Screening for the Antimicrobial Properties of the Leaves of *Calophyllum inophyllum* Linn. (Guttiferae)

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Summary: The various crude extracts (ethanol, chloroform, water and butanolic) and pure compounds (friedelin, canophyllol, canophyllic acid and inophynone) from *Calophyllum inophyllum* Linn. were tested against various fungi (human, animal and plant pathogens) and bacteria (gram positive and negative). It is observed that the chloroform extract can inhibit the growth of tested human and animal pathogens . Among the pure samples, fredelin could be used as growth inhibiting agent against human pathogen.

Introduction

Calophyllum inophyllum Linn. is a common, moderate sized, evergreen, ornamental and medicinal garden-plant. It belongs to the family Guttiferae with forty genera and one thousand species. Its trivial English name is "Alexandrian Laurel". The genus *Calophyllum* consists of about 130 species, distributed in tropical areas of the world, of which five species are cultivated in Pakistan.

The *C. inophyllum* has great medicinal value and all parts (such as bark, leaves, seeds) are used as antiseptic, astringent, expectorant, diuretic and purgative. Oil of seeds and roots is beneficial in wounds and scabies. The plant is also recommended in leprous nephritis, intramuscularly as analgesic.

The chemical literature survey revealed the presence of wide variety of natural products such as flavonoids[1], triterpenoids[2], xanthones[3], coumarines[4], steroids[5] and other bioactive compounds. Although some of the *Calophyllum* species have been examined phytochemically, reports on *C.inophyllum* are scanty.

In view of the medicinal importance and availability in Pakistan, we subscribed to the phytochemical investigation of *C. inophyllum*. Here we report some known and unknown constituents of *C. inophyllum*. Their structures were solved with the aid of spectroscopic techniques. The antifungal and antibacterial efficacy of purified compounds and crude extracts were assessed.

Results and Discussion

Ethanol, butanol, aqueous and chloroform extracts of the leaves of *C. inophyllum* Linn. and the pure compounds (**1-4**) were tested for antibacterial and antifungal activities. A summary of results are shown in tables-1 and 2.

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Table-1 Antibacterial Activity of Crude and Pure Samples from the Leaves of *C. inophyllum*

Samples / Organisms	EtOH Ext.	BuOH Ext.	$CHCl_3$ Ext.	Std. Drug 1	Std. Drug 2	1	4	Std. Drug 1	Std. Drug 2	3	2	Std. Drug 1	Std. Drug 2
Gram Positive													
Staphylococcus aureus	6.51	-	6.01	24.02	25.91	6.60	5.50	17.51	13.59	-	4.51	10.81	20.02
Staphylococcus pyogenes	14.01	-	6.53	23.13	20.03	-	-	14.53	9.53	-	5.03	5.01	10.73
Corynebacterium diptheriae	6.36	-	7.10	23.00	25.03	3.50	4.53	13.01	11.09	-	-	8.51	11.00
Bacillus subtillus	13.00	-	-	23.09	25.77	-	-	11.53	10.00	-	-	-	-
Gram Negative													
Escherichia coli	-	7.59	-	-	-	-	-	13.52	17.51	-	-	-	-
Salmonella typhi	-	-	-	20.03	22.02	3.53	-	11.50	12.01	-	-	-	-
Klebsiella pneumonia	-	-	-	8.51	15.75	4.09	3.00	11.01	10.52	-	-	-	-
Proteus mirabillis	-	-	-	-	-	3.11	3.50	16.99	17.05	4.52	-	10.05	11.23
Pseudomonas aeroginosa	-	-	-	-	-	-	-	11.50	16.03	-	-	-	-

- The values are reflected the zone of growth inhibition in mm
- (-) Means no activity
- Std. drug 1: Ampicillin
- Std. drug 2: Amoxicillin

Table-2 Antifungal Activity of Crude and Pure Samples from the Leaves of *C. inophyllum*

Sample / Organisms	EtOH Ex.	BuOH Ext.	$CHCl_3$ Ex.	Aqueous Ext.	1	4	2
Human Pathogens							
Pseudallescheria boydii	-	-	79.01	-	81.04	-50.03	4.10
Candida albicans	46.01	6.25	48.09	4.30	51.73	15.45	9.09
Aspergillus niger	60.71	-	82.33	-3.44	85.09	-48.52	3.22
Trichophyton schoenleinii	-	-	57.72	-1.11	55.05	-15.35	5.09
Animal Pathogens							
Microsporum canis	56.41	7.10	75.32	58.80	72.93	5.97	3.58
Trichophyton mentagrophytes	57.39	77.91	3.55	-	-	-	-
Trichophyton simii	55.02	84.04	90.11	28.55	92.00	-18.42	5.26
Plant Pathogens							
Fusarium oxysporum var. *lycopersici* (tomato)	-	-	-23.70	-	14.43	1.90	11.81
Fusarium salani var. *lycopersici* (tomato)	-	-	5.13	-	5.04	-12.12	-1.14
Rhizoctonia solani	-	-	62.50	-	56.62	25.93	4.16

- 100 % Growth inhibition shown by std. Drugs
- Std. drugs: miconazole, ketoconazole
- The values reflect the % growth inhibition.

Antibacterial activity

The ethanol extract showed moderate activity against two gram +ve organisms i.e *Staphylococcus aureus* and *Corynebacterium diptheriae*. This extract did not exhibit any activity against gram -ve bacteria. The butanolic extract was found inactive against all gram +ve and -ve strains except *Escherichia coli*. The chloroform extract showed significant activities against gram +ve bacteria: C. *diptheriae, S. aureus, S. pyrogenes*. However, this extract was found inactive against all tested gram -ve organisms.

The isolated pure compounds: canophyllic acid (3)[6], canophyllol (4)[6], friedelin (1)[6] and inophynone (2)[7] were subjected to antibacterial activity. Canophyllic acid had bactericidal activity against *Proteus mirabillis* compared to Ampicillin and Amoxicillin. Canophyllol is a better antibacterial agent than other isolated compounds against gram positive strains, *Staphylococcus aureus* and *Corynebacterium diptheriae. Klebsiella pneumonia, Proteus mirabillis* were also tested with 400 µg/ml of canophyllol. Friedelin showed good activity against two gram positive strains, *Staphylococcus aureus* and *Corynebacterium diptheriae* and three gram negative strains: *Salmonella typhi, Klebsiella pneumonia* and *Proteus mirabillis*. The compound Inophynone at level (400 µg/ml) displayed good activity against *Staphylococcus aureus* when compared with ampicillin and amoxicillin.

Antifungal activity: The ethanolic, butanolic, chloroform and aqueous extracts of *Calophyllum inophyllum* Linn. and the isolated compounds; friedelin (**1**), canophyllol (**4**) and inophynone (**2**) were screened for antifungal activity against various fungi (table-2).

The butanolic extract was examined against different fungal cultures and exerted the significant growth inhibition against the two species of the genus *Trichophyton* named as *simil* and *mentagrophytes*. The chloroform extract displayed an excellent activity against human pathogens: *Pseudallescheria boydii,* and *Aspergillus niger* and moderate activity against *Candida albicans* and *Trichophyton schoenleinii.* Of interest is the growth promoting effect of the extract when it was used for inhibition against the plant pathogen *Fusarium oxysporum*. The aqueous extract had inhibition activity against the fungal culture *Microsporum canis*. It also showed growth promoting activity *Trichophyton schoenleinii* and *Aspergillus nigar*. However, there was no observable inhibition against any tested plant pathogen.

The isolated compounds friedelin (**1**), canophyllol (**4**) and inophynone (**2**) were also examined against various fungi. The compounds were isolated in n-hexane and chloroform from fresh leaves of *C. innophyllum*.

Pure friedelin, was highly active against most of the human pathogenic fungal strains such as *Aspergillus niger, Pseudallescheria boydii* and *Trichophyton schoenleinii,* It comparatively had a low while showed moderate activity against *Candida albicans* which is most common human and animal pathogen, Canophyllol and inophynone did not exhibit any significant antifungal activity against any of the fungal strains used in this exercise. This shows that the active principle in the extract is friedelin.

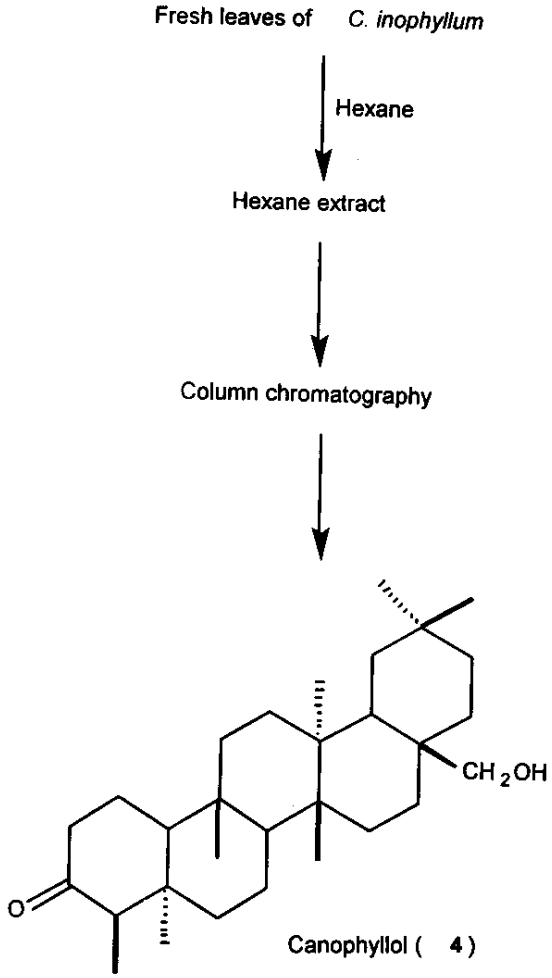
Experimental

Collection and identification

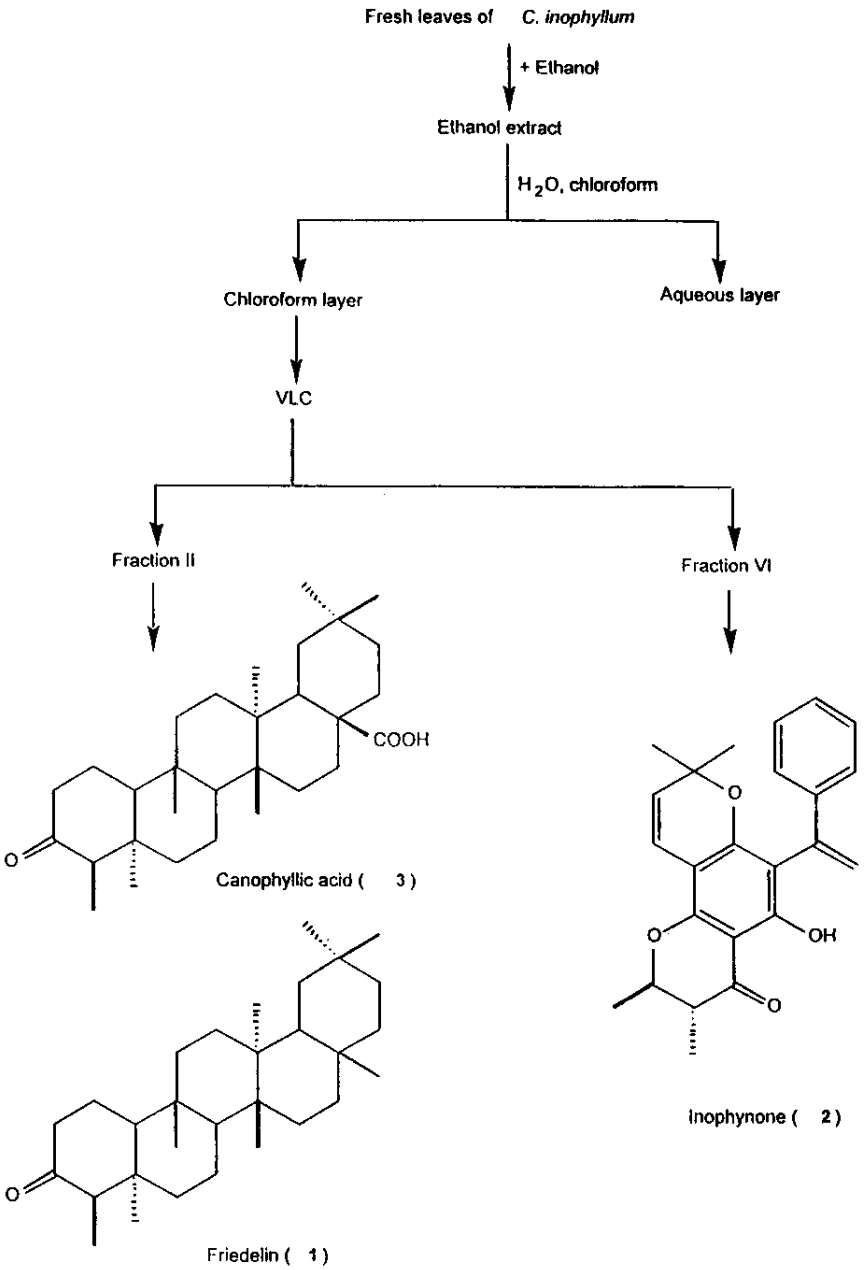
Fresh leaves of *Calophyllum inophyllum* Linn. (5 kg) were collected from Karachi region and identified by Dr. Suryyia Khatoon, Assistant Professor, Department of Botany, University of Karachi, Karachi. The voucher specimen (No. 9170) of plant is deposited in the herbarium.

Extraction and fractionation

The fresh leaves (2.5 kg) of *Calophyllum inophyllum* Linn. were chopped into small pieces and precoated in n-hexane for fifteen days. The hexane extract thus obtained, was evaporated under reduced pressure. The crude hexane extract (50.4 g) was subjected to column chromatography and the elution was carried out by using various solvent systems according to increasing order of polarities such as: n-hexane, n-hexane: chloroform, chloroform, chloroform: methanol and finally pure methanol as mobile phase. The fraction eluted at various polarity of solvents were checked by thin layer chromatography and were mixed on the basis of same TLC profiles (Scheme-1).



Scheme-1



Scheme II

In addition to the extraction with hexane, the fresh leaves of same material (2.5 kg) were soaked in ethanol for fifteen days. The ethanol was removed by evaporation under reduced pressure. The gummy ethanol extract (95.0 g) was partitioned between chloroform and water. The water layer was again dispensed into butanol. The butanol was removed under reduced pressure. The chloroform extract (41.2 g) was concentrated and subjected to VLC (Vacuum Liquid Chromatography). Fractions were drawn using mixtures of hexane, chloroform and methanol (Scheme-2).

From the hexane and ethanol extracts four compounds were purified which are described in Schemes 1 and 2.

Biological evaluations

The antifungal and antibacterial effects of the (chloroform, butanol, ethanol and aqueous) extracts of *Calophyllum inophyllum* Linn. were examined, along with the pure compounds (1-4) were especially assessed for this effects

Antimicrobial assays

Media employed in the tests were Sabouraud Dextrose (SDB, Biolife, Italy) and Nutrient Broth (NB, Oxoid). All pathogenic microbes were clinical isolates and kindly provided by Microbiology Department, University of Karachi, except *Staphylococcus aureus* and *Candida albicans,* which were generously given by Liaqat National Hospital, Karachi. The bacteria were grown overnight in nutrient and sabouraud dextrose broth, respectively, at 37°C. Cell suspensions in sterile media were adjusted to give a final concentration of 10^4 - 10^6 viable cells/ml. In case of fungi, the spore suspensions were obtained by flooding the tubes with 0.85% saline and diluted to make inocula containing 2.5 - 3.0 x 10^6 cells or spores with colony forming ability per milliliter[8].

Anti-microbial assays were determined by photometric microtiter broth dilution protocol. Stock solutions of plant extracts in hexane and ethanol were prepared to give final concentration of 400 mg/ml that was added to each well containing sterile media. Using a multichannel micropipette, an inoculum of 5 ml of the cell / spore suspension was added to their respective wells. The plates were incubated for 24 hrs. to 5 days[9] at 28 - 30°C depending on optimal growth conditions.

Microbial growth in the samples containing media were determined by measuring their turbidity or O.D. values at 540 and 600 nm for fungi and bacteria, respectively using ELISA reader (Spectramax 340, Molecular Devices, USA). Growth inhibition is calculated with reference to the negative control and compared with reference antibacterial drugs: ampicillin $(H_2O)_3$, amoxicillin and anti-fungal drugs: miconazole, ketoconazole (Johnson and Johnson PAK (Pvt.) Ltd.). Various organisms used in this study are mentioned in tables-1 and 2.

Conclusion

In view of the performed biological tests it is concluded that the chloroform extract can be used to inhibit the growth of tested human and animal pathogens and the growth of *Fusarium oxysporum* can be promoted by the same extract. It is also observed that friedelin is an excellent agent to inhibit the growth of tested human pathogens.

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