In vitro antimicrobial efficacy of Securinega leucopyrus (Willd.) Muell. formulations

Foram P Joshi*[1], Saiema Ahmedi[2], Nikhat Manzoor*[2,] TS Dudhamal[3]

[1]Department of Shalyatantra, All India institute of Ayurveda, Goa, India-403513

[2]Medical Mycology Lab, Department of Biosciences, Jamia Millia Islamia, New Delhi,India- 110025.

[3] Department of Shalyatantra, ITRA, Jamnagar, Gujarat, India– 361008

*Corresponding authors

Dr Foram P Joshi: drforamj@gmail.com,

Orcid Id: 0000-0002-5177-4837

Dr Nikhat Manzoor: nmanzoor@jmi.ac.in

Orcid Id: 0000-0002-5855-9806

Abstract:

Background: Herbal medicaments have been practiced since antiquity as natural remedies, for the management of acute and chronic ailments. Diabetic wound healing activity of *Securinega leucopyrus* (Willd.) Muell has been reported in several case studies from India and Sri Lanka. **Objectives**: *In vitro* antimicrobial activity of different formulations of *Securinega leucopyrus* oil was investigated against clinically significant bacterial and fungal strains. **Material and Methods**: Five different samples of *S. leucopyrus* oil and ointments were blinded and studied with standard control Ampicillin (against *S. aureus, E. coli, P. aeruginosa, K. pnumonie*) and Fluconazole (against *C. albicans, C. glabrata, C. tropicalis* and *Aspergillus flavus*) using agar disc diffusion method and Minimum Inhibitory Concentration (MIC) values were estimated using broth dilution method. **Results:** A zone of inhibition (ZOI) of 16 mm was achieved at 14 µl/ml, 16 µl/ml and 12 µl/ml at MIC of dry drug-based *S. Leucopyrus*. oils against *C. albicans*, *C. glabrata*, *C. tropicalis*, respectively, whereas no sample showed antifungal activity against *Aspergillus flavus*. Fresh and Dry drug based *Thumari* oils represented antibacterial activity with a ZOI of 21 mm achieved at an MIC of 26 µl/ml against *Staphylococcus* aureus. Both the fresh and dry drug base ointments did not produce any antimicrobial action against the tested strains. **Conclusion**:

Thumari oils presented a significant antifungal action against all three *Candida* species and antibacterial action against the gram-positive *S. aureus* strains.

Key words: *Securinega leucopyrus,* antibacterial, antifungal, wound healing

1. Introduction:

Wound healing still remains a global question since evolution of mankind. It is one of the most complex phenomena in the human body.[1] Unlike acute wounds, chronic wounds demand a vigilant correction of vascular supplies, neurological supplies, localized tissue infection, and altered metabolic status, to accelerate healing. Diabetic wounds fail to heal due to the presence of colonized microbes. These may result with infective secretions which prolong the inflammatory phase and delay wound repair. [2]

Gram positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram negative (*Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterobacter* species, and *Morganella* species) microorganisms are responsible for the development of chronic wound infections. Among these, *S. aureus* is mostly resistant to antibiotics and contains high virulence, contributing to the pathogenicity of the host. [3] *S. aureus* and *P. aeruginosa* have been found as the most common microbes in non-healing diabetic ulcers. [4]

Prolonged usage of antibiotics has made them ineffective making microbes resistant to treatments. Moreover, conventional antimicrobial drugs are toxic with several side effects. With this background, it was decided to explore the antimicrobial action of herbal medicines that aid healing of chronic wounds in patients with altered metabolic conditions and comorbidities, like diabetes mellitus. Topical antibiotics are not the best choice for the treatment of Diabetic Foot Ulcers due to imbalanced moisture production and contact dermatitis. [5] The microbial adaptations to the microenvironment of the skin leads to virulence and impaired wound healing. The most challenging part of treating chronic wounds is polymicrobial infections and a high tendency to become resistant to prolonged antibiotic treatments. [6]

Hence, it is the need of time to address bacterial and fungal loads of the wound floor while applying local medicaments. *Thumari* (*Securinega leucopyrus* (Willd) Muell.) is a desert climatic weed, commonly used in Shri Lanka and belongs to the *Euphorbiaceae* family. It is widely and wildly available in Saurashtra region of Gujarat, India. [7] Besides *Thumri* (Sanskrit name), it is also known as *Humari* (Hindi name) and *Shinavi* (Gujarati

name) in traditional clinical practice and *Ayurveda*. [8] It has been prescribed for wound management in India for a decade now.[9] Medicated oils exhibit better shelf lives in comparison to powder drugs and fresh plant pastes. [10,11] It was hence planned to evaluate the *in vitro* anti-microbial action of medicated oil of *S. leucopyrus*.

2. Material and Methods

In vitro antimicrobial studies were conducted using medicated oil and ointment of *S. leucopyrus*. Both the formulations were tested against clinically significant bacterial and fungal species. The conventional drugs, fluconazole and ampicillin were included as standard positive controls, for antifungal and antibacterial activity, respectively. After preparation, the study samples were blinded, labeled, and tested in the Medical Mycology lab, Department of Biosciences, Jamia Milia Islamia, New-Delhi [Table 1].

Table 1 Blinding of samples for In-vitro antimicrobial study

Samples	Blind term	Interpretation	Translation
Sample 1	*Th. T. M.*	*Thumari Taila Moorchhita*	*Thumari* Oil from Dry drug paste and Refined (*Ayurvedic* Method) sesamum oil
Sample 2	*Th. T. F.*	*Thumari Taila Fresh*	*Thumari* Oil from Fresh drug paste
Sample 3	*Th. T. D.*	*Thumari Taila Dry*	*Thumari* Oil from Dry drug paste
Sample 4	*Th Mh F*	*Thumari Malhar Fresh*	*Thumari* ointment from Fresh Drug paste
Sample 5	*Th Mh D*	*Thumari Malhar Dry*	*Thumari* Ointment from Dry drug paste

2.1 Chemicals, media, and strains

All Media components, DMSO and ampicillin were acquired from Hi media (India) while fluconazole was procured from Aldrich (Germany). All other chemicals used were of analytical grade and were procured from E. Merck (India). The samples were diluted using 10% DMSO. Bacterial strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *K. pneumoniae* (MDR)) were obtained from Dr. Ram Manohar Lohia Hospital, Department of Microbiology (New Delhi, India) and maintained on Luria broth (LB) at 37 °C. *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. tropicalis* ATCC 750 were maintained on yeast extract, peptone, and dextrose (1:1:2) with 2.5% agar.

2.2 Preparation of Samples

The *Thumari* (*S. leucopyrus*) oil and ointment samples were prepared in the Institute of Teaching and Research in Ayurveda, Jamnagar by classical methods. [12]

2.2.1 Preparation of *Moorchhita Thumari Taila* (MThT) (processed *S. leucopyrus* oil): *Sesamum oil was* heated for 3 consequent days (average 5.23 hrs./day) on medium heat with oil processing ingredients of *Ayurveda* in 1: 4: 16 ratios (i.e., 1 part drug paste (1.819 kg), 4-part oil (7.5 kg) and rest 16-part water (30 kg)) [Figure 1].[13]

Figure 1: *Taila Moorchhana* (Oil Processing)

After complete evaporation of water by intermittent heating method, *Sneha Siddhi Lakshana* (oil formation signs) were achieved and finally the processed oil was filtered at room temperature and stored in a glass container. This *Moorchhita Taila* (processed oil) was used as base oil (7 kg) and heated again with dry *S. leucopyrus* paste (1.75 kg) and water (28kg) to prepare *S. leucopyrus* oil i.e., *Moorchhita Thumari Taila (MThT)* for 3 consequent days (average heating 8 hrs./day) until complete water evaporation. Entire procedure of *MThT* preparation was completed in 6 days duration.

Same procedure was repeated for the preparation of *Thumari Taila (ThT) (S. leucopyrus* oil) and Fresh drug based *Thumari Taila* (FThT) excluding *Moorchhana* (oil processing) procedure [Figure 2].

Figure 2: *Thumari* (*S. leucopyrus*) oil preparation

2.2.2 Preparation of *S. leucopyrus* ointment:

Thumari oil with fresh drug paste (*ThTF***)** was heated with white wax in 2.5:1 ratio (1 part of white wax heated with 2.5 part of *ThTF*) by direct heating method in a metal pot until complete melting of wax. After that it was filtered with dry and sterile cotton cloth and was allowed to condense. Same procedure was followed to prepare another ointment by heating refined sesamum oil base *Thumari* oil (*MThT*) and white wax in 2.5:1 ratio (2.5 parts of *Thumari* oil (*MThT*) with 1 part of white wax.)

2.3 Antifungal efficacy of samples 1-5 against *Candida* species and *Aspergillus flavus*

2.3.1 Minimum Inhibitory Concentration

Antifungal susceptibility of samples 1-5 (*S. leucopyrus* oil and ointments) was studied against three clinically significant *Candida* species (*Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. tropicalis* ATCC 750) by broth dilution method. *Candida* strains were maintained on yeast extract-peptone-dextrose (YEPD) in the ratio 1:2:2 along with 2.5% agar at 4°C. *Aspergillus flavus* ITCC5599 was maintained on potato dextrose agar (PDA) at 4°C. Outcomes were reported in terms of Minimum inhibitory concentration (MIC) as per the guidelines of CLSI reference document M27-A3 and was defined as the lowest concentration that causes 90% decrease in absorbance in comparison to that of the control (without test compound) [Table 2]. [14]

2.3.2 Agar Disc-diffusion assay [15]

Fungal cells were added into molten media with agar at 40°C and poured into 90-mm petri plates. *Candida* cells ($1x10^5$ per ml) and 100 µl of *Aspergillus* conidia suspension was used for inoculation. Sterile filter discs (4mm) were loaded with different concentrations of test compounds and placed on agar plates. [16,17] For higher concentrations, wells were prepared with the help of a sterile syringe. The average diameter of zones of inhibition (ZOI) was measured after 48 h. Fluconazole (10µg/disc in case of *Candida* and 50µg/disc for *Aspergillus*) was also applied to discs as a positive control.

2.4 Anti-bacterial activity of samples 1-5 against four Bacterial species MIC.

Anti-bacterial susceptibility assays of the samples 1-5 (*S. leucopyrus* oils and ointments)

Samples	***C. albicans ATCC 90028***	***C. tropicalis ATCC 750***	***C. glabrata ATCC 90030***	***A. flavus ITCC 5599***

against bacterial species were studied on Four different bacterial species in the present study namely, *Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 27853, Klebsiella pneumoniae (MDR)* and were reported in terms of MIC and disc diffusion. The MIC of the test compound for bacterial strains was determined by broth dilution method as per the guidelines of CLSI reference document M 07 and was defined

	MIC (µl/ml)	ZOI (mm)	MIC (µl/ml)	ZOI (mm)	MIC (µl/ml)	ZOI (mm)	MIC (µl/ml)	ZOI (mm)
1	15	10 (MIC/2) 12 (MIC) 14 (2MIC)	10	9 (MIC/2) 12 (MIC) 14 (2MIC)	- (MIC/2) 14 (MIC) 16 (2MIC)	15	-	-
2	-	-	-	-	-	-	-	-
3	15	- (MIC/2) 11 (MIC) 17 (2MIC)	20	- (MIC/2) 8 (MIC) 10 (2MIC)	- (MIC/2) 12 (MIC) 14 (2MIC)	15	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-		-

as the lowest concentration that causes 90% decrease in absorbance in comparison to that of the control (without test compound). [18]

For **agar disc-diffusion bacterial** cells (10^6 cells/ml) were inoculated into molten LB agar at 37°C and poured into 90mm petri plates. Sterile filter discs (4 mm) were loaded with different concentrations of test compounds and placed on agar plates. [19] For higher concentrations, wells were prepared with the help `of a sterile syringe. The average diameter of ZOIs was measured after 48 h. Ampicillin (20µg/disc) was also applied to discs as a positive control. Dimethyl sulfoxide **(**DMSO) (10%) was used as a solvent and had no antifungal effect of its own.

3. Results:

3.1 Antifungal susceptibility

The *Candida* species gave an MIC of 10 µg/ml for fluconazole while *Aspergillus* gave an MIC of 50 µg/ml, indicating that the fungal strains used in the present study were not resistant to this conventional antifungal drug. Only samples 1 and 3 (*S. leucopyrus* oil with dry drug-based paste) showed significant antifungal activity. MIC of both the effective samples (1 and 3) was 15 µl/ml for *C. albicans* ATCC 90028 and *C. glabrata* ATCC 90030. The same samples gave an MIC of 10 µl/ml for and 20 µl/ml for *C. tropicalis* ATCC 750. Samples 2, 4 and 5 did not display any antifungal property in both solid and liquid media. Similarly, *A. flavus* showed neither a significantly low MIC value nor ZOIs (Table 2).

Table 2. Antifungal efficacy of Samples (1-5) against three clinically significant *Candida* species

(-) implies no activity, MIC*- Minimum Inhibitory concentration, ZOI*- Zone of Inhibition

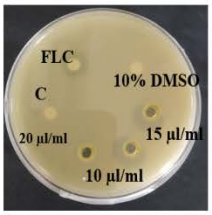
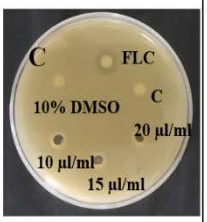
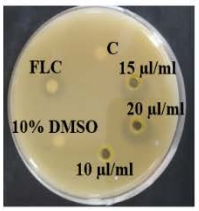
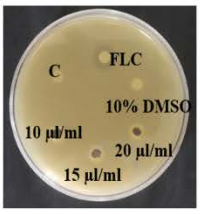
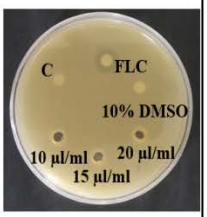
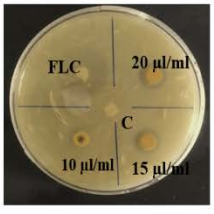
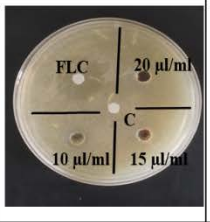
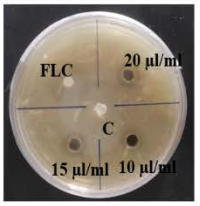
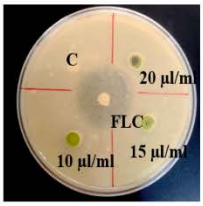
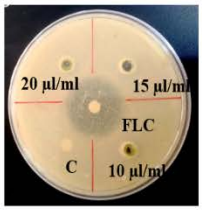
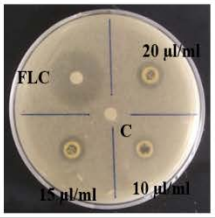
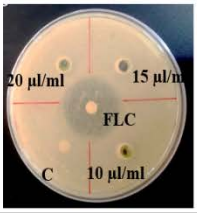
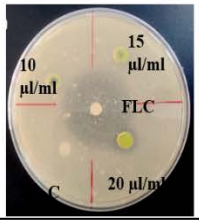
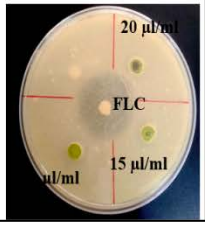
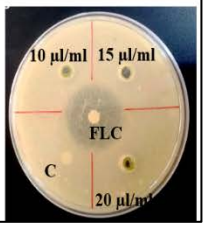
At MIC, sample 1 S. *leucopyrus* oil with dry drug-based paste and refined oil) formed ZOIs of 12 mm, 14 mm and 12 mm for *C. albicans*, *C. glabrata* and *C. tropicalis*, respectively which increased by 2 mm as the MIC was doubled in each strain. Similarly in sample 3, the ZOI increased from 11 mm, 12 mm and 8 mm at MIC to 17 mm, 14 mm and 10 mm at 2MIC in these *Candida* species [Table 2, Figure 3]. It is to be noted that none of the ointments showed antifungal activity.

3.2 Antibacterial susceptibility

The strains *E. coli*, *S. aureus*, and *P. aeruginosa* gave an MIC of 20 µg/ml for ampicillin, indicating that these three bacterial strains, used in the present study, were not resistant to the conventional antibacterial drug. On the other hand, *K. pneumoniae* gave an MIC of >100µg/ml for ampicillin indicating that this clinical strain was resistant. The samples 1, 2 and 3 (*S. leucopyrus* oil) showed significant antibacterial activity against *S. aureus* only giving an MIC of 25 µl/ml, 30 µl/ml and 20 µl/ml, respectively [Table 3]. The remaining two samples 4 and 5 (*S. leucopyrus* ointments) were not sensitive even against *S. aureus*. No samples showed anti-bacterial activity against *E. coli*, *P. aeruginosa* and *K. pneumoniae* [Table 3]. As shown in figure 4 and table 3, the ZOIs formed in *S. aureus* around the discs loaded with sample 1, 2 and 3 were 15 mm, 16 mm and 16 mm, respectively which increased to 21 mm, 18 mm and 17 mm.

Figure 3: Antifungal Activity of *S. leucopyrus* Formulations

Table 3: Antibacterial efficacy of Samples (1-5) against gram positive and gram-negative

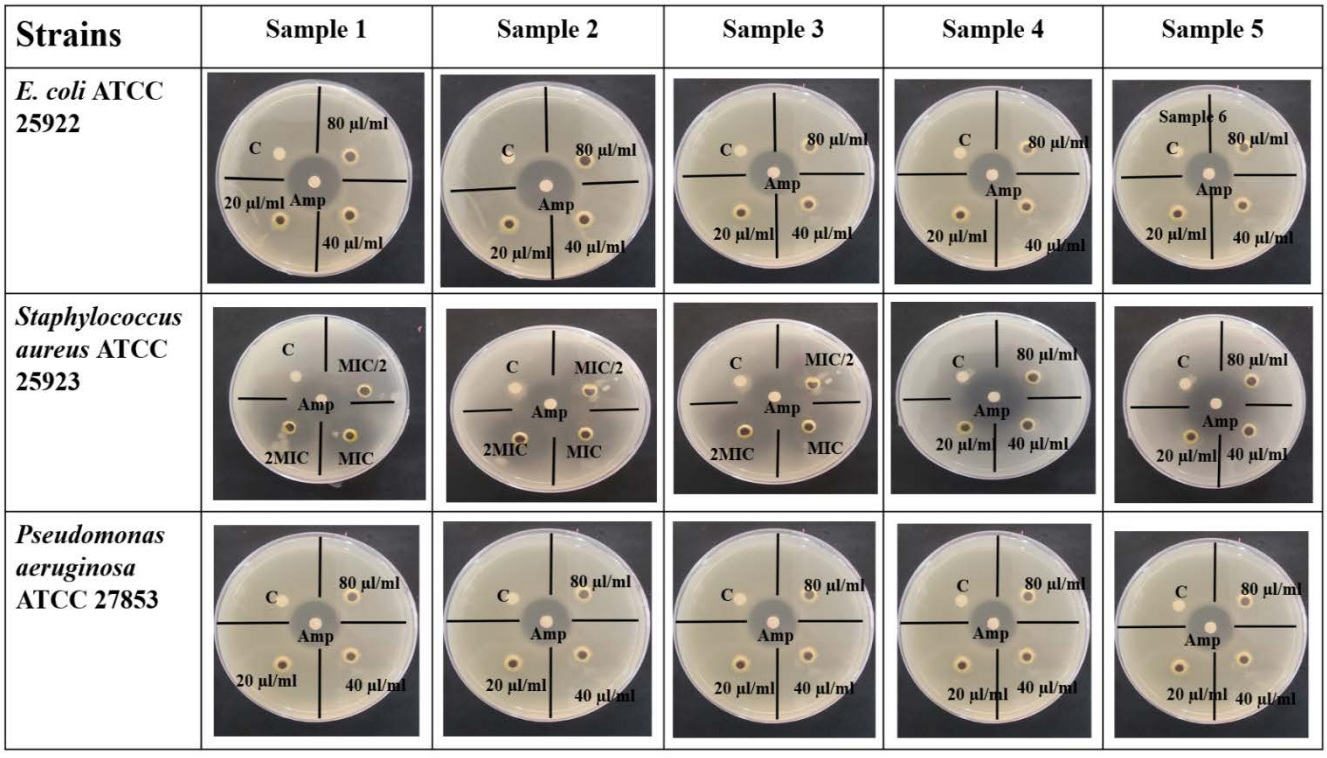
Strains	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
C. albicans ATCC 90028					
C. glabrata ATCC 90030					
C. tropicalis ATCC750					

bacteria

Samples	*E. coli ATCC 25922*		*S. aureus ATCC 25923*		*P. aeruginosa ATCC 27853*		*K. pneumoniae (MDR)*	
	MIC (µl/ml)	**ZOI (mm)**	**MIC (µl/ml)**	**ZOI (mm)**	**MIC (µl/ml)**	**ZOI (mm)**	**MIC (µl/ml)**	**ZOI (mm)**
1	-	-	25	12 (MIC/2) 15 (MIC) 21 (2MIC)	-	-	-	-
2	-	-	30	10 (MIC/2) 16 (MIC) 18 (2MIC)	-	-	-	-
3	-	-	20	09 (MIC/2) 16 (MIC) 17 (2MIC)	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-

(-) implies no activity, MIC*- Minimum Inhibitory concentration, ZOI*- Zone of Inhibition

Figure 4: Antibacterial Activity of *S. leucopyrus* Formulations



4. Discussion

Non healing ulcers contain biofilm layer over the wound floors. These wound beds are the residential areas for microbial colonies which keep on damaging healthy cells and keep using up the essential nutrition. [20] In the infected non healing ulcers and wounds we often find multiple gram-negative and gram-positive bacteria, fungal strains, and parasites. [21] Previous studies have reported a high bacterial load of *S. aureus* in the wound bed of the diabetic ulcers along with some other localized microbial colonies. [22, 23] Moreover, local tissue invasion, localized Reactive Oxygen Species (ROS) activity and delayed wound healing are some consequences that arise due to the formation of microbial colonies in wounds.

S. leucopyrus oils (Samples 1, 2, and 3) were significantly effective against *S. aureus* giving MIC values of 20-30 µl/ml *in vitro.* The anti-bacterial role of the all three varieties of *S. leucopyrus* oils was confirmed, specifically for *S. aureus*, a gram-positive bacterium that is the leading cause of skin and soft tissue infections. [24, 25] Previous studies have also confirmed the antimicrobial activity of methanolic extracts of bark and leaf extracts of *S. leucopyrus*. [26,27] Drug availability, low shelf life, easy transport and approach to distance places are some factors that make these medicinal oils better antimicrobials. Present study confirmed the retained antibacterial activity in oil base formulation, which promotes its utility with increased shelf-life.

To the best of our knowledge, antifungal potential of *S. leucopyrus* has not been reported yet. *C. albicans* constitutes around 33.33% in the fungal loads of Diabetic foot Infections along with other species like *C. tropicalis* and *C. glabrata*, which constitute 21.3% and 4.76%, respectively. [28] Classical method of refining the medicinal oil enhances *Tikshna* property (~ sharpness of the oil) which augments the capacity the oil to penetrate tissues inside the wound bed. Moreover, it helps in disintegrating the microbial biofilms on the wound floor and thus, it ultimately synergizes the process of cellular proliferation and wound healing.

Dry drug paste-based oil [Samples 1 and 3] of *S. leucopyrus* exhibited antifungal activity against *C. albicans, C. tropicalis and C. glabrata.* None of these samples produced antifungal activity against the mold *Aspergillus flavus*. Novel formulations with *S.*

leucopyrus oil need to be prepared which can be more effective and have a broader antimicrobial spectrum.

Host cell membranes are formed of phospholipids which allow drug absorption at different rates. Oil processing (classical method of oil refining) helps in reducing contamination and further helps in making the oil more powerful and improved with respect to its sharpness and natural qualities.

5. Conclusion:

Only samples 1 and 3 i.e., Dry drug-based *S. leucopyrus* oils *(MThT* and *ThT)* have significant antifungal potential. The three samples of oils from dry and fresh drug paste, i.e., *MThT, ThT* and *FThT*) were effective against *S. aureus,* a gram-positive bacterium but were completely ineffective against all the tested gram-negative bacteria namely *E. coli*, *P. aeruginosa* and *K. pneumonia.* Further *in vitro* and *in vivo* studies may elaborate on the effect of these samples on the microbial virulence and pathogenicity. More improved oil formulations are needed that are effective against resistant clinicals bacterial and fungal strains.

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7. Additional Information: None

8. Conflict of Interest: Nil

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