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In-vitro pharmacological screening of alkaloids from the leaves of *Daphniphyllum longeracemosa* as an antifungal

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Abstract
This study was designed as *In-vitro* pharmacological screening of alkaloids from the leaves of *Daphniphyllum longeracemosa* as an antifungal agent. The aqueous and methanol extracts of fresh dried leaves were obtained and subjected for preliminary phytochemical screening and TLC for the determination of phytoconstituents. The dried extract powder was found to be 10 gm and 15 gm respectively and the phytoconstituents like alkaloids, glycosides, steroids, saponins, flavonoids, phenolic compounds, proteins and amino acid were present in the extract. The *In-vitro* antifungal activity different strains were exposed to the plant extracts with known antifungal as positive control by the disc diffusion method and the MIC through the broth dilution method. The methanol extract showed higher inhibitory activity (26.25mm) followed by water extract (08.75mm) and standard drug (27.50mm) against the fungus, *Aspergillus fumigatus.* The methanol extract and positive control group also showed significant inhibitory activity against *Candida albicans* and *Aspergillus niger* respectively, which was ranging between 23.50mm to 15.60mm, and 25.45mm to 22.00mm respectively. However, the water extract showed moderate activity against the fungal species *viz.*, *Candida albicans, Aspergillus fumigates,* and *Aspergillus niger,* respectively. The alcoholic and aqueous leaf extracts exhibited remarkable antifungal activity in respect to MIC, which was ranging between 300–400 µg/mL and 200–300 µg/mL respectively. Finally it was concluded that the new alkaloids of Daphniphyllum with unprecedented skeletons have made a great contribution to resist the growth of microbes.

Keywords: *Daphniphyllum longeracemosa, Candida albicans, Aspergillus fumigates, Aspergillus niger*

1. Introduction
Natural products have been traditionally used since ancient times, especially as food supplements in the healthcare industry providing chemical therapeutics with biological probes and ethnopharmacological action [1]. These have been used as human ailments for long time without any influence of adverse effects or any other functional organ damage. The interest among natural products medication raises their popularity day by day and improves health condition more truthful [2].

Daphniphyllum longeracemosa belonging to family Daphniphyllum is an evergreen, large shrub with fairly substantial leaves and rhododendron-like foliage with red petioles. The flowers look like Mulberries, when in bud and have aristocratic foliage. The leaves have found to be *Daphniphyllum* alkaloids with unique polycyclic fused ring systems along with their extensive bioactivities. These alkaloids derived from squalene and are well reported to have cytotoxic, antioxidant, vasorelaxant, and anti-platelet activating factor effects [3]. Longeracinphyllin A and Longeracinphyllin B have been isolated from the leaves of *Daphniphyllum longeracemosa* shows a wide range of biological activities such as antitumor, antiviral and nerve growth factor-regulating properties [4].

The new alkaloid, daphnilongeridine (1), together with six known alkaloids, daphmacropodine (2), daphmacrine (3), codaphniphylline (4), daphniyunnine A (5), daphniyunnine C (6), and daphniyunnine E (7), were isolated from the leaves and stems of plant also reported to have cytotoxic, antiviral and as a nerve tonic [5]. Successive discoveries of new alkaloids with unprecedented skeletons have made a great contribution to structural diversities of alkaloids elaborated by plants of the genus *Daphniphyllum.* The antifungal effects of alkaloids derived from leaves of *Daphniphyllum longeracemosa* was investigated by agar diffusion method and minimum inhibitory concentration (MIC) method was determined [6].

Plant Profile
Daphniphyllum longeracemosa is a large well branched bushy shrub (4-5m tall) or small evergreen tree with thick shoots which are reddish brown in color [7]. Leaves are dark green, broadly oblong-elliptical round-ended with reddish venation and held on red petioles (Fig.1). Fruits are long-elliptic blooming in late spring with new leaf axils [8].

Fig 1: Leaves and plant of *Daphniphyllum longeracemosa*

2. Material and Methods

2.1 Plant collection and authentication

The leaves of *Daphniphyllum longeracemosa* were collected from the local areas of Mussoorie and Dhanaulti (Dehradun) situated in the middle Himalayas region at the altitudinal range of 2000-2500 meter in the month of October-November. The plant sample was authenticated from Botanical Survey of India (BSI), Dehradun (Uttarakhand), India.

2.2 Chemicals and reagents

Standard drug Fluconazole (Fungicip) 100mg was procured from Cipla limited, Haridwar. All other chemicals and reagents are of L.R. and A.R. grade respectively.

2.3 Preparation of extracts and preliminary phytochemical screening

The fresh shade dried leaves was subjected for cold maceration method using water and methanol thrice for 48 hrs separately [9]. The aqueous extract (AxDL) and methanol extract (MxDL) were obtained and lyophilized under vacuum to make powder form. Both AxDL and MxDL were subjected for preliminary phytochemical screening on the basis of standard available methods.

2.4 Thin layer chromatography

TLC is an analytical tool widely used because of its simplicity, relative low cost, high sensitivity, and rapidity of separation. Both extracts were subjected to TLC using various solvent systems, among them Hexane: Ethyl acetate (1:1) was used and Rf values were calculated [10].

2.5 Procurement and preparation of fungal spore suspension

Different fungal strains were used for experimental study. *Aspergillus niger, Fusarium oxysporum, Aspergillus awamori, Aspergillus nidulans* were kind gift from the National Research Center of Groundnut, Junagadh, Gujarat, India, and *oxysporum–Aspergillus flavus, oxysporum–Aspergillus parasite, Trichoderma virens, Trichoderma harzianum and harzianum– Alternaria sp.* were kind gift from the Department of Biosciences, Sardar Patel University, Vallabh Vidyanagar, Gujarat, India. These strains were transferred to fresh slants in sterile conditions incubated at 28°C till sporulation and were preserved in 250 ml sterile flasks. This sterile spore suspension can be used for antifungal activity [11].

2.6 *In-vitro* Antifungal Activity

Antifungal activity was investigated by the disc diffusion method. Fungal suspension (2x105) was streaked on the potato dextrose agar (PDA) medium containing Petri plates. Then, sterile discs (made from Whatman filter paper) each about 5mm diameter impregnated with the leaf extracts separately were placed on the inoculated plates. Similarly, each plate was placed with a sterile disc, Fluconazole as positive control. All the plates were incubated at 28°C for 24-48 hours. Zones of growth inhibition around the disc were measured after 48 hours. The sensitivity of the fungal species to the plant extracts was determined by measuring the sizes of inhibition zones (diameter of the zone) on the agar surface around the disc [12].

2.7 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined through the broth dilution method. The fungal inoculum (10-5 dilution) was taken in test tubes with (1800µl) nutrient broth supplemented with six different concentrations of leaf extracts 100-800µl/mL separately. The results of the extracts were compared with a standard control (Fluconazole 100µg/mL). All the test tubes were incubated at 35°C for 24-48 hours. The tubes were examined for visual turbidity. The MIC values were taken as the lowest concentration that inhibited the visual growth of the tested organisms [13].

2.8 Statistical Analysis

Antibacterial activity of *Daphniphyllum longeracemosa* leaf extracts was indicated by clear zones of growth inhibition. All experiments were performed in triplicates and the results are presented as mean±SD (Standard Deviation) according to New Duncan's Multiple Range Test.

3. Results

3.1 Preparation of extract and preliminary phytochemical screening

The fresh shade dried leaves (150g) was subjected for triple maceration using aqueous and methanol as a solvent and obtained extract was found to be 10 gm and 15 gm respectively. The obtained extracts were subjected for lyophilizer to make powder form and phytochemical screening was performed as mentioned in Table 1.

Table 1: Preliminary Phytochemical screening of leaves extracts

S.N.	Chemical constituents	Methanolic extract (MxDL)	Aqueous extract (AxDL)
1.	Alkaloids	+	+
2.	Glycosides	+	-
3.	Steroids	-	+
5.	Saponins	+	+
6.	Flavonoids	+	+
7.	Tannins and Phenolic compounds	+	+
8.	Tri-terpenoids	+	+
9.	Proteins and Amino acid	+	+

+: Present, -: Absent

3.2 TLC Profile of leaves extract of *Daphniphyllum longeracemosa*

The several numbers of spots were observed at the TLC plates after sprayed over alcoholic HCl. The TLC was prepared by using silica gel-G, and then dried in oven for 30min. Sample of AxDL and MxDL was put drop by drop with the help of capillary tube on the TLC plates separately. The number of spots under visible and U.V. was given in figure 2 and table 2.

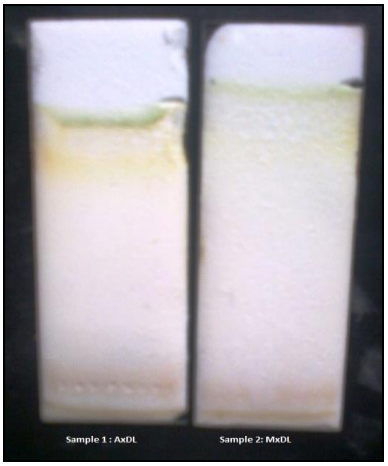


Fig 2(a): Under Visible light after spraying

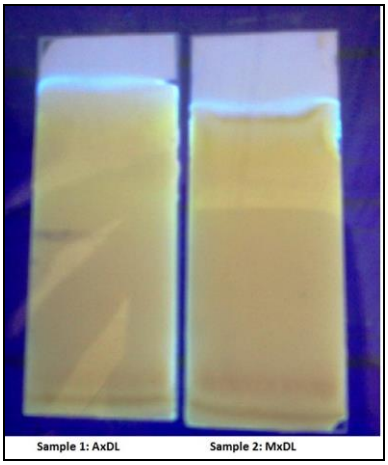


Fig 2(b): In U.V. after spraying

Table 2: TLC profile of AxDL and MxDL extracts

Observation	AxDL		MxDL	
	Under Visible	Under U.V.	Under Visible	Under U.V.
Number of Spots	04	04	02	04
Color of spots	Pink, Red-orange, Yellowish- Green, Brownish- green	Pink, Violet, Yellow, Greenish- Brown	Pink, Greenish- yellow	Pink, Yellowish- Green, Brown, Green
Rf Values	0.18 0.70 0.92 0.93	0.18 0.53 0.70 0.93	0.18 0.93	0.18 0.70 0.92 0.93

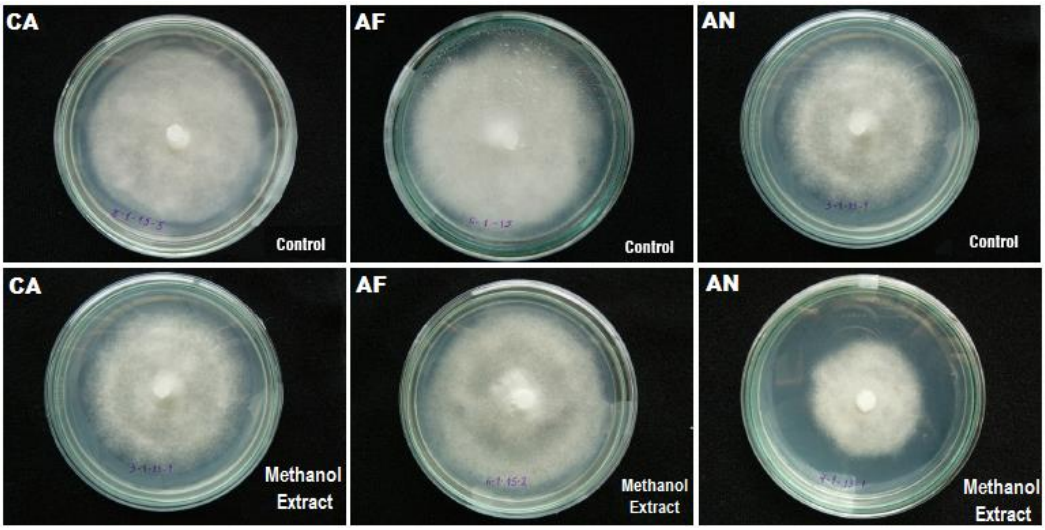
3.3 *In-vitro* Antifungal activity

Among the two solvents attempted, the methanol extract showed higher inhibitory activity (26.25mm) followed by water extract (08.75mm) and standard drug (27.50mm) against the fungus, *Aspergillus fumigatus.* The methanol extract and positive control group also showed significant inhibitory activity against *Candida albicans* and *Aspergillus niger* respectively, which was ranging between 23.50mm to 15.60mm, and 25.45mm to 22.00mm respectively. However, the water extract showed moderate activity against the fungal species *viz., Candida albicans*, *Aspergillus fumigates*, and *Aspergillus niger*, respectively (Table 3).

Table 3: Antifungal activity of alcoholic and aqueous extracts of *D. longeracemosa*

Control/ Extracts	Zone of inhibition (mm)		
	CA	AF	AN
Control (Fluconazole)	25.45 ± 0.15	27.50 ± 0.20	22.00 ± 0.30
Alcoholic Extract (Methanol)	23.50 ± 0.40	26.25 ± 0.50	15.60 ± 0.45
Aqueous Extract (Water)	12.30 ± 0.25	08.75 ± 0.30	03.00 ± 0.35

Values were performed in triplicates and represented as mean±SD; Mean values followed by different superscript in a column are significantly different ($p<0.05$). CA = *Candida albicans*, AF = *Aspergillus fumigatus*, AN = *Aspergillus niger*.



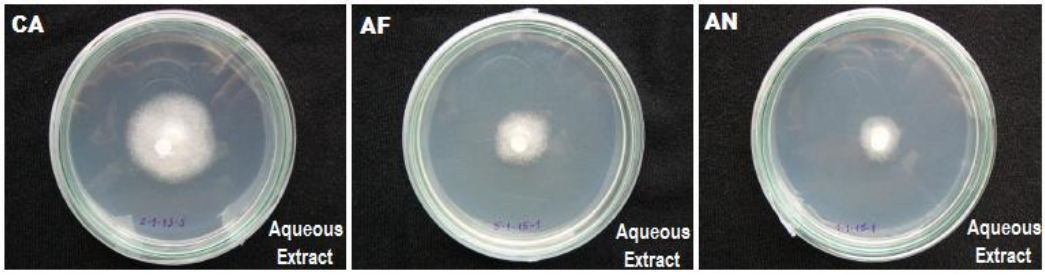


Fig 3: Results showing the *In-vitro* antifungal activity of Alcoholic and aqueous leaf extract of *D. longeracemosa* compared with Fluconazole (Standard drug). CA = *Candida albicans*, AF = *Aspergillus fumigatus*, AN = *Aspergillus niger.*

3.3 Minimum inhibitory concentration

Table 4 presents the data on minimum inhibitory concentration (MIC) of alcoholic and aqueous leaf extracts of *Daphniphyllum longeracemosa.* The leaf extracts exhibited remarkable antifungal activity which was ranging between 300 and 400µg/mL and 200 and 300µg/mL respectively.

Table 4: Minimum inhibitory concentration (MIC) of alcoholic and aqueous leaves extracts

Plant and drug	Minimum inhibitory concentration (µg/ml)		
	CA	AF	AN
Fluconazole	400	300	300
Alcoholic	400	300	300
Aqueous	300	200	200

CA = *Candida albicans*, AF = *Aspergillus fumigatus*,
AN = *Aspergillus niger*

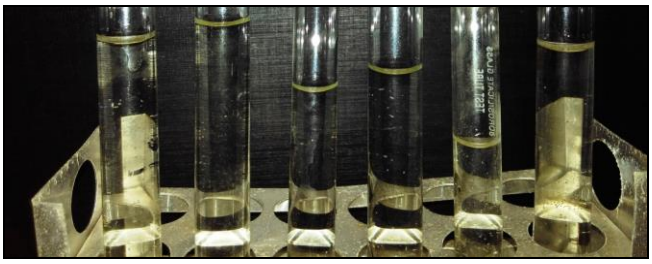


Fig 4: MIC of alcoholic and aqueous leaves extracts before administration of drug

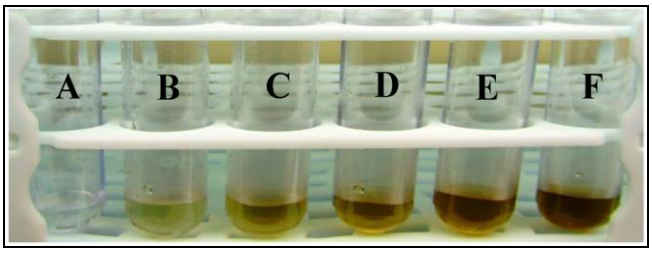


Fig 5: MIC of alcoholic and aqueous leaves extracts after administration of drug

Exploitation of the evaluation of antifungal activity of the present study revealed that the *Daphniphyllum longeracemosa* possess potential antifungal activity against three pathogenic fungal species. From the evaluation it is found that alcoholic extract inhibited the growth of the colonies of all fungal species than the other solvent extracts studied.

4. Discussion

The preliminary phytochemical screening of *Daphniphyllum longeracemosa* reviles that the presence of phytoconstituents in alcoholic leaf extract *viz.* alkaloids, glycosides, saponins, flavonoids, tannins and phenolic compounds, tri-terpenoids, proteins and amino acid. While the aqueous leaf extract contains only alkaloids, steroids and sterols, saponins and flavonoids. These finding suggest that the alcoholic extract has contains more phytoconstituents as compare to water extract of *D. longeracemosa.*

The results obtained from the present investigation revealed that the highest antifungal activity was exhibited by the alcoholic extract and the lowest by the aqueous extracts. Whereas the Fluconazole treatment group also exhibit the comparable antifungal activity as compare to alcoholic treatment group. The basis of varying degree of sensitivity of test organisms of fungi may be due to the intrinsic tolerance of microorganisms and the nature and combinations of phytocompounds presents in the crude extracts.

The antifungal activity of alcoholic (Methanol) extract of *Daphniphyllum longeracemosa* leaf showed highest inhibitory activity against the fungus, *Aspergillus fumigates.* It is a filamentous ascomycete fungus that is ubiquitous in the environment and has been implicated in opportunistic infections of humans. It causes various diseases in plants and animals. In human it causes black mould and rot diseases and in human beings it causes aspergillosis by which pulmonary allergy, bronchopulmonary aspergillosis and pulmonary aspergilloma are common disease of this class.

5. Conclusion

In the present study, alcohol and aqueous extracts of *D. longeracemosa* leaf showed significant antifungal activity against three fungi *viz.*, *Candida albicans, Aspergillus fumigates,* and *Aspergillus niger*, which was significantly inhibited by alcoholic (methanol) extract. Aqueous extract of *D. longeracemosa* leaf inhibited less number of fungus (i.e. *Aspergillus fumigates,* and *Aspergillus niger*) while the inhibition of *Candida albicans* was satisfactory as compare to these fungal species. This could be due to the lack of specific active compounds in the extracts. However, both the extracts of *Daphniphyllum longeracemosa* leaf inhibited the growth of all fungus used in this study.

It was further observed that the inhibitory activities of alcohol extract of leaf against *Candida* sp. and standard drug (Fluconazole) treatment group against same species were significantly greater than that of the water extract treatment group, which indicates the effectiveness and specific inhibitory function of alcoholic solvent by deriving specific compounds against these fungi. Minimum inhibitory concentration (MIC) of methanolic leaf extract was ranging between 300 and 400µg/mL. While aqueous leaf extract was ranging between 200 and 300µg/mL. The

most susceptible species for this extracts was *Candida albicans* (400µg/mL) against Fluconazole treatment.
The unique polycyclic fused ring systems of Daphniphyllum alkaloids, along with their extensive bioactivities, make this family of alkaloids especially attractive targets for total synthesis and biogenetic studies. Successive discoveries of new alkaloids with unprecedented skeletons have made a great contribution to structural diversities of alkaloids elaborated by plants of the genus Daphniphyllum.

6. Acknowledgment
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