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Antifungal, anti-yeast, antioxidant and HPLC analysis of different solvent extracted samples from *Calamus aromaticus* leaves

Bilal Muhammad Khan and Jehan Bakht

Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan.

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Abstract

The antifungal, anti-yeast and antioxidant effects of *Calamus aromaticus* leaves were studied. The extract showed mild activity against two fungal strains and strong activity against the yeast. The results also indicated the presence of extremely high DPPH radical scavenging potential in all tested samples at each of the seven concentrations used. The data further revealed that ethyl acetate and *n*-butanol extracted samples measured highest antifungal (25.0% inhibition each against *Rhizopus oryzae* at 1000 µg/well), anti-yeast (73.5 and 66.4% activity respectively at 2000 µg/disc) and antioxidant (96.6 and 97.7% activity respectively at 250 ppm) potential while samples extracted with water were least potent. HPLC analysis of the extracted samples confirmed ethyl acetate and *n*-butanol as the solvents of choice for the extraction of antifungal, anti-yeast and antioxidant compounds (phenolics) from the leaves of *C. aromaticus*.

Introduction

Calamus aromaticus, generally known as sweet flag, is a member of the family *Araceae* (*Adoraceae*). It is a perennial herb commonly found on the banks of streams and in damp marshy places. Several important biological activities such as antifungal (Lee et al., 2004; Lee, 2007), antibacterial (McGraw et al., 2002; Phongpaichit et al., 2005), anticellular and immunosuppressive (Mehrotra and Mishra, 2003) have been attributed to the rhizomes, roots and essential oil extracted from this plant.

High performance liquid chromatography (HPLC) is frequently used for the quantification of polyphenols from plant extracts due to its accuracy, reliability and repeatability. The same technique was employed in this experiment for the quantification of gallic acid in various solvent extracted samples from the leaves of *C. aromaticus*.

The efforts devoted to the study of antifungal, anti-yeast and antioxidant properties of *C. aromaticus,* however, are no match for the enormous potential of the biological activities of its leaves. Additionally, extraction on the basis of ascending polarity of the solvents was used for the first time in this study. Designing such an experiment, hence, was utmost important in revealing the true potential of the leaves of this enormously promising medicinal plant.

Materials and Methods

Plant material

The present study was conducted at the Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan. Fresh plant material was collected from different localities of District Swat, Khyber Pakhtunkhwa, Pakistan. After thorough washing with running tap water, the leaves were chopped, shade dried and grounded in electric grinder.

Crude extract preparation

One thousand grams of powdered leaves were soaked in five liters of analytical grade methanol, kept in dark

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at 25ºC for one week and agitated three times a day. The mixture was filtered through Whatman filter paper No. 1. The solid residue was soaked in 2.5 L fresh methanol and the whole process was repeated thrice. A rotary evaporator (Stuart, RE 300, Bibby Sterilin Ltd., UK) was used to dry the filtered methanolic solution at 45ºC under vacuum pressure. The semisolid extract so obtained (crude methanol extract) was kept in dark for drying in a glass vial at room temperature. The crude extract was divided into two portions, one to be used as crude extract and the other part was fractionated with different solvents.

Fractionation of crude extract

Fifty grams of the crude extract was dissolved in 500 mL sterile distilled water and was then poured into a separatory funnel. Then 300 mL *n*-hexane was added to it, the funnel was shaken gently and the solutions were allowed to settle for 15-30 min to separate the two phases. The upper *n*-hexane phase was collected in a flask and the lower aqueous phase was re-extracted three times with fresh *n*-hexane. All fractions of *n*-hexane were pooled together and were dried at 45°C under vacuum pressure in the rotary evaporator. The same procedure of fractionation was carried out for ethyl acetate and *n*-butanol. The lower aqueous phase at the end of the process was dried as described previously.

Culture media

All media used in the study were procured from Oxoid Ltd., Basingstoke, Hampshire, England. Potato dextrose agar (CM0139) was used for culturing the fungal strains and the yeast tested in the study, while potato dextrose broth (CM0001) was used for shaking incubation and standardization of the tested organisms.

The required quantities of potato dextrose agar (39 g/L) and potato dextrose broth (13 g/L) were prepared in conical flasks. Some of the potato dextrose broth (approx. 20 mL/test tube) was also poured into test tubes. All the media flasks and test tubes were capped with aluminum foil and autoclaved at 1.5 pounds pressure and 121ºC for 20 min.

After sterilization, potato dextrose agar medium was poured aseptically into sterile disposable petri plates (90 mm; Kartel, Italy) in a laminar flow hood. A sterile environment was maintained during pouring to avoid contamination. The medium was allowed to solidify in petri plates for about an hour before the petri plates were placed in an inverted position (to avoid evaporation of water from the medium within the plates) in an incubator at 37ºC for 24 hours. After 24 hours, uncontaminated plates were used for culturing of the test organisms. The potato dextrose broth in flasks (approx. 20 mL/flask) was used for shaking incubation of microorganisms while potato dextrose broth in test tubes was used for standardization of cultures.

Organisms used for testing the antifungal and anti-yeast activities

Candida albicans (ATCC 10231), *Trichoderma reesei* (ATCC 26921), *Acremonium alternatum* (ATCC 60645), *Aspergillus niger* (ATCC 6275), *Rhizopus oryzae* (ATCC 20344) and *Penicillium chrysogenum* (ATCC 11709) were collected from the Department of Plant Pathology, The University of Agriculture, Peshawar, Pakistan.

Antifungal assay

The method described by Ramdas et al. (1998) was used to study the antifungal potential of the different extracts. Pure and fresh cultures were obtained by sub-culturing the different strains of fungi used in the study. An OD of 0.1 at 600 nm marked standardization of fungal spores in potato dextrose broth. Potato dextrose agar plates were inoculated with 50 µL of standardized fungal inoculums. The inoculum was spread on the surface of agar plates with the help of a sterilized spreader for uniform growth. These plates were then kept at 37°C for 5-7 days for fungal growth and then transferred to 4°C to be used as fungal source in the activities.

Autoclaved potato dextrose agar was poured in sterile disposable petri plates. Upon setting, three wells were made on the surface of agar plates using a sterilized cork-borer (8 mm in diameter) in a triangular manner. Each well represented a distinct concentration of plant extract (500, 1000 and 2000 µg). The plates were left for 20 to 30 min at room temperature for proper diffusion of extracts. Fungal discs (8 mm in diameter) were then kept in inverted position on top of each well. The plates were afterwards incubated at 37°C for five days. Furthermore, fungal discs over wells devoid of plant extract were used as negative control and fluconazole (100 µg/well) was used as positive control in the experiment.

Growth in each extract was compared to fungal growth in the negative control to obtain percent growth inhibition by the following formula (Grover and Moore, 1962; Rusman, 2006).

% Growth inhibition = {(Diameter of fungal colony in control – Diameter of fungal colony in treatment) / Diameter of fungal colony in control} x 100

Anti-yeast assay

Stock cultures of the yeast *C. albicans* were freshened by streaking using a sterile inoculation loop on potato dextrose agar plates in a laminar flow hood followed by incubation at 37ºC for 24 hours. Then, these were again sub-cultured. The second streaked cultures were inoculated into potato dextrose broth in flasks and

subjected to shaking incubation (200 rpm) for 18 hours at 37ºC. Potato dextrose agar plates were inoculated with 50 µL of 18 hours cultures of microbial inoculums (a standardized inoculum 1-2 × 10^7 CFU/mL 0.5 McFarland Standard). The inoculum was spread on the surface of agar plates with the help of a sterilized spreader for uniform colony growth. The inoculated plates were then kept for 20 to 30 min in laminar flow hood.

Disc diffusion assay described by Bauer et al. (1966) was employed for the activity of different extracts against the yeast *C. albicans*. Three discs of Whatman No. 1 filter paper (6 mm in diameter; autoclaved) were placed in triangular manner with the help of a sterile forceps on the media in petri plates. Each disc represented a distinct concentration of plant extract (500, 1000 and 2000 µg). Fluconazole (100 µg) was used as positive control and DMSO (6 µL/disc) as negative control in the study. Inoculated plates were then incubated at 37ºC for 18-24 hours. The diameter zone of inhibition was recorded in mm and percent activity was determined using the following formula.

% Activity = (Inhibition zone of sample / Inhibition zone of control) x 100

Antioxidant assay

The method elaborated by Mensor et al. (2001) was used for the determination of DPPH radical scavenging potential of the plant extracts. All solutions used in the study were prepared using 98.0% analytical grade methanol. 0.3 mM DPPH solution in methanol was prepared which was stored at 4ºC for further use in the study. Gallic acid was used as a standard in radical scavenging analysis of our extracts which was used in a concentration of 500 ppm. Sample stock solutions, in a concentration of 1000 ppm, were diluted to required concentrations of 250, 125, 50, 25, 10 and 5 ppm with methanol. To 2.5 mL solution of the standard and each extract, 1 mL of 0.3 mM DPPH solution was added. The reaction mixtures were shaken and incubated in dark at ambient temperature for 30 min. A UV-visible spectrophotometer (Pharmaspec UV-1700, Shimadzu) was used to measure absorbance of the standard (500 ppm gallic acid), control (0.3 mM DPPH solution) and each reaction mixture (extract + 0.3 mM DPPH solution) at 517 nm. The percent DPPH radical scavenging potency of the extracts was determined using the following relation reported by Elayaraja et al. (2010).

DPPH Scavenged (%) = $[(A_{control} - A_{test}) / A_{control}] \times 100$

In the above equation, '$A_{control}$' means the absorbance shown by control (0.3 mM DPPH solution) and 'A_{test}' indicates the absorbance recorded for the reaction mixture (extract + 0.3 mM DPPH solution).

Quantitative analysis through HPLC

Quantification of the phenolic compound, gallic acid, was done using high performance liquid chromatography (HPLC) technique.

Shimadzu Liquid Chromatograph (LC-6AD) equipped with Shimadzu Prominence diode array detector (SPD-M20A), Shimadzu dualistic pumps (LC-6AD) and Shimadzu communication bus module (CBM-20A) was used during the study. For chromatographic analysis a diamonsil C_{18} column (4.6 mm x 250 mm; 2.5 µm particle size) was used.

Acetonitrile and water (both HPLC grade; procured from Sigma-Aldrich) were the solvents used in standard and sample preparation. Moreover, the mobile phase of HPLC consisted of HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B). The solvents were vacuum-filtered using a nylon filter with pore size of 0.45 µm, and were sonicated to remove air bubbles prior to use.

For the preparation of standard and sample solutions a combination of HPLC grade acetonitrile and HPLC grade water in a ratio of 1:1 (v/v) was used as solvent. The standard gallic acid was prepared in different concentrations (10, 20, 40, 80 and 160 ppm) from the stock solution of 500 ppm. Similarly, each sample was prepared in concentration of 500 ppm which was filtered using a 0.45 µm membrane filter, millipore. Furthermore, the standard and samples were centrifuged at 5,000 rpm for 10 min prior to injection.

Purging was carried out to remove air bubbles from the system and the system was given blank runs, two to three times, to wash the column before injection of either the standard or sample. The standard and samples were injected in a volume of 20 µL each and the temperature of the column was maintained at 25ºC. The HPLC program was run so that the cumulative flow rate of both pumps was 1 mL/min, UV detection wavelength was set at 254 nm, and the complete run time of the system was 25 min.

Statistical analysis

The experiments were repeated thrice and the values were reported as mean ± standard deviation which was calculated using Microsoft Excel 2010.

Results

Antifungal activity

Out of the five fungal strains used in the present study, the extracted samples showed activity only against *R. oryzae* and *A. alternatum*. The crude methanol extract and its fractions in different solvents extracted from the leaves of *C. aromaticus* were tested against *R. oryzae* (Table I). The results revealed that all tested samples were active against this fungus at each of the three concentrations used in the present investigation. The

data suggested that maximum growth inhibition (37.5%) was observed for hexane extracted fraction at 2000 µg/well while minimum growth inhibition at the same concentration was noted for crude methanol extract (16.6%). At 1000 µg/well, however, ethyl acetate and *n*-butanol extracted samples measured maximum growth inhibition of 25.0% each. Ethyl acetate and butanol extracted fractions measured similar degree of growth inhibition (22.9%, 25.0% and 27.0% at 500, 1000 and 2000 µg/well respectively). Moreover, at 500 µg/well, the activity measured by crude methanol extract and by its fractions in hexane and water was the same (16.6%). Both ethyl acetate and butanol extracted samples with 22.9 and 25.0% growth inhibition at 500 and 1000 µg/well respectively were more active than *n*-hexane extracted sample at these two concentrations. *n*-Hexane extracted fraction, however, with 37.5% inhibition left behind ethyl acetate and *n*-butanol extracted samples with 27.0% inhibition at 2000 µg/well.

The different solvent extracted samples from the leaves of *C. aromaticus* were also tested against *A. alternatum* (Table II). The data indicated that *n*-hexane extracted fraction was the sole among the tested samples to which this fungus was completely resistant at all the three concentrations used. *n*-Butanol and aqueous extracted samples, on the other hand, were inactive against the tested organism at 500 µg/well each. The tested fungus was found more sensitive to crude methanol extract which measured 21.8, 45.4 and 52.7% inhibition at 500, 1000 and 2000 µg/well respectively. It was followed by butanol and water extracted samples which inhibited growth of the same fungus to the extent of 34.5% at 2000 µg/well each. The activity shown by *n*-butanol and aqueous extracted fractions against the tested organism was similar with both measuring 23.6 and 34.5% inhibition at 1000 and 2000 µg/well respectively. The data also revealed that ethyl acetate extracted fraction was the least effective in controlling the growth of this fungus with 12.7, 18.1 and 29.0% inhibition at 500, 1000 and 2000 µg/well respectively.

Anti-yeast activity

The data from the anti-yeast activity of different solvent extracted samples from the leaves of *C. aromaticus* against *C. albicans* is given in tabulated form in Table III. The data revealed that all tested samples were active

Table I

Different solvent extracted samples from the leaves against *Rhizopus oryzae*

Plant extract	Conc. of extract (µg)	Z1 Replicates (mm)	Z2 Replicates (mm)	Z3 Replicates (mm)	Mean ± S.D	Negative control (mm)	Growth inhibition (%)
Crude (methanol)	500	20.0	18.5	21.5	20.0 ± 1.5	24.0	16.6
	1000	20.0	20.0	20.0	20.0 ± 0.0		16.6
	2000	18.0	19.0	23.0	20.0 ± 2.6		16.6
n-Hexane	500	20.0	17.5	22.5	20.0 ± 2.5		16.6
	1000	18.0	20.0	17.5	18.5 ± 1.3		22.9
	2000	15.0	13.0	17.0	15.0 ± 2.0		37.5
Ethyl acetate	500	18.5	17.0	20.0	18.5 ± 1.5		22.9
	1000	18.0	16.5	19.5	18.0 ± 1.5		25.0
	2000	17.5	19.0	16.0	17.5 ± 1.5		27.0
n-Butanol	500	18.0	18.5	19.0	18.5 ± 0.5		22.9
	1000	18.0	17.3	18.7	18.0 ± 0.7		25.0
	2000	17.2	17.8	17.5	17.5 ± 0.3		27.0
Aqueous	500	20.0	20.0	20.0	20.0 ± 0.0		16.6
	1000	18.0	22.0	20.0	20.0 ± 2.0		16.6
	2000	19.0	23.0	15.0	19.0 ± 4.0		20.8

S.D means Standard Deviation from the mean

against the yeast at each of the three concentrations used, except water extracted fraction which showed no activity at 500 µg/disc. Crude methanol extract which measured 53.5%, 73.5% and 93.3% activity at 500, 1000 and 2000 µg/disc respectively was the most active extract against the tested organism. It was followed by hexane extracted sample with 53.3%, 66.5% and 80.2% activity at 500, 1000 and 2000 µg/disc respectively. The data also suggested that ethyl acetate and butanol extracted fractions were with 73.5% and 66.4% activity respectively at 2000 µg/disc. The least effective among the tested samples turned out to be water extracted fraction which measured 43.5 and 60.0% activity at 1000 and 2000 µg/disc respectively.

Antioxidant activity

The data in Figure 1 suggested that all extracted samples from the leaves showed DPPH radical scavenging potential at each of the seven concentrations used in the present study. Ethyl acetate extracted fraction measured the best antioxidant activity of 75.4, 76.2, 77.2, 80.6, 87.2, 90.3 and 96.6% at 5, 10, 25, 50, 100, 125 and 250 ppm respectively. It was followed by *n*-butanol fraction, crude methanol extract and water extracted sample with 97.7, 96.6 and 95.6% activity respectively at 250 ppm. The least active of the tested samples in scavenging free radicals turned out to be *n*-hexane extracted sample which measured 75.6, 76.5, 77.5, 78.0, 80.6, 82.5 and 87.7% activity at 5, 10, 25, 50, 100, 125 and 250 ppm respectively.

HPLC analysis

The standard curve and regression equation were obtained by noting the peak areas of the various concentrations of gallic acid with respect to retention time and plotting these against the concentrations used. The following equation was obtained which was employed in calculating the concentration of gallic acid (in ppm) in the different solvent extracted samples from the leaves.

$$y = 46598 x + 62967; R^2 = 0.996$$

Where 'y' is the peak area of the sample, and 'x' denotes the concentration of gallic acid in that particular sample

Moreover, the concentrations (in ppm) so obtained were converted to mg/g (quantity of gallic acid in mg per g of dry extract) using standard mathematical

Table II

Different solvent extracted samples from the leaves against *Acremonium alternatum*

Plant extract	Conc. of extract (µg)	Z1 Replicates (mm)	Z2 Replicates (mm)	Z3 Replicates (mm)	Mean ± S.D	Negative control (mm)	Growth inhibition (%)
Crude (Methanol)	500	21.5	20.0	23.0	21.5 ± 1.5		21.8
	1000	15.0	13.2	16.8	15.0 ± 1.8		45.4
	2000	11.3	13.0	14.7	13.0 ± 1.7		52.7
n-Hexane	500	-	-	-	-		-
	1000	-	-	-	-		-
	2000	-	-	-	-		-
Ethyl acetate	500	24.0	22.0	26.0	24.0 ± 2.0		12.7
	1000	22.5	20.5	24.5	22.5 ± 2.0	27.5	18.1
	2000	21.0	19.5	18.0	19.5 ± 1.5		29.0
n-Butanol	500	-	-	-	-		-
	1000	21.0	19.0	23.0	21.0 ± 2.0		23.6
	2000	16.3	18.0	19.7	18.0 ± 1.7		34.5
Aqueous	500	-	-	-	-		-
	1000	21.0	20.2	21.8	21.0 ± 0.8		23.6
	2000	15.8	18.0	20.2	18.0 ± 2.2		34.5

S.D means Standard Deviation from the mean; '--' indicates absence of activity

Table III

Different solvent extracted samples from the leaves against *Candida albicans*

Plant extract	Conc. of extract (µg)	ZI Mean ± S.D (mm)	ZI Fluconazole (mm)	% ZI	ZI DMSO (mm)
Crude (methanol)	500	12.0 ± 1.3		42.8	-
	1000	17.0 ± 1.3		60.7	-
	2000	23.1 ± 1.5		82.7	-
n-Hexane	500	6.5 ± 1.3		23.3	-
	1000	8.1 ± 1.4		28.9	-
	2000	10.0 ± 1.3		35.7	-
Ethyl acetate	500	8.0 ± 1.3	15.0	28.5	-
	1000	10.1 ± 1.4		36.0	-
	2000	12.0 ± 1.3		42.8	-
n-Butanol	500	0.0 ± 0.0		0.0	-
	1000	8.0 ± 1.5		28.7	-
	2000	11.0 ± 1.3		39.2	-
Aqueous	500	13.5 ± 1.4		48.4	-
	1000	15.6 ± 1.4		55.9	-
	2000	18.1 ± 1.4		64.6	-

S.D means Standard Deviation from the mean; '--' indicates absence of activity

operations. The mean retention time ± Standard deviation of gallic acid was calculated to be 15.3 ± 0.2 min. Furthermore, the gallic acid peaks in samples were identified according to retention time and confirmed by comparing the UV spectra of sample peaks with that of gallic acid.

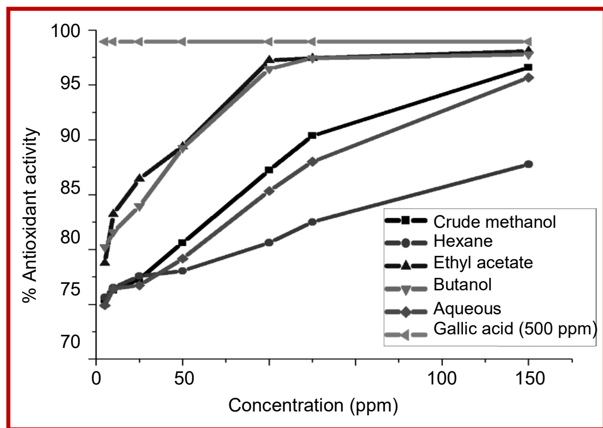

Figure 1: Anti-oxidant potential of crude methanol, hexane, ethyl acetate, butanol and water extracted samples from leaves of *Calamus aromaticus* in DPPH radical scavenging assay

The highest gallic acid content was calculated for ethyl acetate extracted sample from the leaves with total gallic acid content of 10.8 mg/g dry extract (Figure 2). It was followed by *n*-butanol extracted sample which measured gallic acid content of 8.1 mg/g dry extract. Aqueous fraction showed the least quantity of the phenolic compound (5.3 mg/g dry extract).

Discussion

The different solvent extracted samples from the leaves of *C. aromaticus* were tested against five filamentous fungi and one clinically important yeast, *C. albicans*. The extracted samples, however, showed some activity against only two of the five filamentous fungal strains (*R. oryzae* and *A. alternatum*) and the yeast studied in our experiment.

R. oryzae, one of the filamentous fungi studied in this experiment, is the utmost significant and illustrative cause of mucormycosis. Vascular invasion with hyphae, infarction and necrosis of tissue are pathological characteristics of mucormycosis, while rhinocerebral and craniofacial mucormycosis represent the most

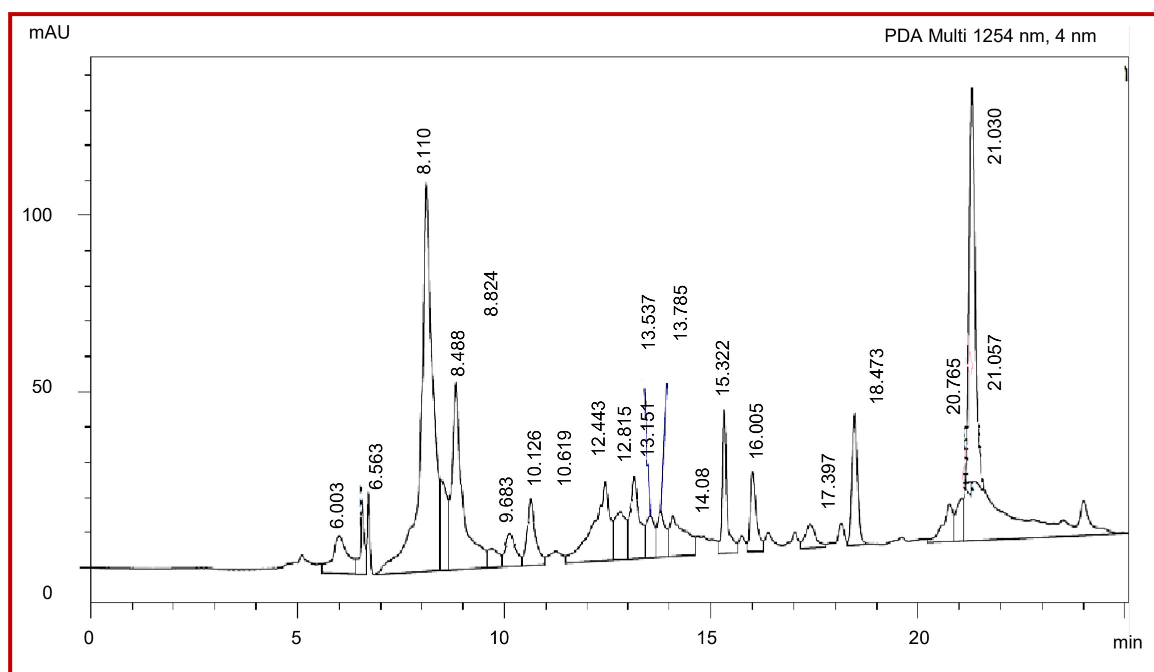

Figure 2: HPLC chromatogram of ethyl acetate extracted sample from leaves

usual form of the numerous clinical exhibitions caused by *Rhizopus* species. If untreated, this usually results in death within the first four weeks of onset (Mucorales Database, Broad Institute). All samples extracted from the leaves showed some inhibitory activity at each of the three concentrations used in the present study. With regards to the order of activity, hexane extracted fraction from the leaves showed comparatively high activity against *R. oryzae*. It was followed by ethyl acetate and *n*-butanol extracted samples, with similar degree of inhibition at all concentrations, and then aqueous fraction which in turn was followed by crude methanol extract.

Human infections due to *A. alternatum*, one of the two filamentous fungi which were found susceptible in our study, are uncommon but usually occur in patients with already suppressed immune system. The most frequent contagion caused by this fungus is *Mycetoma*, which commonly progresses following trauma, while eye infections, diseases caused by colonization of the gastrointestinal tract and lung, and maladies like sinusitis, osteomyelitis, arthritis, and peritonitis may also be caused by this organism (Fincher et al., 1991). All samples extracted from the leaves were active against this fungus except *n*-hexane extracted sample which showed no activity at any of the three concentrations used. The different solvent extracted samples from the leaves according to the descending order of activity were crude methanol extract followed by *n*-butanol and aqueous fractions, both of which showed at par activities at all concentrations used. Ethyl acetate extracted sample followed suit while hexane extracted fraction from this plant part was completely inactive against *A. alternatum*. The polarity of the compounds being extracted with different solvents is a good measure for the rationalization of these results (Manikandan et al., 2010).

To the best of our knowledge, no extract from any plant part of *C. aromaticus* has been studied against either *R. oryzae* or *A. alternatum* in any of the previous studies. However, Mungkornasawakul et al. (2002), Phongpaichit et al. (2005), Devi and Ganjewala (2009), Singh et al. (2011), and Kumar et al. (2014) have reported the presence of antifungal activity in extracts from different parts of this plant.

C. albicans, the yeast studied in this experiment, is a key fungal human pathogen triggering a multiplicity of contagions (Kim and Sudbery, 2011). Intrusive fungal contagions instigated by this yeast have augmented considerably (Cheng et al., 2005). As a source of nosocomial bloodstream contagions in the United States, they currently rank 4th (Jarvis, 1995) and the most usual one at one major infirmary in Taiwan (Chen et al., 1997). In addition to a death rate of thirty to forty percent linked with candidemia, it prolongs the stays in hospitals (Leleu et al., 2002; and Wey et al., 1988) and escalates the expense of treatment (Tortorano et al., 2004). Moreover, most of the existing medications have undesirable characteristics, e.g., amphotericin B, due to its toxicity, produces unwanted side effects, azoles demonstrate drug-drug interactions, and fluconazole and 5-flucytosine bring about resistance-development (White et al., 1998). Hence, the development of a safe plant-based medication for this adamant pathogenic yeast was felt imperative.

Providentially, each sample extracted from the leaves showed inhibitory activity against this yeast, with some revealing extremely promising results. Among the different solvent extracted samples from the leaves, crude methanol extract revealed strikingly high inhibitory activity which in fact almost reached the level of activity displayed by the control (fluconazole) when used in the maximum concentration. It was followed by *n*-hexane, ethyl acetate, *n*-butanol and water extracted samples, all exhibiting surprisingly high inhibitory activities. Similar results are reported by Phongpaichit et al., 2005; Singh et al., 2011; and Somnuk et al., 2014. Devi and Ganjewala (2009), however, reported slightly better activity of ethyl acetate extracted sample from the rhizome against *C. albicans* than that of the same extract from the leaves. The results of our study are also in contradiction with those of Kumar et al., (2014) where better activity of extracts from *C. aromaticus* rhizome against *Aspergillus niger* is reported in comparison to that against *C. albicans*, while samples extracted from the leaves which were tested in our experiment were completely inactive against the former and revealed profound activities against the later.

Samples extracted from the leaves exhibited extremely high antioxidant potency when compared with control in DPPH radical scavenging assay. Such high antioxidant potential of crude methanol extract from the leaves and rhizome of this plant is also reported by Devi and Ganjewala (2011). Shukla et al., (2012) have also reported mild antioxidant activity in *C. aromaticus* essential oil, and Elayaraja et al., (2010) in ethanolic and hydroalcoholic extracts from the roots and rhizome of this plant. Manju et al., (2013), on the contrary, reported extremely lower scavenging potential for aqueous and methanol extracts from *C. aromaticus* rhizome. These researchers, though, employed an extraction technique that was quite different from the one used in our experiment. Furthermore, the radical scavenging potency of samples extracted from all parts increased with increasing concentration.

The different chemical characteristics and polarities of extracting solvents also affect the antioxidant potential of the extracted samples. Acetone, methanol, propanol and ethyl acetate are among the frequently used solvents for the extraction of antioxidants (Mahattanatawee et al., 2006; Alothman et al., 2009). The antioxidant potential of the extracted sample is significantly affected by the solubility of antioxidants in solvent, and the development of a standard solvent which can dissolve all types of antioxidants is unmanageable. Preliminary screening, hence, is of utmost importance in the identification of a suitable solvent which can impart optimal radical scavenging potential to the extracted samples.

In our experiment, we used five different solvents (methanol, *n*-hexane, ethyl acetate, *n*-butanol and water) for determining the scavenging potential of the leaves of *C. aromaticus*. Among different solvent extracted samples from the leaves, ethyl acetate was the one which revealed higher antioxidant potential followed closely by butanol. This in turn was followed by methanol, water and then hexane extracted sample. In a study conducted by Subathraa and Poonguzhali (2012), aqueous extract from *C. aromaticus* rhizome, though extracted in a different manner than the one used here, showed nearly similar antioxidant potential to the aqueous extract from the leaves used in this study. Hence, it is safe to conclude that ethyl acetate is the most suitable solvent for extracting antioxidants from the leaves of this plant.

Found in free state in certain foliage, gallic acid carry the scientific name of 3,4,5-trihydroxybenzoic acid (Condrat et al., 2011). It is a representative member of phenolic acids (Singh et al., 2011) and is reportedly a potent anti-inflammatory (Kroes et al., 1992), antibacterial (Ravn et al., 1989) and antioxidant agent (Gramza et al., 2005; Karamać et al., 2005; Rice-Evans et al., 1996; Brand-Williams et al., 1995). Phenolic compounds are reported to be helpful in the cure of cancer, neurodegenerative and cardiovascular ailments, and also find use in the cosmetic industry in the form of anti-aging products (Gupta et al., 2012). These compounds signify the most deliberated phytochemicals which have been extensively studied as model systems in diverse research areas (Boudet, 2007).

HPLC is frequently used for the quantification of polyphenols from plant extracts due to its accuracy, reliability and repeatability. The same technique was employed in this experiment for the quantification of gallic acid in various solvent extracted samples from the leaves of *C. aromaticus*. Both the chemical composition of plant sample and the polarity of the solvent affect the solubility and retrieval of phenolics. Selection of a suitable solvent, hence, significantly affects the rate and aggregate quantity of extraction. Additionally, other factors like extraction time and temperature also affect the retrieval of phenolics which imitates the conflicting action of solubilization and oxidation induced analyte degradation (Robards, 2003). Development of an efficient extraction procedure which ensures the stability of extracted phenolic compounds, therefore, is also of utmost importance. In our study, we also observed the effect of different solvents on gallic acid extraction.

Among different samples extracted from the leaves, the highest gallic acid content was calculated for ethyl acetate fraction followed by *n*-butanol extracted sample which in turn was followed by aqueous fraction. On the basis of our results, we can conclude that ethyl acetate and butanol are more suitable for the extraction of phenolic compounds, in particular gallic acid, from the

leaves of *C. aromaticus*. Water, on the other hand, cannot be regarded as a solvent of choice for the extraction of gallic acid. Karamać et al., (2005), Singh et al., (2010), Condrat et al., (2011), Deshmukh and Prabhu (2011), Singh et al., (2011), Gupta et al., (2012), and Sharma and Singla (2013) also used organic solvents for the extraction and quantification of gallic acid from certain parts of different plants.

Conclusion

Both ethyl acetate and *n*-butanol extracted samples were found to exhibit strong antifungal, anti-yeast and antioxidant potential, and the water extracted sample trailed behind in the accomplishment of this goal.

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Conflict of Interest

Authors declare no conflict of interest

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Author Info
Bilal Muhammad Khan (Principal contact)
e-mail: ranezai@yahoo.com