

HARNESSING THE ANTIFUNGAL POTENTIAL OF CHRYSANTHEMUM MORIFOLIUM ON CANDIDA ALBICANS: SOLVENT-SPECIFIC EXTRACTION, PHYTOCHEMICAL COMPOSITION, AND EX VIVO STUDY

Biochemistry

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ABSTRACT

Candidiasis is a notable illness, and the unfolding of drug-resistant *Candida* strains demands the probing of novel antifungal drugs. This study assesses the antifungal efficacy of various *C. morifolium* extracts against *C. albicans*. Methods: The Soxhlet extraction method was employed for *C. morifolium* yellow and purple flower. Additionally, a quantitative and qualitative investigation was carried out, followed by an SEM and Confocal imaging study of the antifungal efficacy of the extract variants; SEM imaging of extract-treated cells reveals cavitation, while confocal imaging provides evidence for cell deterioration comparable to fluconazole-treated cultures. This study proves that current study research done using methanolic extract shows significant impact on *Candida* species compared to other solvent extracts. Keywords: *Chrysanthemum morifolium*, *Candida albicans*, candidiasis, phytochemicals, scanning electron microscopy, confocal microscopy

KEYWORDS

1. INTRODUCTION

Candida species are alarming fungal pathogenic agents occurring in the mucosal lining of the digestive tract and vagina. Contrary to bacterial strains occurring in certain parts of the digestive tract, *Candida albicans* can colonize the whole digestive tract from mouth to anus [1]. *Candida albicans* cause two types of clinically significant infections (**Figure 1**) such as mucosal and systemic candidiasis. Mucosal candidiasis primarily affects the vaginal area, oral cavity, esophagus, and nails, whereas systemic candidiasis affects the bloodstream, central nervous system, liver, spleen, heart, and kidneys (with or without bloodstream infections) [2].

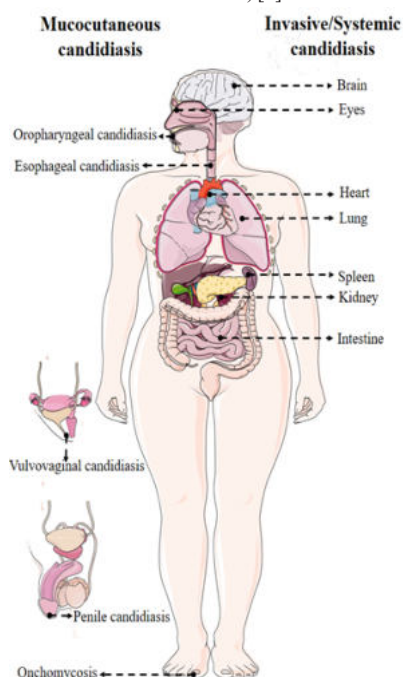


Figure 1. Types of Candidiasis: Organs Susceptible of Infection by *Candida* species and *Candida albicans*.

Candidemia infections spread swiftly among critical patients in Intensive Care Units and other medical settings, raising public concern [3],[4]. Major *Candida* spp (*C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*), which cause invasive candidemia and chronic organ infections, have developed resistance to antifungal drugs such as azoles and echinocandins, thus raising a challenging situation in treating invasive candidemia and candidiasis. [5], [6]

Various plant extracts have shown promising results in multiple in vitro and in vivo studies for their phytochemical composition and potent anticandidal activity. Aqueous extract from *Morinda citrifolia*, ethanolic extract from *Vitis vinifera*, Methanolic extract from *Zingiber officinale* var. *rubrum* and *Curcuma longa* proven to show efficiency in inhibiting *C. albicans* [7]. *C. morifolium* also known as Ju Hua is a floral plant with a major application in medicine and Chinese cuisine for about 3000 years. Present study, investigates the antifungal efficacy of various purple and yellow flower extracts of *C. morifolium* against *C. albicans* [8].

2. MATERIALS AND METHODS

2.1 Collection Of Plant Material

Five kilograms of *C. morifolium* purple (CMP) and *C. morifolium* yellow (CMY) flowers were procured from Krishna Raja market, Mysuru (India). Individually, flowers were sorted, and petals were segregated and collected. Fresh, clean petals from CMP and CMY were washed multiple times with distilled water to remove dust and dirt and air dried at room temperature for further processing.

2.2 Preparation Of CMP And CMY Extract

The *Chrysanthemum morifolium* yellow (CMY) and purple (CMP) flower petals were dried in a controlled environment at 28°C for 15 days. The dried petals were then pulverized into a fine powder, which was subjected to Soxhlet extraction [9]. Yellow and Purple petals (*C. morifolium*) powdered(500g), were subjected to Soxhlet extraction using methanol, water, and diethyl ether as three solvent variants. Each yellow and purple petal yielded three distinct extracts of each, such as yellow methanolic extract (CMYM), yellow aqueous extract (CMYA),

yellow diethyl ether extract (CMYD), purple methanolic extract (CMPM), purple aqueous extract (CMPA), purple diethyl ether extract (CMPD). The extracts are filtered while still warm and then concentrated using a hot oven. The powder was preserved in cold storage for future experiments. The remaining extract residues were then reconstituted in their original solvents for further studies [10].

2.3 Qualitative Analysis

Six different extracts of *Chrysanthemum morifolium* (CMYM, CMYA, CMYD, CMPM, CMPA, and CMPD) were analyzed for their phytochemical content (flavonoids, glycosides, alkaloids, saponins, tannins, and phenols).

2.3.1 Alkaline Reagent Test (Flavonoids)

To assess the presence of Flavonoids, Five drops of dilute NaOH are added to 2ml aliquots of each extract, which instigates the chemical reaction, yielding a yellow color solution. The addition of dilute HCl to the yellow color solution results in a colorless solution. Thus, the test would confirm the presence of flavonoids [11].

2.3.2 Frothing Test (Saponins)

Two ml of each extracted sample is added to 10 ml of distilled water and shaken vigorously for 5 minutes. The formation of steady foam on the surface of the solution shows the presence of saponins in the test extracts [12].

2.3.3 Ferric Chloride Test (Tannins)

A small aliquot of each extract was diluted with distilled water and boiled until the formation of filtrate. To this test solution, 2-3 drops of FeCl₃ solution are added and observed for the appearance of blackish-green color development, which confirms the presence of tannins [13].

2.3.4 Keller- Kilani Test (Glycosides)

A 1:1 ratio of 2ml mixture of glacial acetic acid with each extract is combined. Once the mixture cools down, 2-3 drops of FeCl₃ are added, followed by a slow addition of concentrated H₂SO₄ along the walls of test tubes. A reddish-brown ring formation between two liquid layers shows the presence of glycosides in the extracts [14].

2.3.5 Salkowski Test (Terpenoids)

A few drops of chloroform are slowly added to each extract without mixing both layers, to which 2ml of concentrated sulfuric acid is added along the sides of the test tubes and observed for a reddish brown ring between two layers, which confirms the presence of terpenoids [15].

2.3.6 Test For Phenols

One ml of each extract and one ml of potassium ferricyanide were mixed with three drops of ferric chloride solution, resulting in the subsequent formation of a greenish-blue complex, which shows the presence of phenolic compounds in the extracts [16].

2.4 Quantitative Analysis

2.4.1 Total Phenolic Content Estimation

A 2mg/ml final concentration of Gallic acid was prepared. Varying concentrations (0, 20, 40, 60, 80, and 120µg/70µl per well) of Gallic acid and each CM extract (CMYM, CMYA, CMYD, CMPM, CMPA, and CMPD) were pipetted into each well. Following the addition of 70µl of FC reagent (50%) and 60µl of sodium carbonate (4%). Distilled water was used as a control. Plates were kept away from light for one and a half hours at room temperature for incubation, and at 765nm, absorbance was recorded. The total phenolic content of each extract was estimated by plotting a standard graph for Gallic acid, and the results were expressed as Gallic acid equivalents (GAE) per milligram of CM extracts.

2.4.2 Total Flavonoid Content Estimation

A 2 mL aliquot of 2% AlCl₃ solution was prepared using distilled water. Quercetin and CM extracts (CMYM, CMYA, CMYD, CMPM, CMPA, and CMPD) were prepared to achieve a concentration of 2mg/ml in 98% methanol and distilled water, respectively. Varying concentrations (0, 20, 40, 60, 80, and 120µg/60µl) of Quercetin and extracts were pipetted into wells in triplicate, following the addition of 2% AlCl₃ solution, while distilled water was used as a control. Plates were kept for an hour incubation without light at room temperature, and at 420 nm, absorbance was recorded. The total flavonoid content was represented as quercetin equivalents (QE) per milligram of plant extracts using a standard curve of quercetin [17].

2.5 Antifungal Activity

2.5.1 Agar Well Diffusion Method

Microbial culture *C. albicans* (ATCC 90028) was procured from the American Type Culture Collection (ATCC 10231), Bengaluru. The microorganism was maintained at 4°C on nutrient agar slants. Sabouraud dextrose agar (SDA) medium was pre-inoculated with a 1% *Candida albicans* inoculum and then poured into sterile petri dishes, allowing it to solidify. Five wells with a diameter of 4 mm were constructed using a sterile cork borer in the solidified agar plates, and 10, 30, and 50 µg/100µl of each test sample (CMYM, CMYA, CMYD, CMPM, CMPA, and CMPD) were pipetted into each well. Fluconazole (1mg/ml) was used as a positive control, while distilled water was a negative control. Later, plates were transferred to an incubator (30°C) for 48 to 72 hours. Plates were then subjected to find the zone of inhibition's diameter (in millimeters) [18].

$$\% \text{ Anticandidal Activity} = \left(\frac{A - T}{A} \right) \times 100$$

A = Zone of Inhibition of antibiotic

T = Zone of Inhibition of test sample

2.5.2 Determination Of MIC (Minimum inhibitory concentration)

MIC of extracts (CMYM, CMYA, CMYD, CMPM, CMPA, and CMPD) against *Candida albicans* was performed using the broth dilution assay in triplicates. *C. albicans* culture (1 X 10⁶ spores/ml) was suspended in PDB broth. To estimate the MIC, 100µl of PDB broth was poured into 8 wells of a 96-well plate. In the first well, 100µl of the extract was added. A two-fold dilution was then made to obtain various concentrations in each well. Then, 100µl of the *C. albicans* suspension was added to each well except for the sample control. The microplate was then incubated at 30°C for 2 days. The sample concentration in the well without visible growth (turbidity) was considered the MIC [19].

2.5.3 Determination of MFC (Minimum fungicidal concentration)

After 3 days of incubation, 100µL of culture mixture of varying dilutions from MIC microtiter plates were sub-cultured on SDA (Sabouraud dextrose agar) plates. These sub cultured plates were incubated for 2 days at 30°C until the appearance of visible growth. The minimum fungicidal concentration was the lowest sample concentration that resulted in either no colonies or fewer than three colonies, corresponding to 99 - 99.5% potent fungicidal activity [20].

2.5.4 Determination of IC50 concentration

IC50 is defined as the concentration of the sample required for inhibiting 50% of the fungal strain.

2.5.5 Protease Activity

Each extract was dissolved in 0.5ml 50mM Tris HCl buffer (pH 7.2). 0.5ml of each extract (10, 30, and 50mg/ml) of varying concentration was taken, and 0.5ml of 2% casein in 50mM citrate phosphate buffer (pH 6.8) was added and incubated for an hour at 37°C. The reaction was stopped by the addition of 1 ml 10% Trichloroacetic acid. The undigested casein was separated using centrifugation (8,000rpm/5min). 0.5ml of supernatant was taken, 2.5ml of 2.9% Sodium carbonate and 50% 0.75ml of Folin-Ciocalteu's reagent was added. Test samples were then incubated for 20mn at 37°C and absorbance was read at 650nm using a spectrophotometer. The amount of enzyme that releases 1 µg of tyrosine equivalent per minute was defined as 1U (unit) of protease activity [21].

2.5.6 Lipase Inhibitory Activity

As described by Winkler and Stuckmann et al lipase activity was conducted in triplicates using pancreatic lipase in test tubes and the volume was made up to 400µL using phosphate buffer (0.1M, pH 8.0). 50µl of test samples (CMYM, CMYA, CMYD, CMPM, CMPA, and CMPD) were taken in 10mg, 30mg, and 50mg/ml concentration and to each 50µl of lipase solution was added and incubated for 30min at 37°C. A reaction mixture of 50µl buffer and 50µl lipase served as a control. To all the tubes 400µl of buffer was added followed by 1500µl of p-nitrophenyl acetate and incubated for 30min at 37°C. Absorbance was recorded at 405nm. The same volume of the sample was made as a sample control and added to water to reach the reaction volume. The amount that releases 1nmol/min of free phenol from the substrate (p-nitrophenyl acetate) in 0.1M sodium phosphate buffer (pH=8.0) at 37°C is the definition of one unit of lipase activity [22].

Using the following equation, the percentage of inhibition was determined based on the values of enzyme activity (EA):

$$\% \text{ Lipase Inhibitory Activity} = \left(\frac{E_0 - E_1}{E_0} \right) \times 100$$

E_0 = Enzyme activity without inhibitor

E_1 = Enzyme activity with inhibitor

3. RESULTS

3.1 Extraction Yield And Qualitative Analysis

Table 1. *C. morifolium* flower extraction yield and phytochemical screening

Sample	Solvents	Extracts	Yield	Flavonoid	Phenols	Terpenoids	Glycosides	Tannins	Saponins
Chrysanthemum morifolium yellow flower petal	Methanol	CMYM	35.7 g	+	+	+	+	+	-
	Distilled water	CMYA	44.4 g	+	+	+	+	-	+
	Diethyl ether	CMYD	47.5 g	+	+	+	+	-	+
Chrysanthemum morifolium purple flower petal	Methanol	CMPM	38.2 g	+	+	+	+	+	-
	Distilled water	CMPA	25.4 g	+	+	+	+	-	+
	Diethyl ether	CMPD	27.5 g	+	+	+	+	-	+

(+) indicates the presence of constituents and (-) indicates the absence of constituents

Chrysanthemum morifolium yellow and purple flower (300g) extraction yield (Table 1) shows significant variation between the solvents employed. CMYA and CMYD exhibited higher yields of 44.4 g and 47.5 g, respectively, but CMYM had the lowest yield of 35.7 g. As per the yield results obtained for the purple flower, CMPM had the highest yield of 38.2 g, while the lowest yield was recorded for CMPA (25.4 g) and CMPD (27.5 g). Screening for phytochemical occurrence in these extracts showed varied results. CMYM and CMPM showed positive for flavonoids, tannins, terpenoids, glycosides and phenols, except for saponins. But extracts CMYA, CMYD, CMPA, and CMPD showed positive for all five compounds except for tannins.

Quantitative Analysis

3.1.1 Total Flavonoid Content And Total Phenolic Content

Table 2: Quantification of total flavonoid and phenolic content in different extracts of *C. morifolium* extracts

Extracts	Total flavonoid content (mg QEs/mg extract)	Total phenolic content (mg GAEs/mg extract)
CMYM	56.64 ± 0.58	22.36 ± 0.18
CMYA	33.87 ± 0.36	22.85 ± 0.41
CMYD	41.89 ± 0.27	17.20 ± 0.50
CMPM	59.32 ± 0.38	25.99 ± 0.48
CMPA	27.12 ± 0.26	17.14 ± 0.36
CMPD	40.72 ± 0.43	20.42 ± 0.19

CMPM and CMYM extracts revealed significantly higher flavonoid content of 56.64 ± 0.58 and 59.32 ± 0.38 mg QEs/mg extract, respectively and phenolic content of 25.99 ± 0.48 and 22.36 ± 0.18 mg GAEs/mg extract. This contributes to the fact that certain compound extraction in larger volumes can only be achieved with particular solvents towards which the compounds tend to have more affinity.

3.2 Antifungal Activity

3.2.1 Agar Well Diffusion Assay

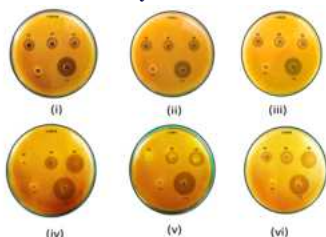


Figure 2: Agar Well Diffusion Assay: Assessment of antifungal activity of CM Extracts (CMYM, CMYA, CMYD, CMPM, CMPA, CMPD) against *C. albicans*

Table 3: *C. morifolium* Extracts: Concentration-Dependent Inhibition of Pancreatic Lipase, Proteolytic Activity and Antifungal Activity against *C. albicans*

Extract	Concentration (mg)	Proteolytic Activity (U)	Inhibition of Lipase (%)	Zone of Inhibition (diameter in mm) (F=20)
CMYM	10	1.33 ± 0.02	27.163	10
	30	4.67 ± 0.35	32.211	10
	50	7.18 ± 0.28	41.526	10
CMYA	10	0.44 ± 0.57	24.999	12
	30	1.38 ± 0.38	33.653	12
	50	2.77 ± 0.19	39.482	12
CMYD	10	1.42 ± 0.31	25.780	10
	30	2.50 ± 0.63	31.730	12
	50	4.35 ± 0.53	37.259	12
CMPM	10	7.72 ± 0.37	52.176	10
	30	10.11 ± 0.49	68.575	12
	50	11.09 ± 0.30	76.446	14
CMPA	10	9.81 ± 0.25	54.621	-
	30	10.11 ± 0.41	59.451	8
	50	10.44 ± 0.23	63.685	12
CMPD	10	8.27 ± 0.39	56.231	-
	30	9.70 ± 0.21	64.281	8
	50	10.55 ± 0.58	68.098	12

Agar well diffusion study revealed that CMPM revealed a 14 mm zone of inhibition while fluconazole retained a 20mm zone (Figure 3), adding further evidence to the potential of CMPM's proteolytic and lipase inhibition, evidencing the regulation of *C. albicans* growth.

Though the inhibition of CMPM is slightly below fluconazole, it underscores the CMPM ability to be used as complementary therapy against *C. albicans* infections [23].

3.3.2 MIC/MFC/IC50

Table 4: MIC, MFC, and IC50 values for against *C. albicans* isolates

<i>C. morifolium</i> extracts	MIC (mg)	MFC (mg)	IC50 (mg)	R ²
CMYM	8.45±0.15	10	1.86±0.38	0.987
CMYA	25.53±0.42	>30	5.18±0.28	0.993
CMYD	17.45±0.50	30	2.99±0.42	0.992
CMPM	28.76±0.36	10	5.20±0.61	0.980
CMPA	27.40±0.23	>30	6.93±0.18	0.972
CMPD	32.48±0.19	>30	6.48±0.36	0.984

*MIC = Minimum Inhibitory Concentration; MFC = Minimum Fungicidal Concentration; IC50 = Half minimum inhibitory concentration.

In MIC evaluation, it was evident that CMYM with MIC of 8.45±0.15 mg, MFC of 10 mg, and IC50 of 1.86±0.38 mg values was proven more efficient, in contrast with other extracts (Table 4).

CMPM, which exhibited exceptional potential in previous studies, had an MIC of 28.76±0.36, an MFC of 10 mg, and an IC50 of 5.20±0.61, which is comparatively better than CMPA and CMPD but lower than CMYM extract efficiency (Figure 4).

The distinct potential of each extract with varied study designs therefore expands the need for evaluation of the efficiency of specific extracts concerning various experimental grounds.[24],

3.3.3 Lipase Inhibition Assay

Table 5: IC50 Lipase Inhibition Profiling of *C. morifolium* Extracts

<i>C. morifolium</i> Extracts	IC ₅₀ (mg)
CMYM	52.804 ± 0.38
CMYA	56.954 ± 0.23
CMYD	54.118 ± 0.42
CMPM	27.337 ± 0.61
CMPA	29.762 ± 0.18
CMPD	31.746 ± 0.36

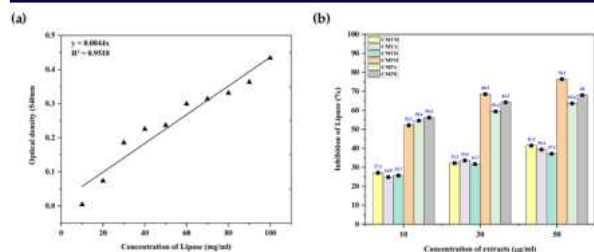


Figure 5: (a) Lipase Activity Standard Curve and (b) Inhibition of Lipase by *C. morifolium* extract: An In-Vitro Assay

3.3.4 Protease Activity

The proteolytic efficiency of each extract was investigated using various concentrations (10, 30, 50 mg) of extracts (Table 3). CMPD extract of purple flower exhibited exceptional proteolytic activity (10.55 U/50 mg) (Figure 6). Following lipase inhibition properties, CMPM and CMPA also showed substantial proteolytic properties, which may significantly affect lipase and proteins of *C. albicans* compared to the other three extracts (CMPD, CMYA, and CMYD). Bioactive components from *C. morifolium* extracts can act as drugs targeting pathways of lipids and proteins in the *C. albicans* lifecycle.

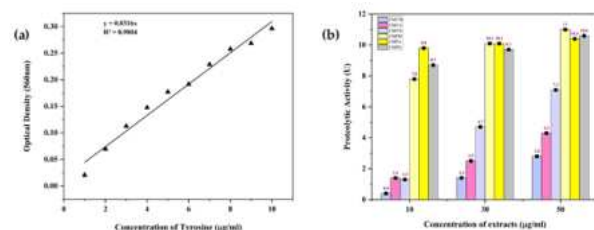


Figure 6: (a) Tyrosine Calibration Curve and (b) Proteolytic Potency of *C. morifolium* extract: An In-Vitro Assay

4. DISCUSSION

Various solvents are used for the extraction of phytochemicals and bioactive components from plant sources. Nevertheless, methanol is the most commonly used solvent. Similarly, in a previous study, the methanolic extraction of rebaudioside A from *Stevia rebaudiana* resulted in a higher yield of glycosides. Apart from the selection of solvent, the specific solvent-to-water ratio also significantly impacts extraction efficiency [25]. It can be correlated with our results, wherein the methanolic extracts of the yellow (CMYM) and purple flower (CMPM) indicated higher flavonoid and phenolic content, which may contributed to their antifungal properties. Followed by diethyl ether (CMYD and CMPD) and aqueous extracts (CMYA and CMPA).

This study presents the antifungal efficacy of *C. morifolium* extracts, which is comparable with the standard drug Fluconazole. The study was focused on In vitro antifungal investigations of various extracts of *C. morifolium*. Further phytochemical profiling using HR-LCMS, NMR, isolation, and characterization of bioactive molecules can give rise to a novel therapeutic drug. Additional studies including combination therapy, in vivo studies, and inhibition mechanism studies including a broad spectrum of pathogens might help develop superior-quality drugs derived from plant sources.

5. CONCLUSIONS

The present study focused on the investigation of the potential of various *C. morifolium* extracts on *C. albicans*. CMYM and CMPM showed noteworthy antifungal activity in lipase inhibition, SEM, Confocal, and Agar well diffusion assays comparable with standard fluconazole. Exceptional inhibitory properties were showcased by methanolic extracts of *C. morifolium* purple flowers. These properties of methanolic extracts in diverse assay conditions warrant further investigation into their phytochemical composition and potential therapeutic applications.

Funding: This research was funded by JSS Academy of Higher Education and Research under the seed money provided to R.R.A (JSSAHER/REG/RES/URG/54/2011-12/14009 dated 06-03-2023) and with JSSAHER PhD Fellowship to G.R.P.(JSSAHER/REG/RES/JSSURF/29(2)/2020-21 dated 22.11.2023)

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

Data Availability Statement: All the data generated and analyzed are included in this research article

Acknowledgments: Part of the work was done following the Memorandum of Understanding between JSS Academy of Higher Education & Research (Mysuru, Karnataka, India) and I.M. Sechenov First Moscow State Medical University of the Ministry of Health of the Russian Federation (Moscow, Russia). The authors thank both organizations for their support towards the MoU activities. The authors thank the DST PURSE-funded confocal imaging facility at the University Sophisticated Instrumentation Center, JSS AHER [SR/PURSE/2021/81 (c)].

Conflicts of Interest: The authors declare no conflicts of interest.

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