid | Ninhydrine test | Purple colour not appeared | Negative |
| 4 | Steroids | Salkowski reaction | Greenish yellow fluorescence not appeared | Negative |
| 5 | Glycosides | Keller –Killiani test | No reddish brown colour appeared at junction of layers | Negative |
| | | Borntrager's test | No pink colour appeared | Negative |
| 6 | Saponin | Foam test | No persistent foam observed | Negative |
| 7 | Flavonoids | Sulphuric acid test | No red colour observed | Negative |
| | | Sodium hydroxide and hydrochloric acid test | No decolourization after addition of acid | Negative |
| 8 | Alkaloids | Dragendorff's test | Orange brown ppt formed | Positive |

Preliminary Phytochemical Screening of Fraction 2 was done and found alkaloids in said fraction-2.

From column chromatography, we obtained 5 different fractions from Chloroform extract.

From HPTLC it is observed that crude extract shows 9 peaks, chloroform extract shows 3 peaks, fraction-1 shows 2 peaks, fraction-2 shows 1 peak, and fraction-3 shows 3 peaks**.** Fraction -2 showed a single peak and quantity was sufficient than other compounds, hence taken for further study. The alkaloid test was positive from fraction 2. Hence compounds from the alkaloid group may be responsible for wound healing. The present study shows a significant improvement in wound healing in rats treated with isolated fraction -2 compared to a reference standard. Herein, the higher dose (4%) of isolated fraction-2 possesses remarkable wound- healing activities in rats.

NMR and LCMS screening of isolated fraction-2:- Fraction -2- was sent for $^{1}$H NMR, $^{13}$CNMR and LCMS screening to sophisticated analytical instrumentation facility Punjab University Chandigarh

Structure Elucidation of compounds from fraction-2:-The fraction-2 was obtained from hexane-ethyl acetate fraction as a white powder; its melting point is186$^{0}$C which is similar with the melting point of α-amyrin and β-amyrin. The IR spectrum showed bands at $V^{max}$ 3384.84 cm$^{-1}$ which shows the presence of hydroxyl group, 2866.02 cm$^{-1}$ which represent C-H stretching ,1458.08 cm$^{-1}$ which represent C-H bending. (12)

$^{1}$H NMR spectral (500 MHz,$CDCl_3$ and Table no -3) data showed $\delta^{H}$ 5.34 (s), $\delta^{H}$5.12 (t), 3.18 (m) ,1.18 (s), 1.00 (s),0.98 (s), 0.92 (s), 0.86 (d), 0.82 (d), 0.80 (s), 0.78 (d). (13)

The $^{1}$HNMR spectrum indicates the 2 bands at 5.12 ppm (H-$_{12}$,1H) and 5.06 ppm (H-$_{2}$,1H) which is related to β-amyrin and α-amyrin. (14)

From $^{13}$C NMR of the fraction-2, it is observed that there are signals at 139.59 ppm and145.19 ppm which corresponds to $C_{13}$, whereas bands at 124.43 ppm and 121.74 ppm which corresponds to $C_{12}$ of α-amyrin and β-amyrin,. The other peaks were allocated as indicated in Table no 4 which are related to available researches. (15-16)

Structures of α-amyrin, β-amyrin and mixture of α-amyrin and β-amyrin are presented in Figure 7. It is observed that the chemical formula of α-amyrin and β-amyrin are same and also have similar molecular weight. The only difference is presence of methyl group at $C_{30}$ and $C_{29}$ carbon positions. While the methyl groups of $C_{30}$ and $C_{29}$ in α-amyrin are linked to two separate carbon positions. (13-14)

From the LCMS spectrum of isolated fraction-2 it is observed that the molecular weight of fraction -2 is verified as dimensionless at 463.37 + 1 m/z and the formula is [$C^{30}H^{50}O$+K].Which is similar to the molecular weight of α-amyrin and β-amyrin i.e 426.73.

**Figure 7: Chemical structures of α- amyrin (A) and β- amyrin (B) and mixture of α- amyrin and β- amyrin (C) (13-14)**

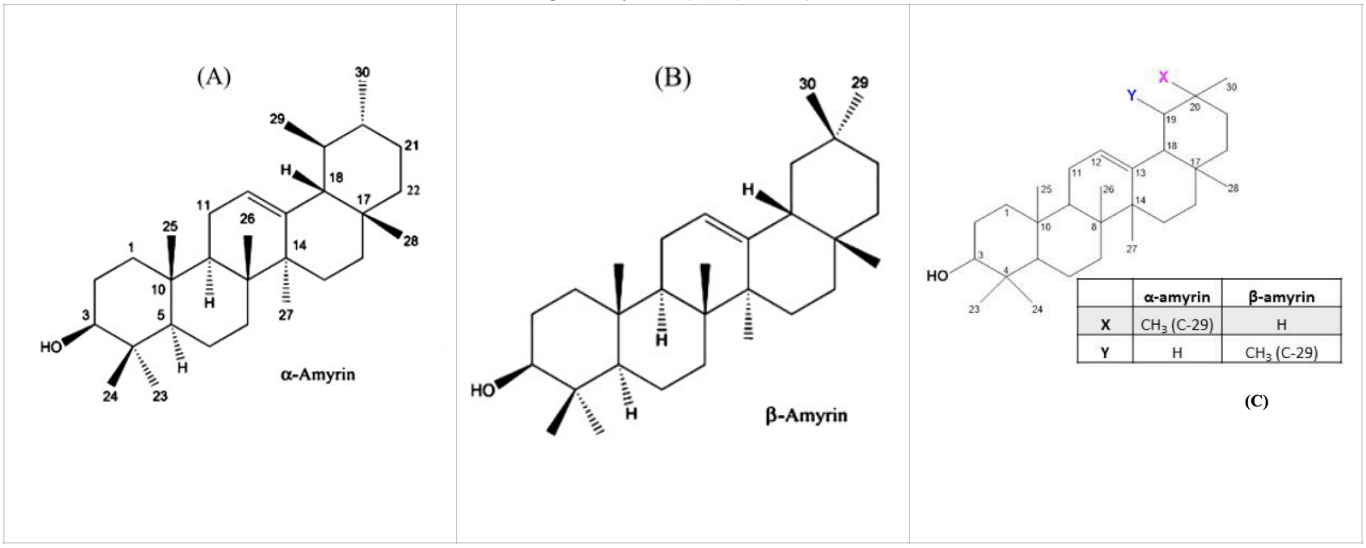


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**Table 3:-The experimental value of $^{1}$HNMR spectrum of isolated fraction-2 in correlation with information from the researches.(17-21)**

| Proton position | Experimental | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|
| 1 | - | - | - | - | - | - |
| 2 | - | - | - | - | - | - |
| 3 | 3.56 (t) | 4.52(m) | 3.16(s) | 3.15 | 4.55 (t) | 4.50(dd) |
| 4 | - | - | - | - | - | - |
| 5 | - | - | - | - | - | - |
| 6 | - | - | - | - | - | - |
| 7 | - | - | - | - | - | - |
| 8 | - | - | - | - | - | - |
| 9 | - | - | - | - | - | - |
| 10 | - | - | - | - | - | - |
| 11 | - | - | - | - | - | - |
| 12 | 5.12(t) | 5.15(d) | 5.15 | 5.12 | 5.15(s) | 5.12(t) |
| 13 | - | - | - | - | - | - |
| 14 | - | - | - | - | - | - |
| 15 | - | - | - | - | - | - |
| 16 | - | - | - | - | - | - |
| 17 | - | - | - | - | - | - |
| 18 | - | - | - | - | - | - |
| 19 | - | - | - | - | - | - |
| 20 | - | - | - | - | - | - |
| 21 | - | - | - | - | - | - |
| 22 | - | - | - | - | - | - |
| 23 | 0.88(s) | 0.88(s) | 0.98(s) | 0.95(s) | - | 0.88(s) |
| 24 | 0.82(s) | 0.82(s) | 0.77(s) | 0.76(s) | - | 0.88 (s) |
| 25 | 1.00 (s) | 1.00 (s) | 0.95 (s) | 0.75 (s) | 0.93 (s) | 1.00 (s) |
| 26 | 0.98 (s) | 0.96 (s) | 1.12 (s) | 0.89 (s) | 0.95 (s) | 0.98 (s) |
| 27 | 1.06 (s) | 1.05 (s) | 0.92 (s) | 1.01(s) | 1.08 (s) | 1.07 (s) |
| 28 | 0.80 | 0.79 (s) | 0.81 (s) | 0.95 (s) | 0.80 (s) | 0.79 (s) |
| 29 | 0.83 (d) | 0.83 (d) | 0.85 (d) | 0.85 (d) | - | 0.88 (s) |
| 30 | 0.85 (d) | 0.85 (d) | 1. 51 (d) | 0.79(d) | - | 0.88 (s) |

**Table 4:- The experimental value of $^{13}$CNMR data of isolated fraction-2 in correlation with $^{13}$CNMR information for α-amyrin and β-amyrin in CDCl$^{3}$(14)**

| Position | α-amyrin | Experimental | β-amyrin | Experimental | Position | α-amyrin | Experimental | β-amyrin | Experimental |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 38.80 | 38.81 | 38.60 | 38.61 | 16 | 26.60 | 26.63 | 26.20 | 26.17 |
| 2 | 27.30 | 27.28 | 27.20 | 27.24 | 17 | 33.80 | 33.76 | 32.70 | 32.67 |
| 3 | 79.10 | 79.03 | 79.00 | 79.03 | 18 | 59.10 | 59.08 | 47.20 | 47.24 |
| 4 | 38.80 | 38.81 | 39.80 | 39.81 | 19 | 39.70 | 39.68 | 46.80 | 46.84 |
| 5 | 55.20 | 55.20 | 55.20 | 55.20 | 20 | 39.60 | 39.62 | 31.10 | 31.09 |
| 6 | 18.40 | 18.39 | 18.40 | 18.39 | 21 | 31.30 | 31.27 | 34.70 | 34.75 |
| 7 | 32.90 | 32.95 | 32.50 | 32.50 | 22 | 40.00 | 40.03 | 37.10 | 37.16 |
| 8 | 40.00 | 40.03 | 41.70 | 41.73 | 23 | 28.10 | 28.12 | 28.10 | 28.12 |
| 9 | 47.70 | 47.73 | 47.60 | 47.65 | 24 | 15.70 | 15.69 | 15.60 | 15.60 |
| 10 | 36.90 | 36.91 | 37.00 | 37.16 | 25 | 15.60 | 15.60 | 15.50 | 15.51 |
| 11 | 23.30 | 23.28 | 23.70 | 23.71 | 26 | 17.40 | 17.49 | 16.80 | 16.82 |
| 12 | 124.40 | 124.43 | 121.70 | 121.74 | 27 | 23.40 | 23.38 | 26.00 | 26.01 |
| 13 | 139.60 | 139.59 | 145.20 | 145.19 | 28 | 28.80 | 28.76 | 28.40 | 28.41 |
| 14 | 41.50 | 41.55 | 42.80 | 42.09 | 29 | 16.90 | 16.88 | 33.30 | 33.35 |
| 15 | 28.10 | 28.12 | 26.90 | 26.96 | 30 | 21.40 | 21.41 | 23.50 | 23.54 |

The fraction-2 isolated from the chloroform fraction of ethanol extract of *Ehretia Laevis Roxb*. responsible for wound healing was able to be confirmed as a combination of α-amyrin and β-amyrin based on FT-IR, $^{1}$HNMR,$^{13}$CNMR studies.

During the wound healing study, there was no infection occurred as *Ehretia Laevis Roxb*. has anti-

microbial properties.(22-23) *Ehretia Laevis* Roxb. was effectively used for various wounds in clinical and pre-clinical studies.( 23-26) Also *Ehretia Laevis Roxb*. has good pain relief activity.(27) Hence isolation of active compounds from *Ehretia Laevis Roxb.* for anti-microbial, wound healing in human beings and pain relief activities can be planned in the future.

**Table 5: Active components isolated from herbal plants shows wound healing activity are as follows**

| Sr no | Name of plant | Active component | Action |
|---|---|---|---|
| 1 | Centella *Centella asiatica*[28] | Asiaticoside | Promote epithelialization and collagen deposition |
| | | Triterpenes | Collagen remodeling, synthesis of glycosaminoglycans |
| | | Madecassoside | Collagen synthesis and angiogenesis |
| 2 | *Wedelia trilobata* [29] | Alcoholic extract | Wound healing |
| | | Luteolin | Antioxidant |
| 3 | *Aloe (*Aloe vera*)*[30] | Acemannan | Wound healing |
| 4 | Ginseng (Panax ginseng)[31] | Ginsenosides Panaxosides Ginsenoside-Rb2 | Wound healing |
| 5 | Neem (Azadirachta indica) [32] | *German chamomile (*Chamomilla recutita*)* | Wound healing |

Limitation of study:

Fractions isolated by the column chromatography were not in sufficient quantity.

α-amyrin and β-amyrin are triterpenes present in many herbal plants. Nkeoma Nkasi Okoye et al (2014) proved its significant anti inflammatory activity, which is responsible for wound healing.(33) Tran Duc Viet et al( 2021) proved its antioxidant property which is responsible for wound healing.(14)

## Conclusion

On comparing experimental data of isolated fraction-2, it is observed that the experimental data matches with research data of α-amyrin and β-amyrin. From this, we can conclude that the isolated fraction-2 is the mixture of α-amyrin and β-amyrin responsible for wound healing. α-amyrin and β-amyrin act as antioxidants, and anti-inflammatory and therapeutics for skin hyper pigmentation so it show fast wound-healing activity.

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