



Antifungal Activity of Flavonoid Isolated from *Lindernia crustacea* (L) F. Muell against Traditionally Claimed Dermatophytes

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SRCD and ABA designed and executed the experimental work, evaluation, analysis of data. Authors TD and FA contributed to the study of phytochemical evaluation of the plant as stated in the current submitted manuscript. Author DS contributed to critical designing and shaping up the manuscript, grammatical and typological error correction, and also responsible for executing the results and data interpretation. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study was aimed to establish antifungal activity of flavonoid isolated from *Lindernia crustacea* (L) F. Muell.

Methods: Potent bioactive constituent from benzene extract was isolated through flash chromatography with solvent Toluene and Acetic acid (4:1). Isolated compound was structurally established by spectroscopic method. Antifungal potential of benzene extract as well as isolated compound was tested by disc diffusion method against two fungal strains *Candida albicans* (*C. albicans*) and *Trichophyton rubrum* (*T. rubrum*).

Results: The isolated compound from benzene extract was found to be flavonoid. Benzene extract as well as flavonoid were shown remarkable zone of inhibition against both fungal strains which indicates strong antifungal activity.

Conclusion: The experimental results concluded that *Lindernia crustacea* (L) F. Muell have significant anti fungal activity due to flavonoid content. It is expected that the results of the present research work would be beneficial in establishing the scientific basis of the traditional claims of medicinal values of *Lindernia crustacea*.

Keywords: *Lindernia crustacea* (L) F; benzene extract; isolation; flavonoid; antifungal activity.

1. INTRODUCTION

Despite advances in antifungal agents and the emergence of new antifungal agents, fungal infections are still a significant cause of morbidity and mortality in human [1]. Candidiasis which is caused by *Candida albicans* increasingly spreading worldwide because it is a frequent opportunistic pathogen in AIDS patients [2]. It is a typical commensal of the urogenital and gastrointestinal tract of human [3]. It is of great concern to find effective antifungal agent for *C. albicans*, as *C. albicans* increasing worldwide with multiple antimicrobial resistance. Therefore, in recent days most of research works are being carried out based on natural resources for developing new drugs against microbial infections without any side effects [4]. Plants are found several times as a source of a number of natural anti fungal agents. Plants are rich in several secondary metabolites like phenolic compounds, flavonoids, alkaloids, tannins etc., which have been found to possess anti fungal as well as anti microbial properties [5]. Isolation and identification of flavonoids from natural origin possesses antibacterial, antifungal and antiviral activity has been challenging for researchers [6]. *Lindernia crustacea* (L) F. Muell, family Linderniaceae was not well explored scientifically for its possible chemical constituents or biological activities, though it has been reported that

Lindernia crustacea is having many ethnomedicinal properties such as antifungal, antimicrobial, analgesic, anti-inflammatory and used widely in traditional system of medicines [7,8,9,10]. During this research work, emphasis has been given to investigate and explore the antifungal activity of extract and isolated compound of *Lindernia crustacea* against fungal infection.

2. MATERIALS AND METHODS

2.1 Chemical and Reagents

Chemicals and reagents used in the study were analytical grade. Silica Gel-G (Merck, India), Benzene (Merck, India), ethyl acetate (Merck, India), ethanol (Merck, India), ammonium thiocyanate (SRL India), Gallic acid (Sigma-Aldrich, India), Rutin (Sigma-Aldrich, India), potassium ferricyanide (Merck, India), ferric chloride (Merck, India) were used during the experimental protocol.

Nutrient agar medium (Himedia, India), Potato dextrose medium, Sabouraud Dextrose Agar medium (Himedia, India) and Whatman No. 1 filter paper were used during the experimental protocol.



Fig. 1. *Lindernia crustacea* (L) F.Muell

2.2 Instruments

Flash chromatography (Teledyne ISCO Combi Flash Rf 150), DSC (Perkin Elmer, DSC 4000), CHN Analyzer (Perkin Elmer, series II 2400), ultraviolet (UV) spectrophotometer (UV-1800, Shimadzu), infrared spectrophotometer (Alpha-E, Bruker) and ¹H NMR and ¹³C NMR (Bruker Avance II 400 NMR spectrometer) and mass spectrometer (Waters, Q-TOF Micromass, ESI-MS, and mass spectrometer).

2.3 Plant Material

The aerial parts of *Lindernia crustacea* (Linn.) F. Muell (Fig. 1) were collected from the paddy field of Dharapur, Guwahati, Assam in April and May and was authenticated by Dr P.P. Baruah, HOD, Department of Botany, Gauhati University, Guwahati, Assam, as *Lindernia crustacea* (L) F. Muell, family Linderniaceae (Specimen accession number 18063). The collected aerial part of the plant was washed with running water, shade-dried and pulverized to get the coarse powder and stored in airtight container for further use.

2.4 Phytochemical Evaluation

2.4.1 Preparation of extract

The powdered aerial part of *Lindernia crustacea* (L) F. Muell was extracted with petroleum ether by Soxhlet extraction to remove fatty material followed by successive extraction by cold maceration in Benzene, Ethyl acetate and Ethanol respectively. The solvents were evaporated under reduced pressure at 40°C by using rotary evaporator and extracts were collected, stored in desiccators [11].

2.4.2 Preliminary Phytochemical screening

Extracts obtained were subjected to preliminary phytochemical analysis for identification of different secondary metabolites such as alkaloids, carbohydrate, glycosides, saponins, steroids, terpenoids, phenolics, flavonoids and protein present in the plant [12,13].

2.4.3 Quantitative estimation of polyphenolic components

A range of phytochemicals was estimated quantitatively based on the observation of preliminary phytochemical analysis. Therefore, estimation of total flavonoid content (where rutin

used as standard) [14], total phenolics content (whereas gallic acid was used as reference standards) [15] of different extracts were determined incorporates with UV spectrophotometer. Results are calculated and expressed as the mean \pm S.E.M using a statistical linear regression method.

2.4.4 Isolation of flavonoid

Sample of each extracts were chromatographed on TLC [16] and it was found that benzene extract showed presence of flavonoid when elution with Toluene: acetic acid (4:1). For the separation of flavonoid, about 10 gm of benzene extract was further chromatographed in flash chromatography by using same eluent [17,18].

The fractions with same R_f value on TLC were pooled together and evaporated to dryness, which was crystallized to give fine powdered crystals. The structure of the isolated compound has been elucidated by DSC, UV, Mass, FTIR, CHN analysis and NMR spectra [19].

2.5 Fungal Strain

The antifungal potency of benzene extract, ethyl acetate extract and ethanol extract of *Lindernia crustacea* (Linn.) F. Muell and isolated compound of most potent extract were selectively tested against two fungal strains *Candida albicans* (*C. albicans*) and *Trichophyton rubrum* (*T. rubrum*). Culture of fungal strains were maintained on nutrient agar slants at 4°C and sub-cultures were transferred to the broth, 24 hours before testing [20].

2.6 Culture Media

Sabouraud Dextrose Agar medium [21] was used for evaluation of antifungal property.

2.7 Screening for *in vitro* Antifungal Activity

Disc Diffusion Method [22] was used to evaluate the antifungal activity. Filter paper discs (Whatman No. 1, 6 mm) were prepared and sterilized in screw-capped Bijou bottles with dry heat at 160°C for an hour. Then the discs were impregnated with 100 μ L of each of the extracts (10 mg/ mL) to give a final concentration of 1 mg/disc and also discs were impregnated with isolated compound (50 μ g/disc) to confirm the antifungal activity of plant extract. Similarly, discs were impregnated with positive and negative

controls. The discs were left to dry under the laminar flow cabinet overnight. For negative control, 10 % v/v DMSO was used. Fluconazole (25 µg/disc) was used as a positive control to confirm that all the microorganisms tested were inhibited by the standard antifungal agents. Before the discs were positioned on the agar surface, the fungal inoculum was spread evenly onto the surface of the Sabouraud Dextrose Agar plates using a sterile cotton bud. Each disc must be pressed down to ensure complete contact with the agar surface. All the plates were incubated for 24 hr at 37° C. The antifungal activity was interpreted from the size of the diameter of zone of inhibition measured to the nearest millimetre (mm) as observed from the clear zones surrounding the discs. The experiments were done with three replicates of each test and developing inhibition zones were compared with positive control.

2.8 Determination of Minimum Inhibitory Concentration (MIC)

Tube dilution method was used to obtain the MIC of plant derived antifungal agents against selected pathogens [23]. A series of dilutions were prepared to contain the same volume of medium inoculated with the test organism (the inoculums may vary from 10^3 to 10^6 cells/mL). The MIC is the lowest concentration of antifungal agent that do not show any growth of tested pathogens.

In present study extracts to be tested were dissolved in 10% DMSO and then serial two-fold dilutions were made to get six different concentrations of 1000, 500, 250, 125, 62.5 and 31.25 µg/ mL in sterile test tubes [24]. Each culture tube contains 2 mL culture medium, 2mL of plant extracts and 0.1 mL inoculums (individual microorganism). These tubes were incubated at 37° C for 24 hours. Similar dilutions were made for isolated compound. Positive control composed of culture medium and inoculums, the only culture medium is taken as a negative control. The tubes were inspected visually to determine growth indicated by

turbidity, the tubes in which the antimicrobial agent is present in a concentration sufficient to inhibit microbial growth, remain clear. The growth of the organism for each dilution was visually observed and thus the MIC was recorded.

3. RESULTS

3.1 Quantification of Extractive Value of Different Extracts

Percentage yield from the different extracts were presented in table 1. It was observed that benzene extract produces the maximum percentage yield.

3.2 Preliminary Phytochemical Screening

Phytochemical parameters were evaluated and the results were shown in Table 2.

3.3 Quantitative Estimation of Polyphenolic Components

Quantitative estimation of total phenolic and flavonoids were estimated (table 3 and 4) from different extracts and it was found that benzene extract contain maximum phenolic content (178.62 ± 0.08 mg GAE/g DW) and maximum flavonoid content (42.63 ± 0.09 mg Rutin/g DW) among all extracts.

3.4 Isolation and Characterization of Flavonoid

Toluene and Acetic acid (4:1) (Fig 2) were used as solvent in flash chromatographic methods for the isolation of flavonoid from benzene extract. Yellow crystals were obtained and confirmed as flavonoid by Shinoda test. The yield of the isolated compound was found to be 2.12% w/w, melting point 314.32 °C, molecular weight is 301. Various methods of spectral analysis of isolated compound were carried out and it was reported as 2- (3',4'- dihydroxy phenyl) - 3,5,7- trihydroxy chromen - 4-one (Fig 3) [25].

Table 1. Percentage of the yield of *Lindernia crustacea* (L) F. Muell extracts from different solvents

Sl. No.	Solvent used	Percentage of yield (w/w)
1.	Petroleum Ether	6.52%
2.	Benzene	12.43%
3.	Ethyl acetate	10.24 %
4.	Ethanol	6.40 %
5.	Water	0.97%

Table 2. Result of preliminary phytochemical analysis

Constituents	Petroleum Ether Extract	Benzene extract	Ethyl acetate extract	Ethanol extract
Amino acid	-	-	-	+
Carbohydrate	-	-	+	+
Alkaloid	-	-	-	-
Flavonoid	-	+	+	+
Phenolic compound	-	+	+	+
Saponin	-	-	-	-
Tanin	+	+	+	+
Steroids	-	+	-	-

Indications: [+] denotes present; [-] denotes absent

Table 3. Total phenolic content: absorbance of extracts

SI.No	Extract	Absorbance (nm)	Phenolic content (mg GAE/g DW)
1.	Benzene	0.236±0.05	178.62±0.08
2.	Ethyl Acetate	0.121±0.05	90.15±0.07
3.	Ethanol	0.053±0.07	37.84±0.07

Values are mean ± SEM of 3 replicates

Table 4. Total flavonoid content: absorbance of extracts

SI. No	Extract	Absorbance (nm)	Flavonoid content (mg Rutin/g DW)
1.	Benzene	0.215±0.06	42.63 ±0.09
2.	Ethyl Acetate	0.082±0.03	18.86±0.07
3.	Ethanol	0.021±0.07	7.92±0.03

Values are mean ± SEM of 3 replicates

**Fig. 2. TLC plate developed with Toluene : Acetic acid (4:1)**

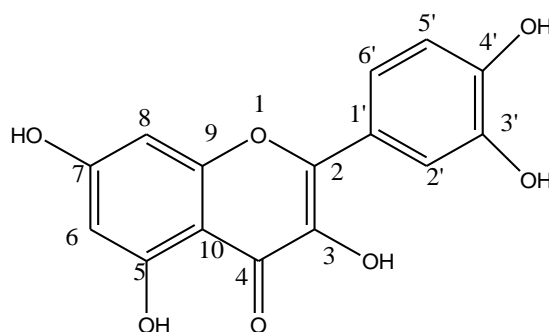


Fig. 3. 2- (3', 4'- dihydroxy phenyl) -3, 5, 7- trihydroxy chromen – 4 - one

Table 5. Antifungal activity (zone of inhibition, mm) of different extract of *Lindernia crustacea*

Fungi	LCBE	LCEE	LCEAE	Isolated compound	Fluconazole
Zone of Inhibition (mm ± SE)					
<i>C. albicans</i>	19.15±0.37	6.79±0.21	9.3±0.19	20.46±0.36	23.01±0.18
<i>T. rubrum</i>	13.16±0.33	0.00±0.00	7.81±0.33	17.53±0.33	22.09±0.22

Diameter of zone of inhibition is a mean of triplicates ± SE (mm), LCBE: *Lindernia crustacea* benzene extract; LCEE: *Lindernia crustacea* ethanol extract; LCEAE: *Lindernia crustacea* ethyl acetate extract.

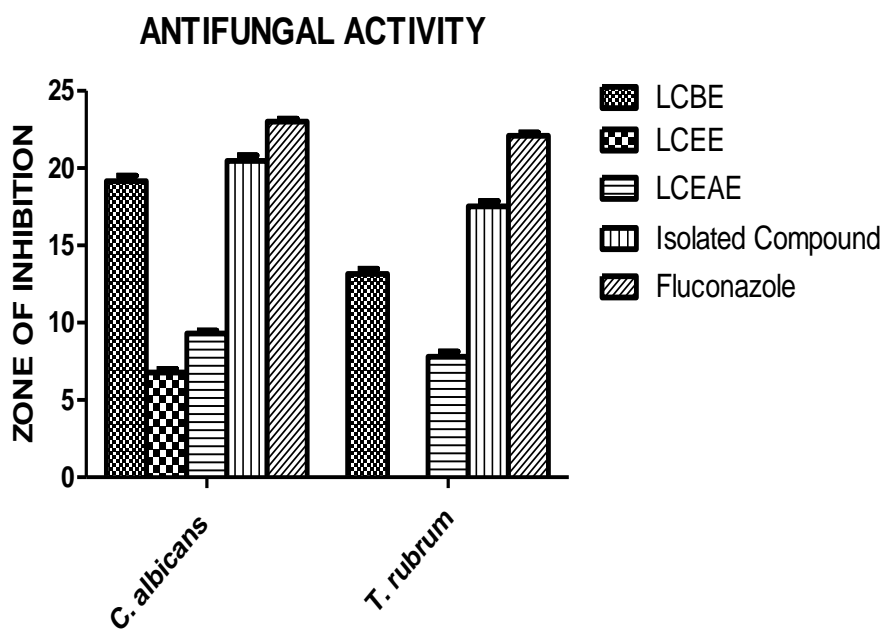


Fig. 4. Antifungal activity of *Lindernia crustacea* (L) F. Muell

Table 6. Antifungal activity (MIC, µg/mL) of different extract and isolated compound of *Lindernia crustacea*

Fungi	Benzene Extract (µg/mL)						Ethanol Extract (µg/mL)						Ethyl Acetate (µg/mL)						Isolated compound (µg/mL)					
	1000	500	250	125	62.5	31.25	1000	500	250	125	62.5	31.25	1000	500	250	125	62.5	31.25	1000	500	250	125	62.5	31.25
<i>C. albicans</i>	-	-	-	-	+	+	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	+
<i>T. rubrum</i>	-	-	-	+	+	+	#	#	#	#	#	#	-	-	+	+	+	+	-	-	-	-	+	+

(+)= Growth; (-) = No growth, (#)= Not determined since there was no activity, All positive control showed (+) and negative control showed (-)

3.5 Screening for *in vitro* Antifungal Activity

The result of the antifungal assay is summarized in Table 5 and Fig 4. Among all three extracts, benzene extract as well as isolated compound were shown significant zones of inhibition against *C. albicans* and *T. rubrum* which indicates strong antifungal activity.

3.6 Minimum Inhibitory Concentration (MIC) for Antifungal Activity

The tubes containing benzene extract of 31.25 and 62.5 µg/mL against *C. albicans* and 31.25, 62.5 and 125 µg/mL against *T. rubrum* exhibited turbidity. Hence, 125 µg/mL and 250 µg/mL were found to be MIC for benzene extract against *C. albicans* and *T. rubrum* respectively. Whereas 62.5 µg/mL and 125 µg/mL were found to be MIC for isolated compound against *C. albicans* and *T. rubrum* respectively (Table 6).

4. DISCUSSION

Preliminary phytochemical screening revealed that benzene extract showed a maximum yield of 12.43% w/w. Phyto-constituent like flavonoid and phenolic compounds were present in the benzene, ethyl acetate and ethanolic extracts. Quantitative analysis reported that flavonoid and phenolic compounds were found to be maximum in benzene extract. The flavonoid may be mainly responsible for the antifungal activities of the plant. This is consistent with several research reports which have shown the relationship between flavonoid and antifungal activities [26,27].

Bioactive compound flavonoid was isolated by using flash chromatography and confirmed by shinoda test [28]. Based on physical properties, chemical test and spectroscopic data the structure of isolated compound was identified as 2 - (3', 4' - dihydroxy phenyl) - 3, 5, 7 - trihydroxy chromen 4 - one. Traditionally *Lindernia crustacea* is used to treat thrush, which is caused by the fungus *C. albicans* [29]. Both benzene extract and isolated compound of *Lindernia crustacea* were shown significant zones of inhibition 19.15 ± 0.37 and 20.46 ± 0.36 respectively against *C. albicans* and 13.16 ± 0.33 and 17.53 ± 0.33 respectively against *T. rubrum* which indicates strong antifungal activity then other two extracts.

5. CONCLUSION

From the experimental results it can be concluded that flavonoid obtained from *Lindernia crustacea* was found to be potent anti fungal agent against two fungal strains *Candida albicans* (*C. albicans*) and *Trichophyton rubrum* (*T. rubrum*). This research work has also established the traditional and ethnomedicinal claims of *Lindernia crustacea*, which provide scope for further investigations.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

We conducted our research after obtaining proper IEC approval.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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