

In-vitro Evaluation of Antibacterial, Antifungal and Anti-HIV Effects of Calophyllum Inophyllum Leaf Extract

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Calophyllum inophyllum is an evergreen tree with ethno-medical value growing along the seashores and islands of the Pacific and Indian Ocean. All parts of the plant such as bark, seeds and leaves have diverse medicinal uses such as an antiseptic, analgesic in wound healing, astringent, diuretic, purgative and expectorant. Although many species of *calophyllum* have been studied phytochemically for pharmacological properties, reports on *inophyllum* species are scanty. Keeping in view its medical importance and availability in India as well as the rapid development of resistance by pathogens to the commonly used synthetic antibiotics, the pharmacological effects of *C. inophyllum* leaf extract (CIE) on HIV, bacteria and fungi causative of many human diseases was assessed in this study. Isolation of the pure compounds from the ethanolic CIE was performed by gross column chromatography and tested against lyophilized forms of 8 fungal and 14 bacterial strains grown on Sabouraud's dextrose agar and nutrient agar media respectively. Fractions and pure compounds isolated from CIE were evaluated against HIV by the HIV-RT inhibition assay by using the RT assay kit (Roche). The results were tabulated and analysed. Among the purified compounds, *Inophyllum* C & E exhibited significant antibacterial and antifungal properties. Moreover, *Inophyllum* E was more potent than *Inophyllum* C in inhibiting the tested strains of bacteria and fungi whereas *Inophyllum* B shows highest antiretroviral activity. We conclude that CIE is an effective antimicrobial agent against common human pathogens tested in this in-vitro pharmacological evaluation of CIE.

Keywords: *Calophyllum Inophyllum*, leaf extract, antibacterial, antifungal and anti-HIV.

Background

The genus *Calophyllum* belonging to the Clusiaceae family consists of approximately 127 species worldwide with a pantropical distribution. In the American continent, *Calophyllum* is represented by 8 species and 7 species are found in India.¹ *Calophyllum inophyllum* is the most abundant species of this genus. *Calophyllum*

inophyllum L. (*Calophyllaceae*) is native from East Africa, having wide distribution. *Calophyllum inophyllum* (locally called Tamanu) is an evergreen tree with ethno-medical value growing along the seashores and islands of the Pacific and Indian Ocean.^{2,3} It has elliptical leaves, fragrant white flowers and large round nuts.^{1,2,3} All parts of the plant such as bark, seeds and leaves have diverse

medicinal uses such as an analgesic, antiseptic, astringent, diuretic, purgative and expectorant.^{1,3,4} Although many species of calophyllum have been studied phytochemically for pharmacological properties, reports on inophyllum species are scanty.⁴

The chemical literature of calophyllum describes various fractions isolated from it such as flavonoids, triterpenoids, xanthenes, coumarins, steroids, and other bioactive compounds which act as natural source for pharmacologically active drugs.⁴ Numerous dipyrancoumarins with strong anti-HIV-1 Reverse transcriptase activity were found among the chemical constituents of *Calophyllum* species collected from Malaysia and Sri Lanka. For instance, inophyllums B and P from *C. inophyllum*; soulatrolide from *C. inophyllum* and *C. teysmanii*, (+)-calanolide B(7) from *C. lanigerum* var. *austrocoriaceum*.² In 1992, Kashman *et al.* isolated a strong anti-HIV-1 coumarin namely (+)-calanolide A, from the Malaysian tree *C. lanigerum* var. *austrocoriaceum*.^[5] One year later in 1993, Patil *et al.* isolated (+)-inophyllum B and other newer compounds possessing anti-HIV-1 reverse transcriptase activity from the Malaysian tree *C. inophyllum*.^[2,3] Also, compounds containing 4-methylpyranocoumarins isolated from *C. cordato-oblongum*, an endemic species of Sri Lanka demonstrated inhibition HIV-1 reverse transcriptase [10]. Other HIV-1 inhibitory dipyrancoumarins have been isolated from *C. brasiliense* leaves.^{2,6,7,8} Furthermore, four coumarin derivatives isolated from crude extract of nuts of calophyllum inophyllum have shown activity against multi-drug resistant *Staphylococcus aureus*.^{3,9}

Chronic bacterial and fungal infections persist as a major health problem in tropical areas where high temperature and humidity promote their growth. Increase in bacterial resistance to commonly used synthetic antimicrobials and poor access to available drugs further aggravates the problem.

Since years, plants have been used in traditional medicine to treat numerous diseases in human beings. Among the natural products extracted from plants, antimicrobial agents draw particular attention. Ongoing efforts strive to find a new sustainable source of antimicrobials which would be clinically effective, cheap, derived from

renewable sources and easy to produce locally. Indeed, new treatment options which can accelerate control of infections and at the same time help in preventing them arouse interest, particularly in tropical areas.³

We selected *Calophyllum inophyllum* for our study elucidating its pharmacological properties keeping in view its medical importance and availability in India. The antibacterial, antifungal and anti-HIV properties of crude extracts and purified compounds from calophyllum inophyllum were assessed in this study.

Materials and Methods

Plant material

The leaves of *C. inophyllum* were collected from Tirunelveli, Tamil Nadu. The plant name has been checked with <http://www.theplantlist.org> The identification and authentication of plant material was done by the Botany division of Central Drug Research Institute, Lucknow. The voucher specimen is preserved in the Medicinal Plant Herbarium at the Central Drug Research Institute, Lucknow (voucher No. 2044) and Indian Council of Medical Research (ICMR), New Delhi, India (voucher No. ERIH-72) for future reference. After authentication, fresh leaves from mature plants were collected in bulk. The leaves were washed, shade-dried and milled to a coarse powder with the help of a mechanical grinder. The powdered material was then passed through sieve mesh number-40 and then used for further studies.

Preparation of extract

Crushed and dried *C. inophyllum* leaves (10kg) were placed in glass percolator with 25L of 95% ethanol and allowed to stand at room temperature for 24 hours. This process was repeated four times. The percolate was collected, filtered and concentrated under vacuum at 40°C using rotavapor. The weight of extract was found to be 1200gm which was further purified on silica Sep-pak for HPLC analyses.

Sub fractionation of ethanolic extract

The ethanolic extract (C002) was fractionated between toluene and water.

The toluene soluble fraction was separated using separating funnel and concentrated under reduced pressure. The weight of toluene soluble fraction (F003) concentrated was 500 gm.

The aqueous fraction was partitioned with ethyl acetate (F004) in separating funnel and ethyl acetate soluble fraction was concentrated under reduced pressure using rotavapor at 40°C. The weight of ethyl acetate fraction was found to be 160gm. Aqueous fraction was again fractionated with butanol (F005) and its weight was found to be 100gm. The water-soluble fraction (F006) was concentrated and weighed.

Toluene soluble fraction was chosen for further isolation of compounds by Gross column chromatography.

Compound identifications

Identification of pure compounds was performed by nuclear magnetic resonance (NMR) and high-resolution MS-FAB spectroscopic methods (500 MHz for ¹H-NMR and 125MHz for ¹³C-NMR). All the compounds were identified from their spectral data along with their structure confirmation by comparison with published literature. Some peaks remained unidentified.

The compounds isolated from *C. inophyllum* extract (Table 1) were then evaluated for their antimicrobial activity and potency against bacteria (Gram positive and Gram negative) and representative fungal strains in agar well diffusion assays using Mueller Hinton agar and Sabouraud's dextrose agar respectively. Various fractions and pure compounds were evaluated for anti-HIV activity by the HIV-RT inhibition assay using RT assay kit (Roche).

Antimicrobial Screening¹⁰

The lyophilized forms of fungal and bacterial strains were grown on Sabouraud's dextrose agar and nutrient agar media respectively. After incubation, fungal and bacterial growth were suspended in normal saline and maintained at 1.0–5.0 × 10³CFU/ml. The antimicrobial activity of compounds was determined by the NCCLS method for fungus using RPMI-1640 media buffered with MOPS(3-[N-Morpholino] propanesulfonic acid)_(Sigma Chemical Co.) and Mueller–Hinton broth for bacteria. The 96-well tissue culture plates were used for 2-fold serial dilution. The drug control, growth control and the blank were adjusted onto the plate. Compounds were dissolved in 1% Dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml, out of which 20µl of was added to 96-well tissue culture plate having 180 µlRPMI-1640 (Roswell-Park-Memorial-Institute) medium buffered with

MOPS (3-[N-Morpholino Propane Sulfonic acid)_(Sigma Chemical Co.) and Mueller–Hinton broth for bacteria so that the maximum concentration of the compound was 100 µg/ml.

From here, the solution was serially diluted to prepare concentrations of 25, 50, 100µg/ml, and then poured in Petri dishes followed by addition of inoculums with (10⁸-10⁹) colony-forming units per ml. Each time, a fresh stock suspension was prepared, the experimental conditions were maintained constant. Inoculation was done from the lowest to the highest concentration plates. Microtiter plates were then incubated at 35°C in a moist-dark chamber for 24 hours and MICs were recorded spectrophotometrically. Growth inhibition of the fraction under study was calculated with reference to negative control and compared with that of reference standard antimicrobial drug.

Determination of zone of inhibition

Pure ciprofloxacin was taken as a standard antibiotic for comparison of the results while determining zone of inhibition. Two sets of three dilutions (25,50, 100 µg/ml) of *C. Inophyllum* leaf extract and ciprofloxacin (50, 100 and 200 µg/ml) were prepared in double distilled water in Mc Cartney bottles. One sterile nutrient agar plate without extract but with equal volume of the solvent served as the control plate. Sterile nutrient agar plates were prepared and incubated at 37°C for 24hrs.

For each sample (1 mg/ml), two sterile filter paper discs (Whatmann no.1) of 6mm diameter were soaked in two different dilutions of DMSO solution and allowed to evaporate at room temperature. They were placed in appropriate position on the surface of the flooded plate marked as quadrants at the back of the Petri dishes. The Petri dishes were incubated at 37 °C for 24 hrs for bacterial pathogens and room temperature (28°C) for 72 hours for fungal strains. Similar procedure was adopted for the pure ciprofloxacin.

Anti-HIV activity evaluation by In vitro HIV-RT kit assay¹¹

For anti-HIV activity, fractions and pure compounds isolated from *Calophyllum inophyllum* were evaluated by the HIV-RT inhibition assay by using the RT assay kit(Roche). Each fraction was dissolved in DMSO and tested at 50µg/ml. Concentration tested for pure compounds was 1mM. IC₅₀ values were calculated for most active

compounds.

The procedure for assaying RT inhibition was performed as described in the protocol of Roche Kit.

Briefly, the reaction mixture consists of template/primer complex, 20-deoxy-nucleotide-50-triphosphates (dNTPs), and reverse transcriptase (RT) in the lysis buffer with or without inhibitors. After 1-hour incubation at 37°C, the reaction mix was transferred to streptavidine-coated microtiter plate (MTP). The biotin-labelled dNTPs that are incorporated in the template were bound to streptavidine due to activity of RT. The unbound dNTPs were washed using wash buffer and anti-digoxigenin-peroxidase (DIG-POD) was added in MTP. The DIG-labeled dNTPs incorporated in the template were bound to anti-DIG-POD antibody. The unbound anti-DIG-POD was washed followed by addition of the peroxide substrate (ABTS) to the MTP. A coloured reaction product was produced during the cleavage of the substrate by the peroxidase enzyme.

Nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI) was used as negative control. The absorbance of the sample was determined at OD 405nm using microtiter plate ELISA reader. The resulting colour intensity is directly proportional to the actual RT activity. The percentage inhibitory activity of RT inhibitors was calculated by comparing colour intensity to a sample that does not contain an inhibitor. The

percentage inhibition was calculated by the formula given below:

$$\% \text{ Inhibition} = 100 - \left[\frac{\text{OD}_{405\text{nm}} \text{ with inhibitor}}{\text{OD}_{405\text{nm}} \text{ without inhibitor}} \times 100 \right]$$

The experiment was repeated in triplicate and values were tabulated and analysed.

Standard antibacterial drug - Ciprofloxacin

Standard antifungal drug- Ketoconazole

Standard antiretroviral drug: Nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI)

RESULTS

Table 1. List of Isolated compounds from *Calophyllum inophyllum*

S. No.	Compound Isolated
1.	3-oxo-friedelin-28-oic-acid
2.	Calophyllic acid
3.	Isocalophyllic acid
4.	Inophyllum C
5.	Inophyllum E
6.	Calaustralin
7.	Inophyllum B
8.	Inophyllum P
9.	Inophyllum A
10.	Calophyllolide 3
11.	Calanolide A
12.	Calanolide B

Table 2. The antibacterial activity of Inophyllum C and E derived from *Calophyllum inophyllum* leaf extract

S. No.	Name of bacteria	Minimum inhibitory conc. (MIC) in µg/ml	
		Inophyllum C	Inophyllum E
1	<i>E. coli</i> (ATCC 8739),	>50	50
2	<i>Pseudomonas aeruginosa</i> (ATCC BAA-427),	50	<50
3	<i>Staphylococcus aureus</i> (ATCC 25923),	<50	<<50
4	<i>Klebsiella pneumonia</i> (ATCC 27736)	>50	50
5	<i>Bacillus subtilis</i>	>50	50
6	<i>Salmonella typhi</i>	>50	>50
7	<i>Flavobacterium</i>	>50	>50
8	<i>Proteus vulgaris</i>	>50	>50
9	<i>Shigella flexneri</i>	>50	>50
10	<i>Shigella boydii</i>	>50	>50
11	<i>Staphylococcus epidermidis</i>	<50	<50
12	<i>Salmonella typhimurium</i>	>50	>50
13	<i>Vibrio cholerae</i>	>50	>50
14	<i>Corynebacterium diphtheriae</i> ,	>50	>50

The results of the antibacterial and antifungal activity of Inophyllum C and E derived from leaf extracts of *Calophyllum Inophyllum* are indicated in Table-2 and 3.

The minimum inhibitory concentration (MIC) was determined by observing the Zone of inhibition (ZOI) on 14 different bacterial strains (both Gram +ve and Gram -ve) and 8 fungal strains. The MIC of test compound is compared with control group.

Our results show that *Calophyllum Inophyllum* is the most active against Gram+ve bacteria at low concentration whereas many gram-negative bacteria are inhibited at higher concentration.

Inophyllum E has antimicrobial activity against the *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*,

E.coli, *Trichophyton mentagrophytes*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Candida albicans*, *Pseudoallescheria boydii*, at conc. $\leq 50 \mu\text{g/ml}$. Rest of the tested bacteria and fungi were inhibited at higher conc. of $> 50 \mu\text{g/ml}$.

Inophyllum C was found be relatively less active as the concentration needed to inhibit all the 14 strains was much higher than inophyllum E.

For anti-HIV activity, Isolation of the pure compounds from these fractions was performed by gross column chromatography, obtaining inophyllum B, P C, E, A, Claustraline(CI-32), Calophyllic acid(CI-20a), Isocalophyllic acid (CI-20b), 3-oxo-friedelin-28-oic-acid(CI-18).

Most of the fractions obtained from the C. inophyllum leaves extract showed low HIV-1 RT inhibition (Table no. 4). However, fractions 12 and 18 showed high inhibition of HIV-1 RT (Table

Table 3. The antifungal activity of Inophyllum C and E derived from *Calophyllum Inophyllum* leaf extract

S. No.	Name of fungus	Minimum inhibitory conc. (MIC) in $\mu\text{g/ml}$	
		Inophyllum C	Inophyllum E
1	<i>Candida albicans</i> ,	>50	50
2	<i>Cryptococcus neoformans</i> ,	>50	50
3	<i>Sporothrixschenckii</i> ,	>50	50
4	<i>Trichophyton mentagrophytes</i> ,	<50	<50
5	<i>Aspergillus fumigatus</i> ,	>50	>50
6	<i>Candida parapsilosis</i> (ATCC-22019)	>50	>50
7	<i>Pseudoallescheriaboydii</i>	50	50
8	<i>Aspergillus niger</i>	50	50

Table 4. In vitro Anti HIV-RT activity of various fractions derived from *Calophyllum Inophyllum* leaf extract

S. no.	Fraction / Code no.	Bioactivity	Sample	% inhibition at $10 \mu\text{g/ml}$	% inhibition at $100 \mu\text{g/ml}$
1	F-7	Anti HIV-RT activity	Natural	25.4	22.28
2	F-12	Anti HIV-RT activity	Natural	0	77.93
3	F-18	Anti HIV-RT activity	Natural	0	48.29
4	F-23	Anti HIV-RT activity	Natural	20.165	15.55
5	Claustraline(CI-32)	Anti HIV-RT activity	Natural	0	22.54
6	InophyllumC	Anti HIV-RT activity	Natural	0	0
7	InophyllumE	Anti HIV-RT activity	Natural	0	28.39
8	Inophyllum B	Anti HIV-RT activity	Natural	18.25	78.30
9	Inophyllum P	Anti HIV-RT activity	Natural	0	40.21
10	Inophyllum A	Anti HIV-RT activity	Natural	0	18.46
12	Calanolide A	Anti HIV-RT activity	Natural	10.24	28.36
13	Calanolide B	Anti HIV-RT activity	Natural	16.46	24.64
14	Nevapirine control	Anti HIV-RT activity	Control	99.0	100

4). From the above results, it is evident that the fraction-12 (F-12) of gross column chromatography of toluene fractions showed maximum anti-HIV-RT activity at 100µg/ml. Among the pure compounds isolated from these fractions, inophyllum B, inophyllum P showed a potent inhibition on HIV-1 RT. Inophyllum B shows highest antiretroviral activity followed by Inophyllum P. IC50 were calculated for these compounds were 1.6 and 1.8 mM/ml, respectively. Compound Claustralin, inophyllum E and Calanolide A, B exhibited low inhibition of HIV-1 RT. (Table no. 4). Inophyllum C, Inophyllum A, rest of the fractions and pure compounds, have not shown the considerable anti-HIV-RT activity against HIV RT-1.

Our study suggests the presence of effective chemotypes in *Calophyllum Inophyllum* which could be used as plant source for anti-HIV-1 drugs.

DISCUSSION

The use of a natural products and their derivatives as antimicrobials plays a key role in health care. These have been used to treat diseases from ancient times and are relatively safe, environment-friendly and stood the test of time. The importance of natural derivatives as a therapeutic modality has increased nowadays due to rapidly developing resistance to several commercially used synthetic drugs and their metabolites as well as the risk of water pollution from the hospital waste besides being expensive for low-income countries.^{11,12} Hence, there is an urgent need to discover appropriate bio-based products that will circumvent the shortcomings of synthetic products. Ongoing efforts strive to find new source of antibiotics that are cheap and derived from renewable sources and are easy to produce locally.

The use of natural products and their derivatives as an antimicrobial agent is an important aspect of phytochemistry with the prospects of discovering and developing new drugs which are biodegradable with lesser incidence of drug resistance. Screening their extracts for antimicrobial activity against various organisms enables us to identify the lead molecule for further development as potent antimicrobial agent.

Calophyllum Inophyllum is one such

natural source that has shown antimicrobial activity against many Gram positive organisms such as *Staphylococcus aureus*, *Bacillus subtilis*, Gram-negative organisms such as *S. typhi*, *E. coli*, *Klebsiella* and *P. aeruginosa* and some fungal strains.

Staphylococcus aureus, *Pseudomonas aeruginosa* and *Salmonella typhi* are responsible for majority of the nosocomial and community-acquired infections. *S. aureus* can cause wide range of disorders ranging from superficial skin infections like boils, styes; localized abscesses, to more deep-seated infections such as osteomyelitis and endocarditis, food poisoning, toxic shock syndrome, urinary tract infections especially in females. *S. aureus* is also a major cause of hospital-acquired (nosocomial) infection of surgical wounds. *S. epidermidis* causes infections associated with catheters and other indwelling medical devices.¹³ *P. aeruginosa* is a Gram-negative bacterium that typically infects burns, other wounds, and also causes pulmonary tract and urinary tract infections.¹⁴ *S. typhi*, a Gram-negative bacterium, transmitted through food and water causes typhoid fever. Certain strains of *S. typhi* have already developed resistance to known drugs such as chloramphenicol, ampicillin, and trimethoprim.¹⁵ *E. coli* is a Gram-negative bacterium, causes gastroenteritis, urinary tract infections, and neonatal meningitis.¹⁶ High incidence of development of resistance by *Staphylococcus aureus*, *pseudomonas aeruginosa* and *salmonella typhi* to the commonly used antibiotics prompts the quest for development of new and effective antimicrobials to which resistance has not developed.

In our study, *C. inophyllum* (Tamanu) fresh leaves were randomly collected. Isolation of the pure compounds from various fractions was performed by chromatography. Compounds identified are given in Table 1. The extract was analysed using three serial chromatographic silica columns to separate the different fractions using an UV-DAD detector.

Our study found that Inophyllum E derived from extract of leaves of *Calophyllum Inophyllum* showed significant antibacterial properties by agar dilution technique and was more potent than Inophyllum C against the tested Gram +ve and Gram -ve bacteria. The MIC values obtained

show that *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, were inhibited at the concentration of <50 µg/ml and were highly sensitive to *Inophyllum E* derived from the extract. The strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Bacillus subtilis* were inhibited at the concentration of 50 µg/ml and were moderately sensitive. The remaining bacterial strains were found to be inhibited with the concentration >50 µg/ml and were less sensitive to the extract. These results are in conformity to a previous study conducted by Mishra *et al.*¹

This may be attributed to the fact that cell walls of Gram-negative bacteria are less permeable to antimicrobial compounds. This is in agreement with antimicrobial studies by Léguillier T *et al.* in five *C. inophyllum* oils used to treat infected wounds where a dose dependent microbicidal effect in vitro against Gram-ve bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*) and yeasts (*Candida albicans*).² A. Adewuyi *et al.* also reported similar findings in the study of *Calophyllum inophyllum* and *Pterocarpus osun* and their derivatives as antimicrobial agents where the acetone extracts from *C. inophyllum* showed activity against *E. coli*, *Salmonella typhi*, and *P. aeruginosa*, whereas its oil had inhibition against *Bacillus subtilis* and *S. aureus*.¹² Similar results were reported by many antimicrobial study by Ha *et al.*, 2009.¹⁷ *Calophyllum inophyllum* dried fruit peels reported that *C. inophyllum* extracts demonstrated promising antibacterial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis* while the gram negative *Pseudomonas aeruginosa* was resistant to these extracts.¹⁷

These microbicidal effects of CIE in-vitro against Gram-ve bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*) and yeasts (*Candida albicans*) could be due to dose dependent increase in production and release of β -defensin-2 peptide from macrophages. If these results are confirmed in-vivo, it would mean that CIE could indirectly participate to the elimination of Gram-ve bacteria and yeast in skin infections by stimulating innate immune defences.²

On comparing the relative size of ZOI obtained during this antibacterial study, the results indicate that the antibacterial activity of *C. inophyllum* was in the order of *Staphylococcus aureus* > *Staphylococcus epidermidis* > *Bacillus*

subtilis > *Pseudomonas aeruginosa* > *Escherichia coli* > *Klebsiella pneumoniae* > *Proteus vulgaris* > *Shigella flexneri* = *Shigella boydii* > *Salmonella typhi* = *Salmonella typhimurium* > *Vibrio cholera*. The results are comparable to a study conducted by Uma Shankar Mishra *et al* on methanol and chloroform extracts of *calophyllum inophyllum* stem bark. The results against *Pseudomonas aeruginosa* were in conformity with a study conducted by Sikder MA *et al* where the highest zone of inhibition was observed by the pet-ether and carbon tetrachloride soluble fractions of *Calophyllum inophyllum* leaf extracts against *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa*, respectively and the chloroform soluble fraction also showed strong zone of inhibition against *Sh. boydii* and *V. parahaemolyticus*.¹⁸ This antimicrobial activity of *C. inophyllum* is also comparable with that of monocyclic and bicyclic β -lactams, whose activities were reported previously.¹⁹ Also, *Inophyllum C* exhibited antibacterial pattern similar to *Inophyllum E* but was less effective than *Inophyllum E* as most of the microbes were inhibited at much higher concentration than *Inophyllum E*.

In recent years, the scientific community emphasized that the current antibacterial drug development was insufficient to address the problems posed by antibiotic resistance among pathogenic bacteria.²⁰ In our study, the results have shown that as compared to ciprofloxacin, *C. inophyllum* fractions are active against the resistant strains of microbes which is largely involved in nosocomial and skin infections such as *Staphylococcus aureus*. It is less active against Gram-ve bacterial strains but highly active against all tested Gram+ bacteria strains with similar or lower MIC values in comparison with ciprofloxacin suggesting that *C. inophyllum* extract could be topically used for prevention and/or treatment of Gram + skin infections. In this context, CIE appears as a promising source of new antibiotics notably to fight multi-drug resistant bacteria implicated in skin infections.

Fungal diseases are another serious health problem worldwide. Fungal infections have substantially increased in the last two decades and are generally associated with significant morbidity due to increasing antibiotic resistance and population's susceptibility to opportunistic

infections precipitated by immune system suppression. The genus *Candida*, *Aspergillus* and *Trichophyton* species cause majority of the fungal infections in tropical developing countries. Humid weather, over population and poor hygiene are the ideal conditions for the growth of dermatophytes.²¹

C. albicans is a yeast (fungus) that causes candidiasis. This yeast lives as harmless commensal in many different body locations like the skin, the intestines, vagina, in almost half of the population.²² However, in response to a change in the host environment, *C. albicans* can convert from a benign commensal into a disease-causing pathogen, leading to infections in the oral, gastrointestinal and genital tracts.²² Tinea capitis (also known as “Herpes tonsurans”, “Ringworm of the hair,” “Ringworm of the scalp,” “Scalp ringworm”, and “Tinea tonsurans” is worldwide fungal infection (dermatophytosis) of the scalp. The disease is primarily caused by dermatophytes in the *Trichophyton* and *Microsporum* genera that invade the hair shaft. Drugs like terbinafine 250mg, nystatin, and use of selenium or ketoconazole shampoo along with oral antifungal agents like terbinafine, fluconazole and itraconazole are used in treatment of tinea capitis. Griseofulvin is known as the gold standard therapy for tinea capitis but its efficacy is decreasing with time.²³

Currently prescribed antifungal drugs such as polyene antibiotics or azole analogues suffer from certain limitations due to their adverse effects ranging from nausea, vomiting, headache, rash, to peripheral neuropathy, hepatotoxicity, nephrotoxicity during the use of amphotericin B, and drug-drug interactions in the use of azoles.²⁴ There is an urgent need for new effective and less toxic alternative antifungal drugs of plant origin for the treatment of fungal infections in the light of the significant toxicities, failure rates of the currently available systemic antifungal agents and emergence of resistant strains. Use of medicinal herbs in the treatment of skin diseases including mycotic infection is an age-old practice in many parts of the world especially in developing countries. Plants contain a spectrum of secondary metabolites such as triterpenoids, phenols, flavanoids, quinines, tannins and their glycosides, alkaloids and their essential oils having antimicrobial properties.²¹

In our study, *Inophyllum* E shows good antimicrobial activity against many fungal strains

at $\leq 50 \mu\text{g/ml}$ conc but *Inophyllum* C was found to be relatively inactive at this concentration. The MIC values obtained show that *Trichophyton mentagrophyte* was inhibited at the concentration of $< 50 \text{ ig/ml}$ and was highly sensitive to *Inophyllum* E, C derived from the extract. The strains of *Candida albicans*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus niger*, *Pseudoallescheria boydii* were inhibited at the concentration of $50 \mu\text{g/ml}$ and were moderately sensitive. The remaining fungal strains were found to be inhibited with the concentration $> 50 \mu\text{g/ml}$ and were less sensitive to the extract.

Our results show that *Calophyllum Inophyllum* is the most active against *Trichophyton mentagrophyte* at low concentration, *candida albicans*, *aspergillus niger*, *Cryptococcus neoformans*, *Sporothrix schenckii*, *Pseudoallescheria boydii* at moderate concentration whereas many other fungal strains are inhibited at higher concentration. Similar findings are reported by Sundur S, Shrivastava B.²⁵ The results of our study are supported by similar results against *candida* and *aspergillus niger* obtained by Rana MR, *et al.*²⁶ The results of our study are in consonance with those also obtained by Ali MS, *et al* against *Trichophyton mentagrophyte*, *Pseudoallescheria boydii*, *Aspergillus niger* and *candida albicans*.²⁷ Also, triterpenes isolated from leaves and twigs of *calophyllum inophyllum* have shown comparable antifungal spectrum in a study against *candida albicans*, *aspergillus niger*, *pseudallescheria boydii*, and *trichophyton schoenleinii*.²⁸ Many studies have shown that extracts of other *Calophyllum* species, such as *Calophyllum caledonicum*,²⁹ *Calophyllum brasiliensis*,^{30,31} *Calophyllum symingtonianum*,³² *C. Uvarioidendron*³³ harbor promising antifungal activity which strengthens the findings of present study.

In the search for plant-derived antifungal agents, ethanolic extracts from *Calophyllum Inophyllum* leaves shows promising antifungal activity towards *Candida*, *Aspergillus* and *Trichophyton* species.

In recent years, all other forms of immunodeficiency syndromes have been overshadowed by an epidemic of severe immunodeficiency caused by a retrovirus namely Human immunodeficiency virus type-1 or HIV-1. Till date, no cure for HIV infection has been found.

Thus, the present research is aimed at developing chemotherapy against HIV. Chemotherapy for AIDS has progressed steadily in the past decade. However, with the rising incidence of failure of synthetic antiretroviral drugs due to development of drug resistant strains, coupled with their adverse effects, there's an urgent need of newer, more effective, and less toxic chemotherapeutic agents. Therefore, it becomes necessary to screen natural sources for anti-HIV activity. Plants, particularly anti-infective or immunomodulating herbal medicines, can serve as source of active lead compounds which can further be developed as anti-AIDS drugs.

Recent studies from plant sources and their analogues—synthetic coumarins, indicate that some of them serve as potent non-nucleoside RT-inhibitors, others as inhibitors of HIV-integrase or HIV-protease. Ethanolic and water extracts from the calophyllum bark were reported to have significant anti-HIV-IN (HIV-1 integrase) enzyme inhibition.³⁴

The results of our study suggests the presence of effective chemotypes in *Calophyllum Inophyllum* which could be used as plant source for anti-HIV-1 drugs. Inophyllum B, inophyllum P showed a potent inhibition on HIV-1 RT among all the pure compounds tested. Inophyllum B shows highest antiretroviral activity followed by F-12, 18 of gross column chromatography of toluene fraction followed by inophyllum P. Compound Claustralin, inophyllum E and Calanolide A, B exhibited low inhibition. (Table no. 4). However, Calanolide A has also shown good anti-HIV property in a previously reported study conducted by Gomez-Verjan J *et al.*³⁵ In our study, inophyllum B, P surfaced as the potent HIV reverse transcriptase inhibitors among several coumarins present in CIE. The results are in accordance with the results reported by Kostova I and Yu D.^{36,37} Another study conducted by Taylor PB *et al.* which evaluated the ability of Inophyllum B to inhibit actively replicating virus has shown that inophyllum B is part of a subclass of non-nucleoside inhibitors that inhibits template uncompetitively, and is active against the mutant Y181C, which is known to be resistant to nevirapine, TBA, pyridinone L-693,593, and TIBOs R82913 and R82150.³⁸ Other derivatives

including pyranocoumarins such as soullatrolide (inophyllum P) isolated from the latex of certain *Calophyllum* species have also reported to be active against HIV.³⁹

The medical practitioners constantly look forward to new drugs and combinations for treatment of HIV infection effective for first-line treatment, as well as against drug-resistant mutants. The formulation of rational combination therapy for HIV requires the identification of compounds that interact with differing sites of the viral and cellular target macromolecules. Since the inophyllums appear to inhibit RT with a unique mechanism, it is conceivable that they could play a role in combination therapy in AIDS where by using several inhibitors that act by different mechanisms decrease the possibility of viral resistance.³⁸ Results from our study find inophyllum B as the most promising coumarin for anti-HIV-1 drug. Further multicentre trials should be conducted to establish its clinical efficacy.

Taken together, antimicrobial activities of *C. inophyllum* extract arouse great interest in *C. inophyllum* as a valuable candidate in developing new drugs for the treatment of many diseases. Our study also emphasizes the need for continued search for new antimicrobial drugs primarily from plant sources, due to the probability of discovery of new drugs relatively free from the challenges of toxicity and cross-resistance associated with commonly-used synthetic drugs.

CONCLUSION

This study was conducted to evaluate antimicrobial properties of CIE traditionally used to treat infected wounds in tropical areas.

In view of performed in-vitro pharmacological evaluation of the ethanolic extract of *C. Inophyllum* leaves, we conclude that it can be used to inhibit many common human pathogens tested in this study. Our study lends support to traditional uses of CIE in the wound healing process particularly for infected wounds.

We highlighted the pharmacological effects of *C. inophyllum* leaf extract (CIE) against HIV, bacteria and fungi causative of common human diseases and propose it as an effective antimicrobial agent. To the best of our knowledge,

this is comprehensive report of antibacterial, antifungal and anti-HIV prospects of the subject plant *Calophyllum Inophyllum*. Further clinical studies are needed to establish its efficacy in vivo and decorticate its mechanism of action at molecular level.

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Conflict of interest

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