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TITLE

WOUND HEALING ACTIVITY OF TRIDAX PROCUMBENS BY PREPARING MOTHER TINCTURE AND ITS CRUDE EXTRACT – A COMPARATIVE STUDY

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WOUND HEALING ACTIVITY OF PLANT TRIDAX PROCUMBENS BY PREPARING MOTHER TINCTURE AND ITS CRUDE EXTRACT – A COMPARATIVE STUDY

INTRODUCTION:

Early healing of the wounds depends on the way the wound is managed; however, slight negligence on the part of the surgeon makes the treatment a complete failure and endangers the life of the animal [1]

Though various agents have been identified as a wound healer, still there is a need for cheaper therapy which can accelerate the rate of healing, without affecting the normal physiological process along with its socio-economical acceptance and minimal side effects.[2]

Tridax procumbens **(Fig:1)** is a common weed native to tropical America and distributed in tropical Africa, Australia and Asia[4]. It belongs to the Asteraceae family.

It is extensively used in the Indian Ayurvedic system of medicine for the treatment of diarrhea, as an insect repellent, hair tonic and wound healer,i.e. the leaf juice is used to check hemorrhage from cuts and bruises.[5]

The therapeutic properties of various plants are mainly due to their Phytoconstituents. Development of science and technology has brought to light many mysterious entities and mechanisms of nature in recent years. Previous studies have revealed remarkable phytoconstituents from *Tridax procumbens L.* with significant antioxidant activity.

Numerous active compounds are identified from leaves of the *Tridax* plant twenty-three flavonoids- mainly **procumbenetin** has the wound healing property,Thirty-nine alkaloids (mainly **akuammidine)** hydroxycinnamates, tannins and phytosterols, moderate benzoic acid derivatives and lignans, and low carotenoids content. [4,5]

Fig:1 ***Tridax procumbens***

Though the medicine of this plant is not available in the Homoeopathic system of medicine yet various other systems of the medicine and studies have proven its wound healing activity. This study promotes a new pathway to introduce this as a medicine in homoeopathy.

REVIEW OF LITERATURE:

B.Yaduvanshi *et al.,* (2011) evaluated the effect of topical formulation of *Tridax procumbens* leaf juice by using wound excision model over mice.The topical ointment form of *Tridax procumbens*(50 *mg* of either 1 or 4 *mg/g*) was compared with the VEGF(50mg of 1ug/g). The *Tridax procumbens* (4 mg/g) induced inflammation, edematous tissue and decreased vascularity. [6]

Mathiazhagan Suryamathi *et al.,* (2019) conducted a study regarding *Tridax procumbens* nanoparticles to be infused with polycaprolactone (PCL), which can be used as an effective wound dressing material. The PCL nanofiber and *Tridax procumbens* extract immobilized nanofibers were characterized by SEM, XRD and EDAX. The morphology, porosity, swelling and weight loss percentage of the electrospun nanofibers have been investigated. The *Tridax procumbens*-PCL nanofibers were analyzed for its antibacterial activity. The results of the work confess that the scaffolds act as an enhancer of wound healing and treating surfaces that contain pathogenic microorganisms especially in hospital environments. [7].

Katarina Hostanska *et al.,* (2021) conducted a study of wound healing activity of various homoeopathic wound healing remedies like Arnica 4X, Calendula 4X, Hypericum 4X and Symphytum Officinals 6X. An ethanolic preparation was prepared by adding equal parts of these medicines.This preparation was then categorised into sucussed hydroalcoholic solvent and un-sucussed hydroalcoholic solvent. Then its effect was studied on NIH 3T3 fibroblasts. This study showed that the low potency homeopathic remedy exerted *in vitro* wound closure potential in NIH 3T3 fibroblasts. This effect resulted from stimulation of fibroblasts motility rather than of their mitosis. [8].

AIMS AND OBJECTIVES:

AIM:

The aim of this research study is to investigate and compare the wound healing activity of the plant *Tridax procumbens* by preparing mother tincture and its crude extract, with the goal of providing a comprehensive understanding of their therapeutic potential and differences.

OBJECTIVES:

- To prepare mother tincture of the plant of *Tridax procumbens* using the methodology of Calendula Officinalis Ø.
- To prepare its crude extract by using Soxhlet apparatus.
- To assess the wound healing potential of Tridax procumbens Ø and its crude extract in in vitro wound scratch assays.
- To analyze the obtained data comprehensively and draw meaningful conclusions regarding the wound healing potential of Tridax procumbens Ø and its crude extract.

MATERIALS AND METHODS:

Methodology

The plant Tridax procumbens was collected and was identified by a botanist. It was designated with a number J.L.S 001. Then the plant's leaf crude extract and its mother tincture were prepared and labelled as Sample A and Sample B respectively. The Sample A and the Sample B were then subjected to *in vitro* study for comparing the wound healing activity of both samples in human dermal fibroblasts. The detailed methodology was shown in **Fig:2**.

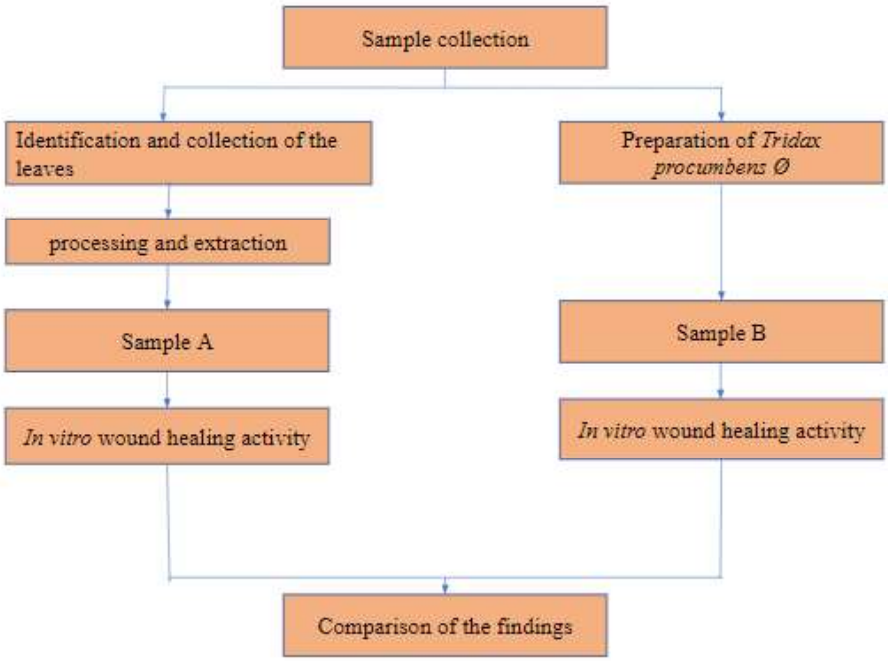

Fig:2 Detailed Methodology

Tridax procumbens crude extract- Sample A

Sample collection:

The *Tridax procumbens* whole plant was collected from the natural habitat. The leaves were separated and dried **(Fig:3)** in shade to remove the moisture and processed for hot solvent soxhlet extraction. The solvent used in the study was ethanol.

Fig:3 Dried leaves of *Tridax procumbens*

Sample Processing

The dried whole plant was powdered and treated with ethanol in a conical flask and left undisturbed for 48 hrs. This mixture will be then subjected to filtration **(Fig 4)**. The solid remnant was retained in a linen bag inside the thimble of the Soxhlet apparatus and the filtrate was placed in boiling flask. The filtrate in the boiling flask was heated for ten cycles until a colourless solvent was obtained in the extractor **(Fig:5)**.

Fig:4 *Tridax procumbens leaves* – Processing/ crude solvent extraction

Fig:5 Hot solvent extraction of *Tridax procumbens* using Soxhlet Apparatus

After obtaining the colourless solvent, the solvent was again heated by removing the solid remnant for deriving the crude extract of *Tridax procumbens* (Sample A). This crude extract was cooled and transferred to an air tight bottle.

Calculation and time duration:

Weight of dry powder of sample taken in linen bag =10.039g

Initial time= 10.45 am

I cycle - 10.54-11.10 am

II cycle - 11.11-11.19 am

III cycle - 11.20-11.28 am

IV cycle - 11.29-11.37 am

V cycle- 11.48- 11.58 am

VI cycle- 11.59 am-12.08 pm

VII cycle - 12.09-12.18 pm

VIII cycle- 12.19-12.27pm

IX cycle - 12.28-12.36 pm

X cycle -12.40-12.50 pm

Tridax procumbens Ø - Sample B

Sample Collection and Processing -

The *Tridax procumbens Ø* (Sample B) was prepared from the leaves using the methodology of Calendula Ø with reference to ENCYCLOPEDIA OF HOMOEOPATHIC PHARMACOPOEIA & DRUG INDEX VOLUME I. **(Fig:6)**

Fig:6 Preparation of *Tridax procumbens*

Moisture content determination:

Some quantities of leaves were cut into minute pieces and subjected to determine the moisture content present in these leaves. The entire procedure was performed on the basis of Homoeopathic pharmacopoeia of India.

Procedure:

The cut leave pieces were put in a porcelain crucible, before weighing it the weight of the empty crucible was weighed. Later the crucible containing the sample was also weighed. Then this sample in the crucible was placed over the water bath for heating and at an interval for one hour the weight was calculated. This procedure was repeated until two successive values remained the same.

Time duration of the process:

10.45am- 21g

11.45am- 19.7g

12.45pm- 19.3g

1.45pm- 19.3g

Calculation:

Weight of the empty crucible=19g

Weight of the crucible with plant=21g

Thus, the weight of the leaf sample=21-19 g=2g (weight of the crucible with plant - weight of the empty crucible)

Net amount of moisture present= Final value - initial value =19.3-2.0= 17.3g

$$\% \text{ of moisture content} = \frac{\text{net amount of moisture present}}{\text{intial value}} x100$$

$$= 89.64\%$$

Procedure for tincture preparation:

The mother tincture is prepared on the basis of the procedure of calendula mother tincture. It is prepared with reference to ENCYCLOPEDIA OF HOMOEOPATHIC PHARMACOPOEIA & DRUG INDEX VOLUME I. The reference formula for tincture preparation is

For 1000 ml of tincture:

6800g of magma and 3200 ml of alcohol are added to obtain 1000ml of the tincture.

Then, for a 10 ml tincture preparation:

6.8g of magma and 3.2 ml of alcohol are added

Wound healing activity- In vitro study

Requirements:

- To measure the wound healing capacity on HDF cell lines, 2 test compounds were received.

  The samples which were used for the cell line study are listed in **(Table:1)**:

Table:1 The details of the sample:

SL No.	Sample name/Code	Concentrations used for the study	Cell line
1.	Untreated	-	HDF
2.	TP(Sample-B)	1:2, 1:3, 1:4	HDF
3.	CE(Sample-A)	1:2, 1:3, 1:4	HDF

Materials:

- HDF -Human dermal fibroblast cell lines (Cat No:106-05A, Sigma)
- Cell culture medium: Fibroblast growth medium - (#116-500, Sigma)
- Adjustable multichannel pipettes and a pipettor (Benchtop, USA)

- Fetal Bovine Serum (#RM10432, Himedia)
- D-PBS (#TL1006, Himedia)
- Test compounds: 2 (provided by client)
- 12 well cell culture plate (Biolite - Thermo)
- 50 ml centrifuge tubes (# 546043 TARSON)
- 1.5 ml centrifuge tubes (TARSON)
- 10 ml serological pipettes (TARSON)
- 10 to 1000ul tips (TARSON)
- 70% ethanol
- Antibiotic-Antimycotic solution (Cat No: A002, Himedia)

Equipments:

1. Centrifuge (Remi: R-8C).
2. Pipettes: 2-10µl, 10-100µl, and 100-1000µl.
3. Inverted Biological Binocular Microscope CKX415F, Olympus, Japan)
4. 37°C incubator with humidified atmosphere of 5% CO2 (Healforce, China)
5. Biosafety Laminar Hood (Healforce, China)

Software:

1. Image J or FIJI
2. Windows Paint
3. MICAM software

Maintenance of cell lines:

The HDF (Human dermal fibroblast cell line) was purchased from Sigma Aldrich, USA. The HDF cells were maintained in DMEM-high glucose media supplemented with 10% FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO2, 18-20% O2 at 37oC temperature in the CO2 incubator and sub-cultured for every 2-3 days.

Experimental Outflow:

1. Grow cells in Fibroblast growth media supplemented with 10% FBS until the cells reach 70-80% confluence.
2. Seed cells into 12 well tissue culture plate at a density of 0.25 million cells per well until they reach ~80-100% confluence as a monolayer for the incubation period of 24hrs.
3. Do not change the medium. Gently and slowly scratch the monolayer with a new

200ul pipette tip across the centre of the well. While scratching across the surface of the well, the long-axial of the tip should always be perpendicular to the bottom of the well.

4. The resulting gap distance therefore equals the outer diameter of the end of tip. The gap distance can be adjusted by using different types of tips. Scratch a straight line in one direction.

5. Scratch another straight line perpendicular to the first line to create a cross in each well.

6. After scratching, gently wash the well twice with medium to remove the detached cells.

7. Treat the cells with desired concentrations of given compound prepared in media and incubate at 37oC with 5% CO2 in the incubator.

8. Grow cells for an additional 48 hours (or the time required if different cells are used).

9. Capture the cell images at different time intervals (ex: 0, 48hr)

10. Set the same configurations of the microscope when taking pictures for different views of the monolayer. The gap distance can be quantitatively evaluated using software such as Image J. To reduce variability in results, it's suggested that multiple views of each well should be documented, and each experimental group should be repeated multiple times.

Formula for calculating percentage of wound healing scored:

% of wound healing scored= $\frac{\text{final area}-\text{initial area}}{\text{initial area}}\ x100$

The procedure is summarised in the following flowchart **(Fig:7)**:

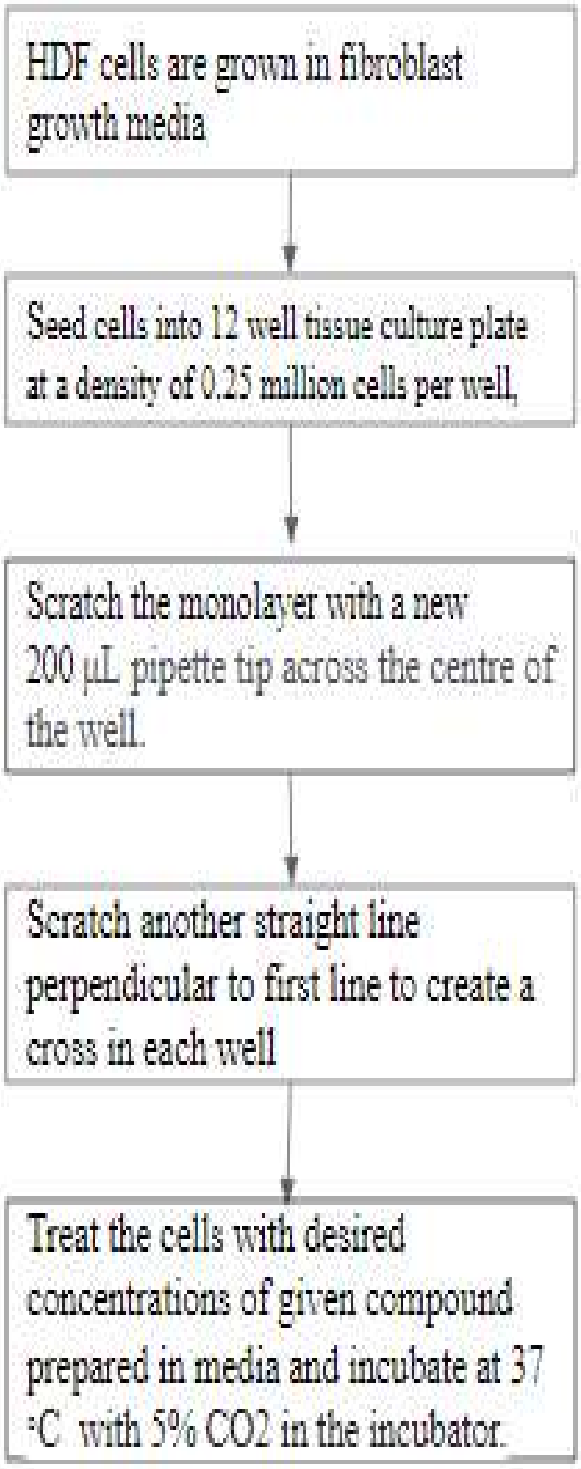

Fig:7 The overview of the procedure

OBSERVATIONS AND RESULTS:

Observation

The 35 ml of sample A was prepared using soxhlet apparatus and the 10 ml of sample B was prepared using the homoeopathic mother tincture maceration process. Both of these samples were subjected for comparative study of wound healing activity in Human dermal fibroblasts cell line in three different ratios -1:2,1:3,1:4 and were observed at 0 and 48 hours.

Observations of the Sample A Tridax procumbens crude extract (CE) at 0 and 48 hours:

The following observation between the untreated sample and the Sample A in the ratios 1:2,1:3 and 1:4 at 0 hour and 48 hours.

In 0 hours the area of the scratch wound in untreated sample was 899200μm.

Different observations in the sample A at the three different ratios that:

In the ratio 1:2, the area of scratch wound was 897577μm

In the ratio 1:3, the area of scratch wound was 933693μm

In the ratio 1:4, the area of scratch wound was 898640μm

In the end of 48 hours, the area of the scratch wound in untreated sample was 733866μm. Different observations in the sample A at the three different ratios that:

In the ratio 1:2, the area of scratch wound was 855466μm

In the ratio 1:3, the area of scratch wound was 878933μm

In the ratio 1:4, the area of scratch wound was 643257μm.

It was observed that there is much closure of the wound in the sample A at the ratio 1:4.

The area of the scratch wound was indicated with arrow marks. **(Fig:8)** and **(Table:2).**

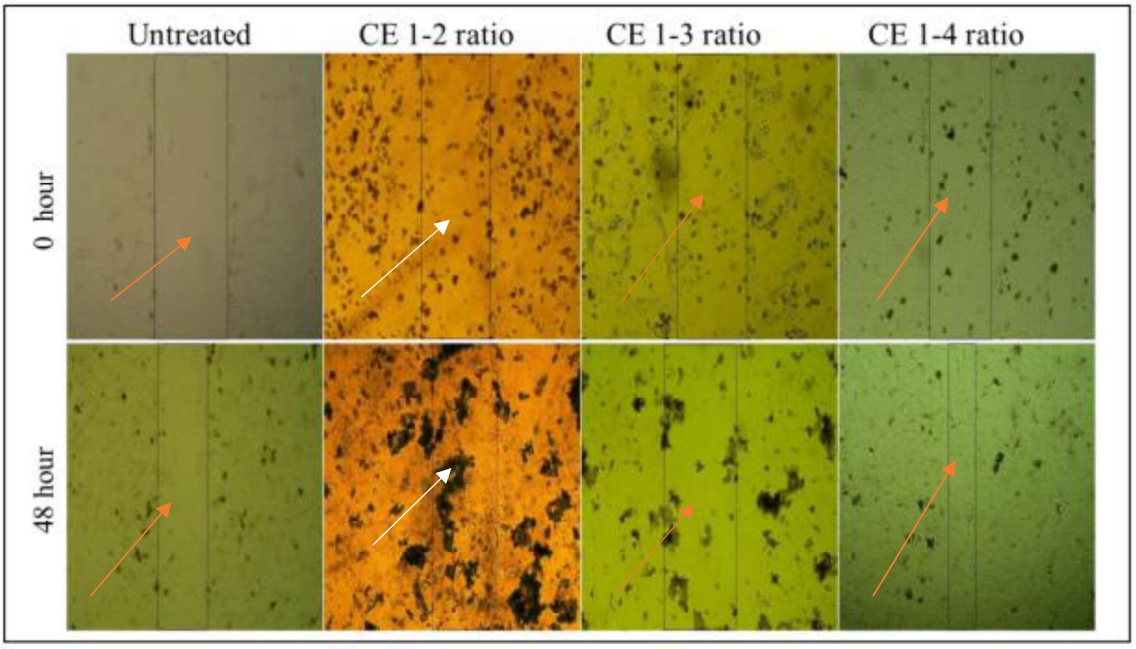

Fig:8 *In-vitro* wound healing activity of Sample A *Tridax procumbens* Crude Extract (CE)

Table:2 In-vitro wound healing effect of CE with different ratios (1:2, 1:3 and 1:4) against the HDF cells at different intervals in terms of area.

Wound Area Covered Overlay-HDF vs CE (Average area (µm) ±SD				
Incubation	**Untreated**	**CE 1-2 ratio**	**CE 1-3 ratio**	**CE 1-4 ratio**
0 hour	899200±45191	897577±41139	933693±20363	898640±26395
48 hour	733866±46280	855466±9688	878933±24265	643257±94483

The area of wound closure was calculated in terms of µm. Those values of the untreated sample and sample A at the different ratios with regard to time period at 0 and 48 hours was represented graphically in terms of bar graph. **(Fig 9)**

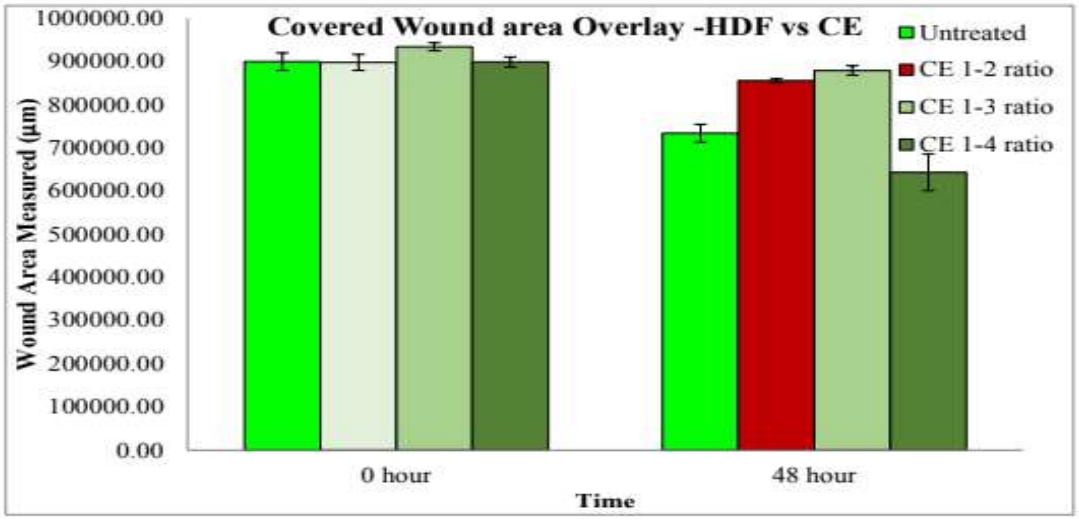

Fig:9 Overlaid bar graph represent the wound healing effect of CE with different ratios of treatment against the HDF cells at different intervals in terms of wound area.

Observations of Sample B Tridax procumbens Ø (TP) at 0 and 48 hours:

The following observation between the untreated sample and the Sample B in the ratios 1:2,1:3 and 1:4 at 0 hour and 48 hours.

In 0 hours the area of the scratch wound in untreated sample was 899200μm.

Different observations in the sample A at the three different ratios that:

In the ratio 1:2, the area of scratch wound was 933693μm

In the ratio 1:3, the area of scratch wound was 903951μm

In the ratio 1:4, the area of scratch wound was 898649μm

In the end of 48 hours, the area of the scratch wound in untreated sample was 733866μm. Different observations in the sample A at the three different ratios that:

In the ratio 1:2, the area of scratch wound was 809413μm

In the ratio 1:3, the area of scratch wound was 739306μm

In the ratio 1:4, the area of scratch wound was 76480μm.

It was observed that there is much closure of the wound in the sample A at the ratio 1:4.

The area of scratch wound was indicated with arrow marks **(Fig:10)** and **(Table:3)**

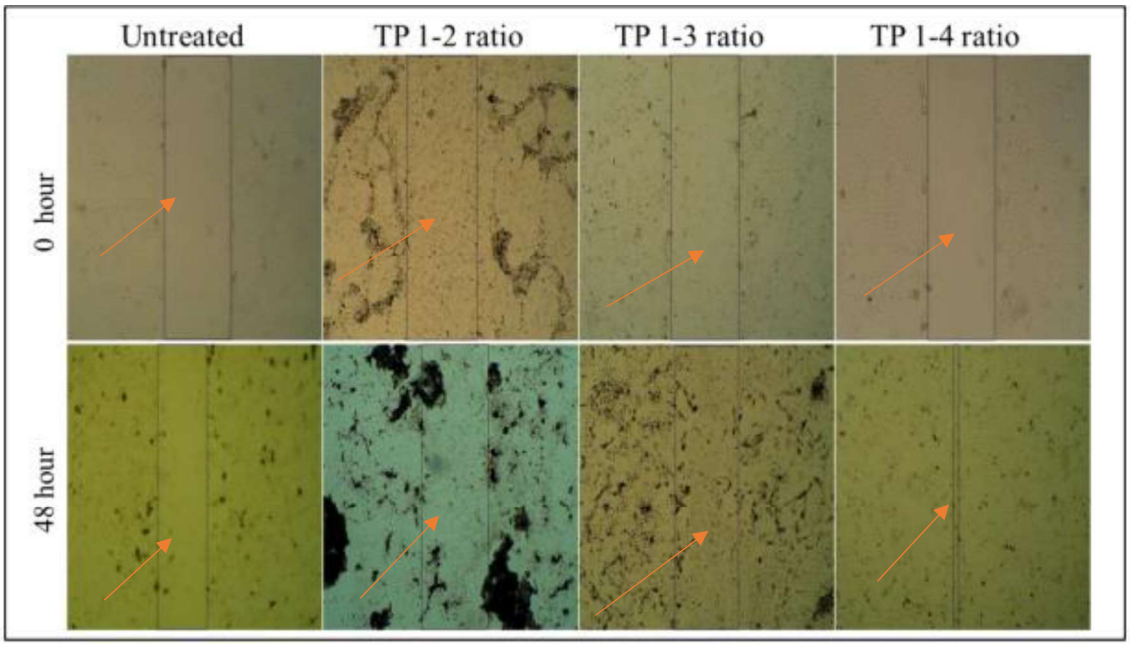

Fig:10 In-vitro wound healing activity of Sample B (TP)

Table:3 In-vitro wound healing effect of TP with different ratios (1:2, 1:3 and 1:4) against the HDF cells at different intervals in terms of area.

Wound Area Covered Overlay-HDF vs TP (Average area (µm) ±SD				
Incubation	**Untreated**	**CE 1-2 ratio**	**CE 1-3 ratio**	**CE 1-4 ratio**
0 hour	899200±45191	933693±26924	903951±26395	898649±12111
48 hour	733866±46280	809413±67453	739306±63556	76480±28253

The area of wound closure was calculated in terms of µm. Those values of the untreated sample and sample B at the different ratios with regard to time period at 0 and 48 hours was represented graphically in terms of bar graphs **(Fig:11)**

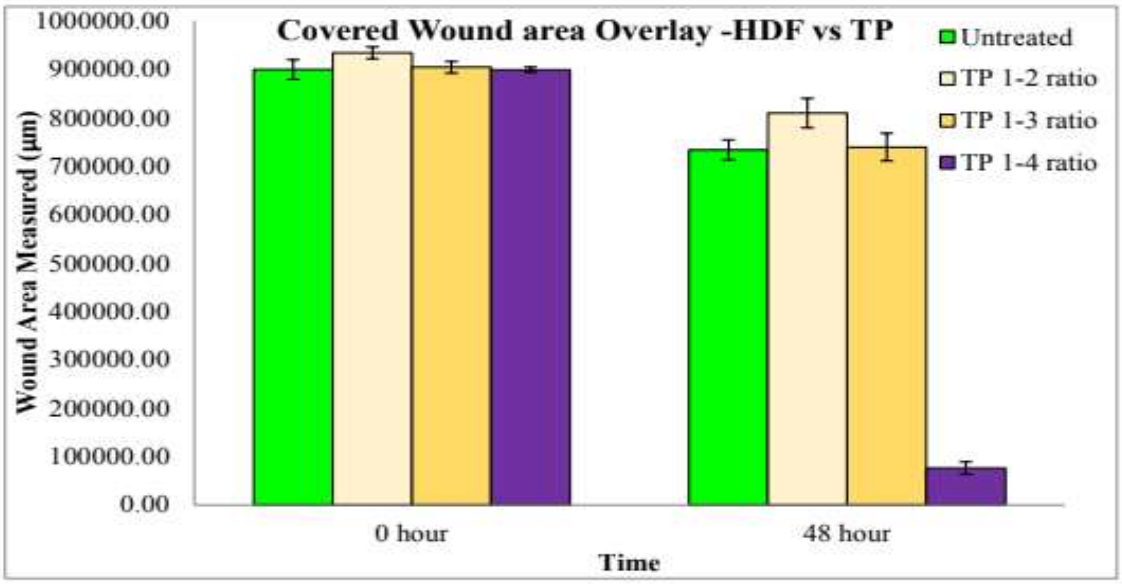

Fig:11 Overlaid bar graph represent the wound healing effect of TP with different ratios of treatment against the HDF cells at different intervals in terms of wound area.

Observation of the area of wound closure by sample A Tridax procumbens crude extract at the different ratios and the untreated sample in terms of percentage:

The area of wound closure was individually observed for Sample A in the ratios 1:2,1:3 and 1:4. The area was then calculated in terms of percentage. Among the values obtained the sample at the ratio 1:4 has 28% of area of wound closure. (**Table:4)**

Table:4 In-vitro wound healing effect of CE with different ratios (1:2, 1:3 and 1:4) against the HDF cells at different intervals in terms of percentage.

Overlay % Wound Closure scored – HDF vs CE				
Incubation	**Untreated**	**CE 1-2 ratio**	**CE 1-3 ratio**	**CE 1-4 ratio**
0 hour	0	0	0.00	0
48 hour	18.39	4.69	5.86	28.41874637

Observation of the area of wound closure by sample B Tridax procumbens Ø at the different ratios and the untreated sample in terms of percentage:

The area of wound closure was individually observed for Sample B in the ratios 1:2,1:3 and 1:4. The area was then calculated in terms of percentage. Among the values obtained the sample at the ratio 1:4 has 94% of area of wound closure. **(Table:5)**

Table:5 In-vitro wound healing effect of TP with different ratios (1:2, 1:3 and 1:4) against the HDF cells at different intervals in terms of percentage.

Overlay % Wound Closure scored – HDF vs TP				
Incubation	**Untreated**	**CE 1-2 ratio**	**CE 1-3 ratio**	**CE 1-4 ratio**
0 hour	0	0	0.00	0
48 hour	18.39	13.31	18.21	91.4893617

The percentage of wound closure of the sample A at different ratios and the untreated sample was represented graphically in terms of line graph in contrast with time period 0 hour and 48 hours. **(Fig:12)**

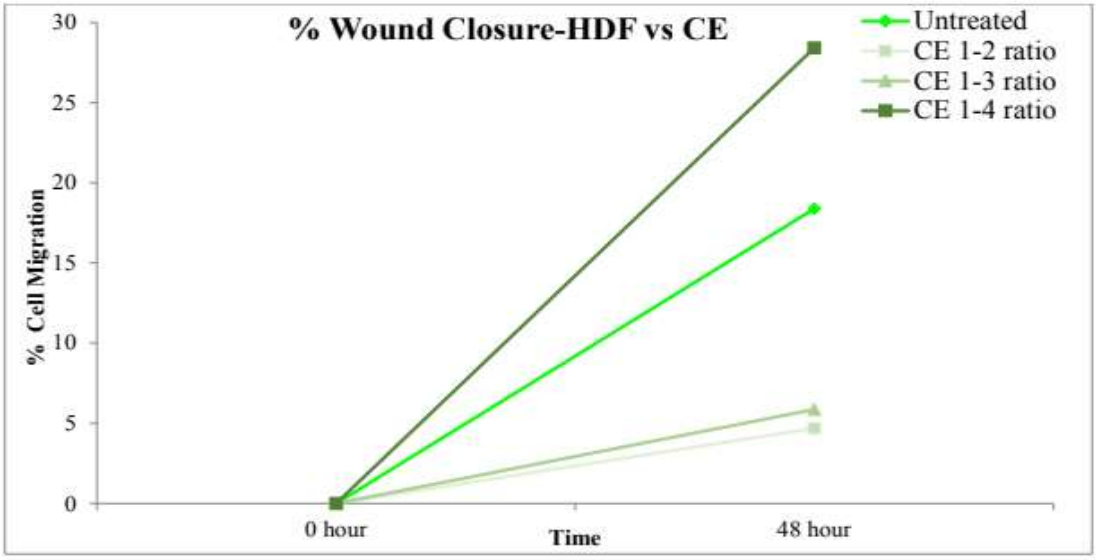

Fig:12 Comparative scatter graph represents the wound healing effect of CE with different ratios against the HDF cells at different intervals in terms of % wound closed area.

The percentage of wound closure of the sample B at different ratios and the untreated sample was represented graphically in terms of line graph in contrast with time period 0 hour and 48 hours. **(Fig:13)**

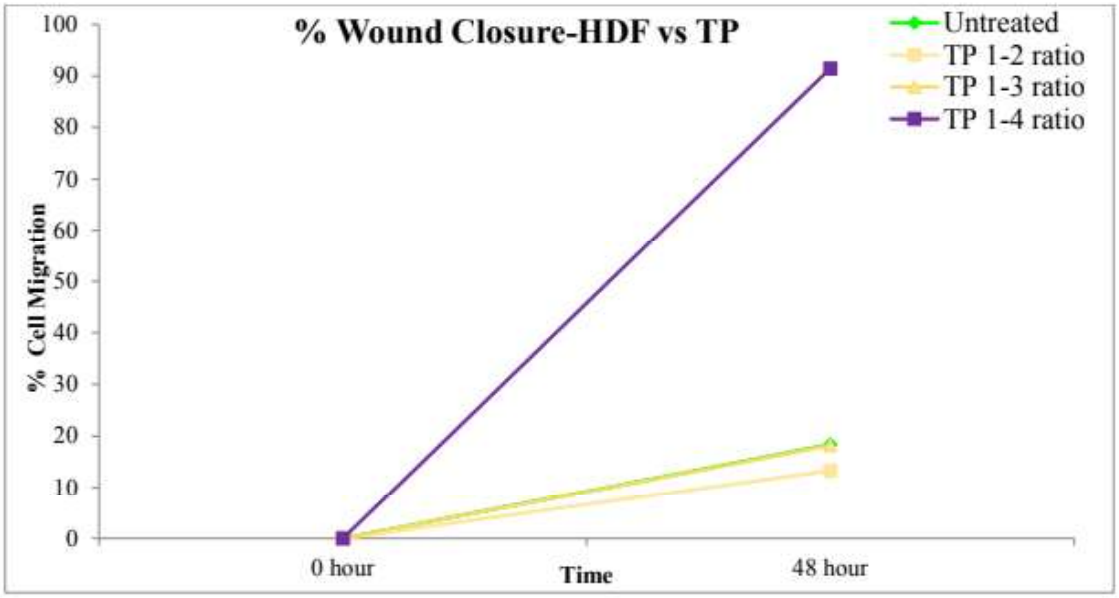

Fig:13 Comparative scatter graph represented the wound healing effect of TP with different ratios against the HDF cells at different intervals in terms of % wound closed area.

Results:

On comparison of the wound healing activity between the both the samples A and B in the ratios 1:2,1:3 and 1:4 with the negative control at 0 and 48 hours, the sample B(TP) has remarkable action over the wound at the ratio 1:4 after 48 hours. The area of wound closure in terms of percentage is 91.49%.

DISCUSSION

The discussion section of the study provides valuable insights into the preparation of *Tridax procumbens Ø,* wound healing potential of *Tridax procumbens Ø* and its crude extract using a scratch assay as the experimental approach. Here, we delve into the significance of the findings and their implications for the broader understanding of wound healing.

Tridax procumbens's Wound Healing Properties:

The research findings establish that both *Tridax procumbens* ethanolic crude extract and *Tridax procumbens Ø e*xhibit wound healing properties. The successful demonstration of these properties is a significant step in understanding the therapeutic potential of *Tridax procumbens* in the context of wound care. The promotion of fibroblast proliferation and migration is crucial in the wound healing process, contributing to wound granulation and re-epithelialization. The ability of *Tridax procumbens* to stimulate these processes suggests its promise as a natural remedy for enhancing wound healing.

This study has also been enhanced previously by one of the well-known homoeopathic remedy *Arnica montana* which is well effective for external wounds in L929 cell line. The study was conducted between four samples- Sample A: *Arnica montana* crude extract, Sample B: *Arnica montana Ø*, Placebo and negative control at the time intervals of 6^{th} hour, 12^{th} hour and 24^{th} hour. In comparison of the findings, the wound healing activity of all the four samples was a very slow progress at 6^{th} hour. Then at the 12^{th} hour there was slower progress than previous observation in all the four samples. Finally, at the 24^{th} hour the sample A and B had very good results over the closure of the wound compared to the placebo whose action was very slow. The results between these samples A and B at the 24^{th} hour, sample A was showing a larger healing effect than sample B. [9].

CONCLUSION:

In conclusion, the findings of this study provide valuable insights into the wound healing potential of *Tridax procumbens Ø* and its crude extract, affirming their ability to promote fibroblast proliferation and migration.The study sorts out the efficacy of the therapeutic power of the *Tridax procumbens Ø* . These results serve as a foundation for future studies aimed at unlocking the full therapeutic potential of *Tridax procumbens* in wound care and beyond. Overall, this research contributes to our understanding of natural remedies and their potential applications in wound healing, offering a promising direction for future investigations in both the realms of phytomedicine and molecular biology.

The current findings validate that the wound healing using scratch assay is a convenient and inexpensive method that gives vigorous and reproducible results for the proliferation as well as the migration of fibroblasts in an artificial wounded area. This was successfully shown with *Tridax procumbens* ethanolic crude extract and *Tridax procumbens Ø* for its wound healing properties.The results can be a starting point for further studies aiming at the elucidation of the molecular processes and signalling pathways underlying proliferation and migration of the fibroblasts induced by *Tridax procumbens* preparations[10].

SUMMARY:

The study was conducted with the tropical plant Tridax procumbens which belong to the family Asteraceae. Various studies were conducted over this plant as it has phytochemical components like flavonoids which promotes wound healing.The part used were leaves. Those leaves were subjected for crude extract and homoeopathic mother tincture preparation. These both samples were then sent for wound healing scratch assay over human dermal fibroblasts. The mother tincture was subjected to the study at three different ratios. Both the samples were assessed at 0 and 48 hours. This study resulted that the mother tincture has better wound healing property and much percentage of wound closure comparative to crude extract.The study's findings affirm the scratch assay as a cost-effective and reliable method for evaluating wound healing by measuring fibroblast proliferation and migration within artificially wounded areas.

This study also revealed the efficacy of the plant to treat the wounds in its prepared homoeopathic form.These results provide a promising foundation for future research, with the potential to uncover the molecular processes and signaling pathways involved in the fibroblast responses induced by *Tridax procumbens* preparations. Overall, this study enhances our understanding of *Tridax procumbens*'s wound healing potential and paves the way for more comprehensive investigations into its therapeutic mechanisms. This study promotes the latent powers of the homoeopathic remedies and helps to elevate our system of medicine.

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LIST OF ENCLOSURES:

- Report Attestation Form (RAF)