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FORMULATION AND EVALUATION OF ANTIBACTERIAL ACTIVITY OF HERBAL OINTMENT CONTAINING LEAVES EXTRACT OF AEGLE MARMELOSE

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Abstract: Aegle marmelos (Linn) Corea commonly known as Bael belonging to family Rutaceae, is a moderate sized, slender and aromatic tree, available in India with great known medicinal uses. The known potential pharmacological activity of the leaves of *Aegle marmelos* are hypoglycemic, anti-inflammatory, antimicrobial, anticancer, chemo preventive and anti-oxidative activity due to its rich content. It was thought worthwhile to explore its application in skin care. The objective of present study was to analyze the phytochemical constituents from leaves of *Aegle marmelos* to determine its antibacterial potential against *Staphylococcus aureus* and to apply this antibacterial potential in preparation of ointment. The antibacterial activity and minimum inhibitory concentration (MIC) Of *Aegle marmelos* was investigated against *Staphylococcus aureus*. Our study focuses on preparation of herbal ointment that consists of antibacterial with anti-inflammatory activity by combination of *Aegle marmelos* leaves extract with turmeric.

Key words: - *Aegle marmelos (Linn) Corea*, Antibacterial activity, Herbal ointment, Extraction Method

INTRODUCTION

Skin is the largest multi-layered organ of a body and it covers area of about 22 square feet and weighs about 4.5-5kg, about 7% of total body weight. Skin serves as a shield against physical and chemical attack. Material such as poisonous gases like mustard gas, nickel ion can penetrate the barrier. The skin act as a thermostat in maintaining body temperature, protect against ultraviolet rays and important role in maintaining blood pressure. It acts as a first line defence against toxic and harmful substances. Skin consists of network of nerve cells and multiple receptors which are responsible for the senses such as heat, cold, pain and touch.

Functions of skin:

- Regulates body temperature and vital sign.
- Detection of tactual sensation.
- Excretion and absorption of drugs.

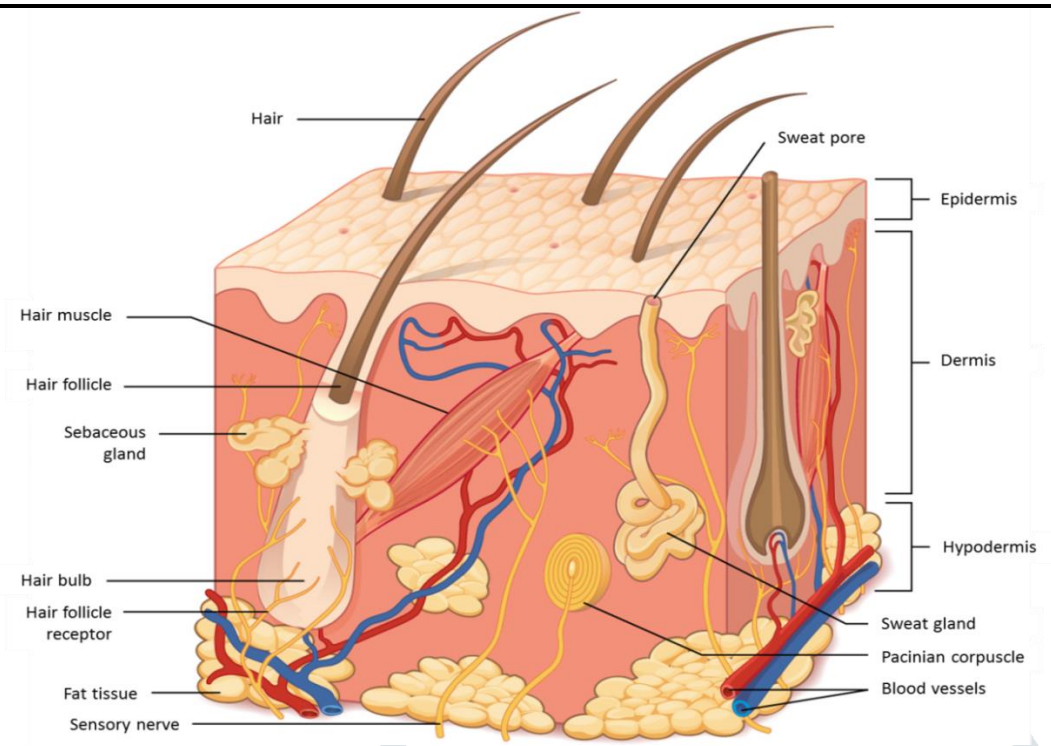

Fig. 1 Scheme of skin components and layers

Skin comprises of three major parts.

- Epidermis
- Dermis
- Hypodermis

A skin wound that does not heal, heals slowly or heals however tends to recur is thought as a chronic wound. Several numerous causes of chronic skin wounds will include trauma, burns, skin cancers, infection or underlying medical conditions.

Some of the numerous causes of a chronic skin wound will include:

- Being immobile (pressure injuries or bed sores), wherever persistent localized pressure restricts blood flow
- Significant trauma injury to the skin
- Surgery – incisions (cuts created throughout operations) could become infected.
- Trophic ulcers

Wound is the set of certain events which causes the damage to the skin due to some external forces such as accidents, injuries or internal bleeding. The healing of wounds occurs in three phases, they are

- Inflammatory phase
- Migratory phase
- Proliferative phase
- Maturation phase

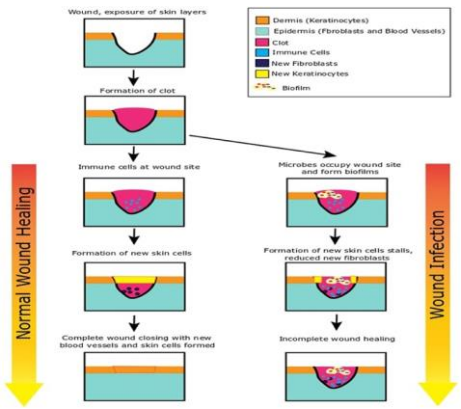

Fig 2. Two possible outcomes after the skin is wounded

Left side Figure represents that after the skin is wounded, inner layers of skin are exposed. Blood clot is formed immediately to stop bleeding. Immune cells are migrating towards the injured site and release certain chemicals. This chemical signal stimulates epidermis and dermis to grow new cells and fill the gap in wound. These new formed cells closing the wound completely and restore the skin integrity. On the right-side figure represents that

Microbes are occupies the open wound site and start growing in increasing manner and formation of biofilms in the wound. They also secrete certain chemicals which inhibit protective system of our skin. As the immune cells can not diminish the bacteria and formation of new cells are paused. That results in incomplete wound healing.

Once bacterium enters or chooses broken skin, the wound provides the required nutrients for them to extend in range and unfold the infection. In these ideal conditions, individual bacterium grows to create large-scale microorganism communities called biofilms. Biofilms carries with it more numbers of bacterium clustered along to create dense, mat-like structures that cover the whole surface of the wound. Biofilm bacterium is held together by a mesh of protein and sugars, resembling glue, typically created by the bacterium themselves. This mesh not solely strengthens the biofilm structure from among however additionally is a protecting covering for the bacterium, close the entire biofilm. once bacterium grow among the wound, they turn out the tiny chemicals that are used as signals to communicate with other bacteria and to effect on immune cells and blood vessels of the host they infect. Immune cells will counter to these signals in an endeavor to kill the bacterium and limit spread of the infection. However, the high density of bacterium and the toxic chemicals the bacterium produce can reduce the ability of immune cells to kill the bacterium, creating the body unable to get rid of the biofilm. This ends up in more growth and spread of the biofilm, that successively more complicates, and delays wound healing. The removal of wound biofilms needs medical intervention, typically within the variety of treatment with antibiotics. Antibiotics are applied as ointments and bandages. Wound biofilms typically need multiple applications of antibiotics, over many days. Even if the right treatment is given, wound biofilms will persist and result in a wound that fails to heal.

The main two active drugs in this formulation are BAEL and TURMERIC

Bael obtained from the unripe or half-ripe fruits or their slices or irregular pieces of *Aegle marmelos* Corr., belonging to family Rutaceae.

The main constituents of the drug are mamelosin A, B and C (0.5%), which is a furocoumarin. Also, some other coumarins are marmesin, psoralin and umbelliferone. The drug also contains carbohydrates (11–17%), protein, volatile oil and tannins. The pulp also contains good amount of vitamins C and A. Two alkaloids O-methylhalfordinol and iso-pentylhalfordinol. Other alkaloids present in the drug are angelenine, marmeline and decamine.

Turmeric is obtained from dried rhizome of Curcuma longa Linn. (syn. C.domestica Valeton)., belonging to family Zingiberaceae.

Turmeric contains yellow colouring matter called as curcuminoids (5%) and essential oil (6%). The chief constituent of the colouring matter is curcumin I (60%) in addition with small quantities of curcumin III, curcumin II and dihydrocurcumin. The volatile oil contains mono- and sesquiterpenes like zingiberene (25%), α-phellandrene, sabinene, turmerone, arturmerone, borneol, and cineole. Choleretic action of the essential oil is attributed to β-tolylmethyl carbinol.

The volatile oil also contains α-and β-pinene, camphene, limonene, terpene, terpinolene, caryophyllene, linalool, isoborneol, camphor, eugenol, curdione, curzerenone, curlone, AR-curcumenes, β-curcumin, γ-curcumin. α- and β-turmerones, and curzerenone.

Ointments are semisolid preparations meant for external application to the skin or mucous membrane. They may be medicated or non-medicated. Ointments are generally used for their: -

1) Emollient action (Lubricating effect).
2) Protective action.
3) Therapeutic action (Topical effect).
4) Prophylactic action on the skin (to treat skin diseases).

Ointment base is a substance or a part of an ointment, which serves as a carrier or vehicle for the medicament, in the preparation of ointment.

Classification of ointment bases: -

There are four types of ointment bases such as,

1) Hydrocarbon bases (oleaginous bases).
2) Absorption bases.
3) Emulsion (water miscible) bases.
4) Water soluble bases.

Preparation Of Ointments

Ointments are prepared by four methods.

1) Trituration method
2) Mortar and pestle method
3) Fusion method
4) Emulsification method

MATERIALS AND EXPERIMENTAL WORK

PREPARATION OF PLANT EXTRACTS

Collection Of Leaves From Local Area:
The Aegle marmelos leaves were collected from local area of Vapi, Gujarat in the month of December 2021.
The samples were washed with distilled water to clean the adhering dust particles.
Then the leaves were kept in incubator at 37°C for 3-4 days and grinded into fine powder using mortar pestle.

Solvent Extraction Method:

Plant material was dissolved in 70% ethanol and 80% methanol, (1:10); 1 g sample should be dissolved in 10 ml of solvent.
Mixtures were kept in the dark for 3 days at room temperature in sterilized beakers wrapped with aluminium foil to avoid evaporation and exposure to sunlight.
After 3 days, mixtures were filtered through Whatman no.1 filter paper and kept in water bath at 40°C till all the solvent had completely evaporated from mixtures.

Aqueous Extraction Method:
The plant materials were washed with tap water and then with sterile water.
They were then macerated using mortar and pestle using sterile double distilled water at a concentration of one gram of tissue per millilitre on water [1:1 w/v] and filtered through gauze and the filtrates were evaporated at 45^{o}c.
The concentrated extracts were weighed and dissolved in 5% dimethylformamide [DMF] individually.

PHYTOCHEMICAL ANALYSIS

(1) Test for Carbohydrates:
(a) For Reducing Sugar: Fehling's Test**:** Mix 1ml of Fehling's A And 1ml of Fehling's B solutions, boil for one minute. Add equal volume of test solution. Heat in boiling water bath for 10 min. First yellow, then brick red ppt. is observed.

(2) Test for Tannins:
(a) To 2-3ml of aqueous or alcoholic extract, add few drops of 5% FeCl3 solution. It will give deep blue-black colour.
(b) To 2-3ml of aqueous or alcoholic extract, add few drops of Lead acetate solution. It will give white ppt.
(c) To 2-3ml of aqueous or alcoholic extract, add few drops of dilute iodine solution. It will give transient red colour.

(3) Test for Alkaloid:
Evaporate the aqueous, alcoholic and chloroform extract separately. To residue, add dilute HCL. Shake well and filter. With filtrate, perform following tests
(a) Mayer's Test: 2-3ml filtrate, add few drops Mayer's reagent gives ppts.
(b) Hager's Test: 2-3ml filtrate with Hager's reagent gives Yellow ppts.
(c) Wagner's Test: 2-3ml filtrate with few drops of Wagner's reagent gives reddish brown precipitates.

(4) Test for Saponins:
(a) Foam Test:
Shake the drug extract or dry powder vigorously with water, persistent stable. Foam observed.

(5) Test for Cardiac Glycosides:
Legal's Test: (Test for Cardenolides):
To aqueous or alcoholic extract, add 1ml pyridine and add 1ml sodium nitroprusside. Pink to Red colour appears.

ANTIBACTERIAL PROPERTIES OF EXTRACT

The antibacterial activity of the extracts was tested by well diffusion using cup plate method against *Staphylococcus aureus.*

Table 1: Preparation of Agar Plate

Chemicals	Quantity Taken
Nutrient Agar	15gm
Beef extract	1gm
Pectin	1gm
Nacl	0.5gm
Distilled Water	100 ml

Procedure:

Each ingredient except agar is dissolved in the appropriate volume of distilled water. The pH of fluid is determined by pH meter and adjusted by using 1N Hcl or NaOH.

1.Add agar powder and medium is heated to dissolve the agar to form a clear liquid, the medium is dispersed.
2.Plug the flask and test tube containing medium by using non-absorbent cotton.
3.Sterilize the media at 121 degree Celsius, 15 lb. pressure for 15 minutes in autoclave.
4.Allow the flask to cool up 50 degree and pour the medium quickly into sterile Petri plates under the aseptic condition.
5.Allow medium to cool and to produce solid agar plates.

Formulation of Ointment

Table 2: Formulation Table for simple Ointment

Serial no	Ingredients	Quantity taken
1	Wool fat	0.5gm
2	Hard paraffin	0.5gm
3	Cetosteryl Alcohol	0.5gm
4	White soft paraffin	8.5gm

Table 3: Formulation Table for *Aegle marmelos* Ointment

Serial no	Ingredients	Quantity taken
1	Bael extract	0.4 ml
2	Turmeric	0.2 gm

Procedure: (By fusion method)

- Since there will be little wastage (loss) of ingredients during weighing and preparing, to manipulate these practical losses, calculate the ingredients for at least one or two grams extra, then prescribed.
- Weigh all the ingredients according to calculation.
- Melt hard paraffin and cetostearyl alcohol in a porcelain dish, by keeping on a water bath.

- To this melt, incorporate wool fat and white soft paraffin, stir well during melting.
- After melting of all the ingredients, remove the foreign matter if present by decanting or straining into another hot dish. Stir the mixture thoroughly, until it becomes cooled and a semisolid base is obtained.
- Herbal ointment was prepared by mixing accurately weighed bael and Turmeric extract to the ointment base by levigation method to prepare a smooth paste with 2 or 3 times its weight of base, gradually incorporating more bases until to form homogeneous ointment, finally transferred in a suitable container.

ANTIBACTERIAL PROPERTIES OF EXTRACT AND OINTMENT

The antibacterial activity of the extracts was tested by well diffusion using pour plate method against *Staphylococcus aureus.*

Preparation Of Agar Plate

Table 4: Preparation of Agar Plate

Chemicals	Quantity Taken (for 10g)
Nutrient Agar	2gm
Beef extract	1gm
Pectin	1gm
Nacl	0.5gm
Distilled Water	100 ml

Procedure:

Each ingredient except agar is dissolved in the appropriate volume of distilled water. The pH of fluid is determined by pH meter and adjusted by using 1N Hcl or NaOH.

1. Add agar powder and medium is heated to dissolve the agar to form a clear liquid, the medium is dispersed.
2. Plug the flask and test tube containing medium by using non-absorbent cotton.
3. Sterilize the media at 121 degree Celsius, 15 lb. pressure for 15 minutes in autoclave.
4. Allow the flask to cool up 50 degree and pour the medium quickly into sterile Petri plate under the aseptic condition.
5. Allow medium to cool and to produce solid agar plates.

Microbial Evaluation of Bael Extract

Cup plate method:

- Each Petri plate was filled to a depth of 4-5 mm with a nutrient agar medium that was previously inoculated with suitable inoculums of suitable test organism.
- Then allowed to solidify.
- The petri plate was specially selected with flat bottom and was placed on level surface to ensure that the layer of medium is in uniform thickness.
- The petri plates were sterilized at 160-170°C in hot air oven for 30 mins before use.
- Small sterile borer of uniform size was placed approximately at 10 cm height, having an internal diameter of approximately 6-8 mm and made of aluminium (or) stainless steel.
- One cylindrical cavity was made in medium with the help of sterile borer.
- Cavity was filled with the beal extract.
- The petri plates were incubated at 37°C for 18 hours.
- Diameter of the zone of inhibition was measured and the average diameter for each sample was calculated

Microbial Evaluation OF Bael Leaf Extarct Containg Onitment

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- One cylindrical cavity was made in medium with the help of sterile borer.
- Cavity was filled with the beal extract containing ointment.
- The Petri plates were incubated at 37°C for 18 hours.
- Diameter of the zone of inhibition was measured and the average diameter for each sample was calculated

EVALUATION PARAMETERS FOR OINTMENT

1) Colour and Odour: -

Physical parameters like colour, odour was inspected by visual examination.

2) Consistency: -

The consistency of the finished ointment was checked by manual method.

3) pH: -

2.5gm *Aegle marmelos* ointment, sample was taken in 100 ml dry beaker, 50 ml water was added to it.

Beaker was heated on water bath maintained at about 60ºC to 70ºC for 10 minutes, cooled to room temperature. The pH of water extract was measured by using pH meter. The pH was measured by using a digital type pH meter by dipping the glass electrode into the ointment formulation.

4) Spreadability: -

The spreadability was resolute by placing ample amount of sample between two clean and neat slides which was pressed to even thickness by placing a certain weight for certain time. The time required to separate the two slides was measured as spreadability. Lesser is the time for separation of 2 slides, better is the spreadability.

Spreadability was calculated by:

$S=M\times L/T$

Where,S= Spreadability

M= Weight tide to the upper slide

L= Length of glass slide

T= Time taken to separate the slides

5) LOD: - (Loss on Drying)

LOD was evaluated by placing the prepared formulation in china dish on water bath and then dried at the temperature 105ºC.

6) Solubility: -

The ointment was placed in the boiling water and miscible with alcohol, ether, chloroform.

7) Washability: -

Finished product was applied on the surface of the skin and then ease extend of washing with water was checked.

8) Non irritancy Test:-

Ointment was prepared and applied to skin of humans and is observed for the effect.

9) Stability study:-

Physical stability test of prepared ointment was carried out for 4 weeks at several temperatures such as 2^{o}C, 25^{o}C and 37^{o}C.

10) Viscosity:-

The measurement of viscosity of prepared ointments was carried out with Brookfield Viscometer.

11) Extrudability:-

Extrudability test is the measure of the force required to extrude the material from a collapsible tube when certain amount of force has been applied on it in the form of weight. In the present study the quantity in percentage of ointment extruded from the tube on application of certain load was determined.

The Extrudability of ointment was calculated by using following formula,

$$\text{Extrudability} = \frac{\text{Amount of ointment extruded from the tube} \times 100}{\text{Total amount of ointment filled in the tube}}$$

RESULT AND DISCUSSION

RESULT OF COLLECTION OF LEAVES AND MAKING THE POWDER

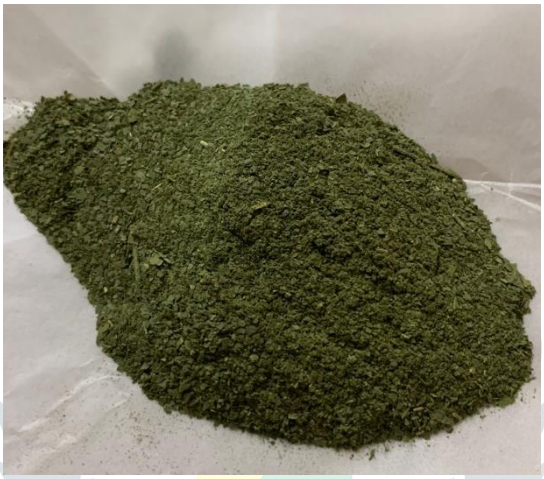

Fig. 3 Dry powder of Bael leaves

RESULT OF LEAVES EXTRACT

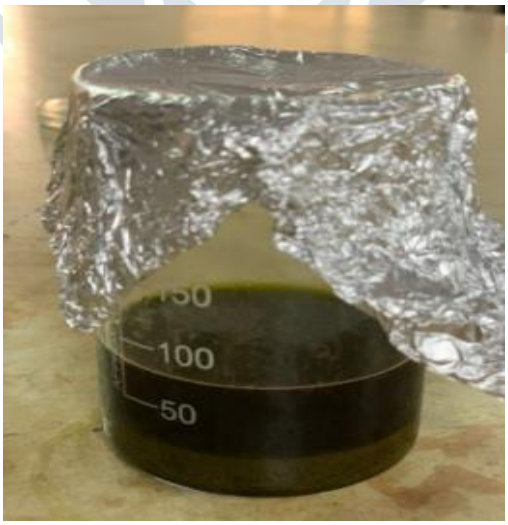

Fig. 4 Alcoholic extract of Bael leaves

Fig. **5** Aqueous extract of Bael leaves

ORGANOLAPTIC EVALUATION OF EXTRACT:

Table 5: Table showing the evaluation of extract

Sr. No:	Parameter	Observation
1	Colour	Greenish brown
2	Taste	Bitter
3	Odour	Pungent
4	Form	Liquid

PHYTOCHEMICAL EVALUATION OF EXTRACT

1.Test for Carbohydrate

- Fehling's test: Brick red precipitate was observed

Fig. 6 Positive result shown by Fehling's test

2. Test for tannins

- $Fecl_3$ test: Black colour was observed.

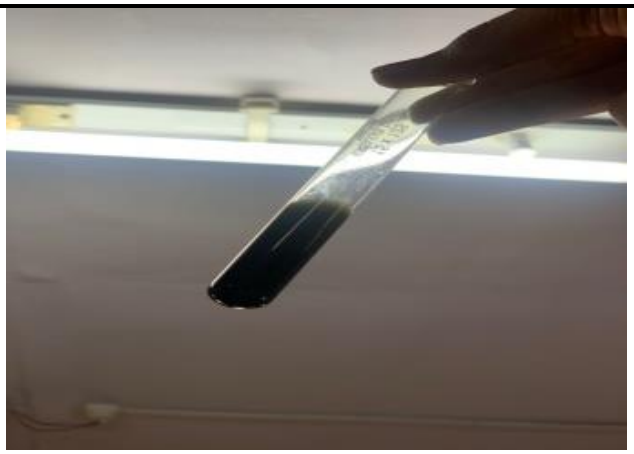

Fig. 7 Positive result shown by Fecl3 test

- Lead acetate test: White precipitate was observed.

Fig. 8 Positive result shown by Lead acetate test

- Iodine test: Red precipitate was observed

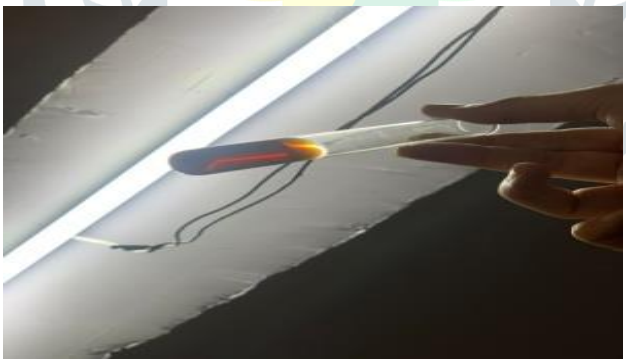

Fig. 9 Positive result shown by Iodine test

3. Test for Alkaloids:

- Mayer's test: White precipitate was observed

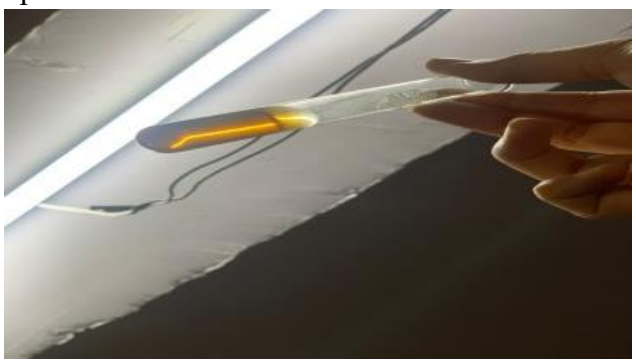

Fig. 10 Positive result shown by Mayer's test

- Hager's test: Yellow precipitate was observed

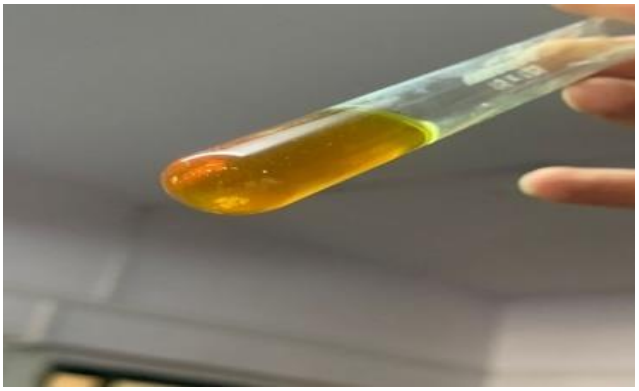

Fig. 11 Positive result shown by Hager's test

- Wagner's test: Reddish-brown precipitate was observed

Fig. 12 Positive result shown by Wager's test

4. Test for Saponin

- Foam test: Foam was observed

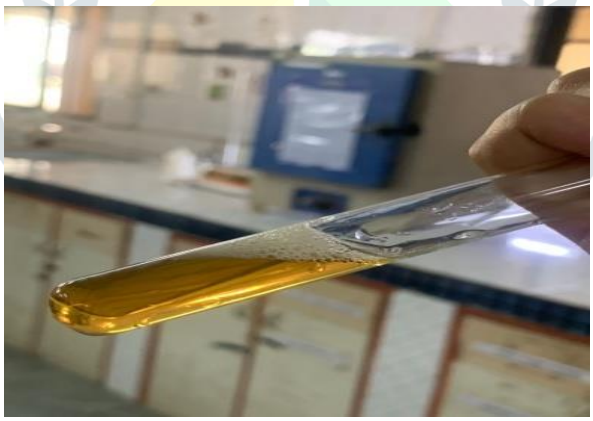

Fig. 13 Positive result shown by Foam test

5. Test for Cardiac glycoside

- Legal's Test: Red precipitate was not observed.

Fig. 14 Negative result shown by Legal's test

RESULT OF AGAR PLATE FORMATION BY CUP PLATE METHOD

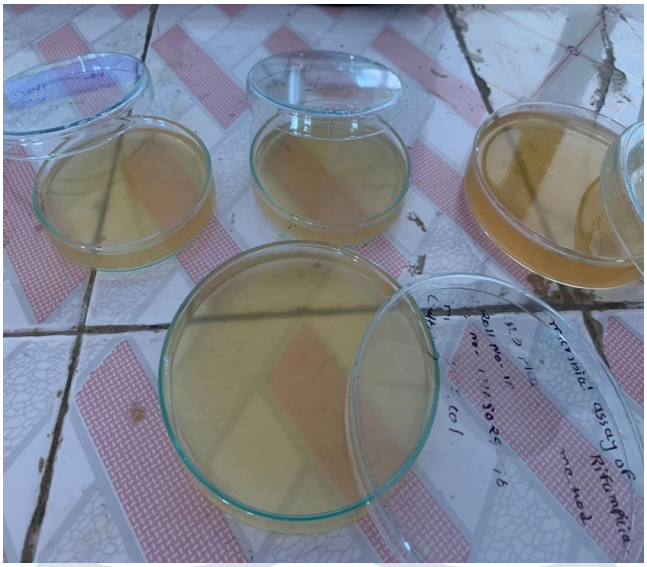

Fig. 15 Figure showing the agar plates

ANTIBACTERIAL ACTIVITY

1. In Extract

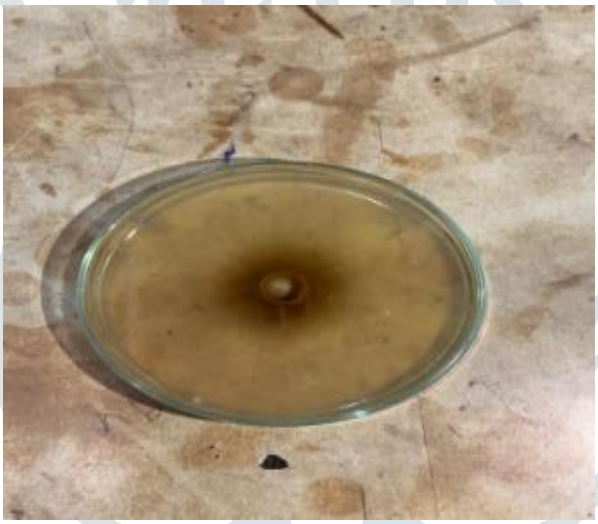

Fig. 16 Shows ZOI caused by the extract

2. In Ointment

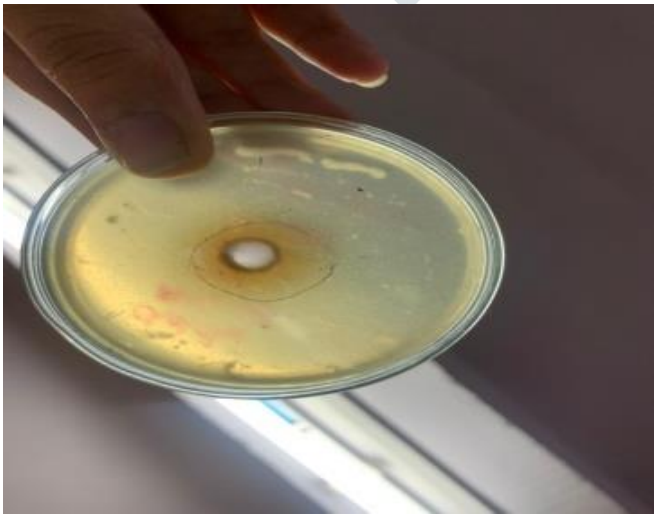

Fig. 17 Shows ZOI caused by the Ointment

EVALUATION OF OINTMENT

Table 6: Table shows the results of evaluation of ointment

Physicochemical parameters	observation
Colour	Yellow
Odour	Characteristic
Consistency	Smooth
pH	7.6
Spreadability (seconds)	7
Loss on drying	30%
Solubility	Soluble in boiling water and miscible with alcohol, ether, chloroform
Washability	Good
Non-irrintancy test	Non-irritant
Stability study(2^{o}C, 25^{o}C, 37^{o}C)	Stable
Extrudability	0.5 g

FINAL FINISHED PRODUCT

Fig. 18 Shows the final product prepared

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