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Antifungal Activity of *Curcuma longa* L. (Zingiberaceae) Against Degrading Filamentous Fungi

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Curcuma (Curcuma *longa* L.) belongs to the family Zingiberaceae and is a popular spice with a strong taste and remarkable yellowish color which has been increasingly interesting considering its possible to replace synthetic preservatives for containing compounds, such as curcumin, with high antifungal and antimicrobial activities. The goal of this paper was to assess the effect of the aqueous extract from the rhizome powder of *Curcuma longa* L. at different concentrations inhibiting the development of fungi isolated in bread. We analyzed the minimum inhibitory concentration (MIC), percentage of fungal biomass inhibition, and mycelium diameter of *Penicillium panemun, Penicillium citrinum, Cladosporium oxysporum, Cladosporium subliforme,* and *Aspergillus chevalieri*. *C. oxysporum* and *C. subliforme* showed lower values for CIM, with 3.12 and 6.25 mg/ml of extract for growth inhibition, while the remaining strains were inhibited at 25 mg/ml. *A. chevalieri* was the most resistant strain to curcuma extract and suffered no interferences during its mycelium and biomass development processes. *Cladosporium subliforme* proved higher sensibility to the extract at 5%, with 85.3% and 98.5% of inhibition of mycelial and fungal biomass growth, respectively, followed by *P. panemum*, P. *citrinum*, and *C. oxysporum* with significant sensitivity to curcuma aqueous extract. Our study reveals that in addition to being used as an ingredient to provide food with color and flavor, the antimicrobial properties of curcuma can be studied as an alternative to the use of synthetic antifungal substances and benefit from expanding the shelf life of foods.

1. Introduction

Saffron (***Cu**rcuma longa* L.), also known as curcuma, is a species native to Southeast Asia and belonging to the family Zingiberaceae. It is largely cultivated in Asian countries and not restricted only to its use as food, but also present in many different areas of industry, medicine, and agriculture (Vilella & Artur, 2008). The properties of curcuma are related to both the prevention and treatment of diabetes, cancer, and inflammatory processes, as mentioned by Tripathi and Misra (2016), in addition to degenerative processes such as osteoarthritis (Chin et al., 2016), antimicrobial activity against *Staphylococcus aureus* (Teow et al., 2016), *Exserohilum turicicum, Fusarium oxysporum, Colectrotrichum cassiicola. Pseudomonas aeruginosa*, *Bacillus subtilis, Escherichia coli, Candida albicans* (Petnual et al., 2010) and *Aspergillus flavus* (Hu et al., 2015).
The substance responsible by the color of curcuma was first isolated during the 19th and named curcumin. Curcuminoids constitute a group of phenolic compounds present in the curcuma which are chemically related to its main ingredient, curcumin, which along with desmethoxycurcumin and bisdemethoxycurcumin represent the main biomolecules in this chemical group (Jayaprakasha et al., 2005).

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The World Health Organization (WHO) established that in order to be acceptable, the ingestion of curcuminoids as food additive must range 0–3 mg/kg. Curcuminoids and derived-curcuma products were considered safe by the Food and Drug Administration (FDA) of the United States of America (Amalraj et al., 2017).
Biopreservation has been highlighted among the technologies of alternative food preservation to prolong shelf life and improve sanitary quality minimizing the impact on nutrition and sensory properties of perishable products. Biopreservation explores the antimicrobial potential of different natural origins (Garcia et al., 2010) rationally. Because of their antioxidant and antimicrobial potential, essential oils from aromatic plants have been of great interest of the systematic research for the development of biocontrol herbal agents such as safe, economical products which are easy to use (Kumar et al., 2016).
In this scenario, the goal of our paper was to study the antifungal capacity of the *Curcuma Longa* L. aqueous extract through tests *in vitro* using five strains of degrading filamentous fungi

2. Material and Methods

2.1 Culture media and reagents

Microbiological tests were performed used the following culture media: potato dextrose agar (BDA) Kasvi Laboratories, malt extract agar (MEA) Kasvi Laboratories, Sabouraud Dextrose Agar (ASD) by Biomark Laboratories Pune 411041 India. RPMI (Roswell Park Memorial Institute Medium), MOPS (3-(N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid). All reagents ranged analytical grade.

2.2 Aqueous extract collection and curcumin determination

In order to obtain the aqueous extracts, rhizome powder of *Turmeric longa* L. (cultivated in Votuporanga – São Paulo – Brazil) was diluted in sterile distilled water with 0.1% Tween 80 both directly (without thermal treatment rhizome powder) and via sterile (autoclave of the rhizome powder to obtain the aqueous extract).
Curcumin content was determined in the rhizome powder through the adapted method described by Fulekil & Francis (1968). Total curcumin content was expressed in percentage.

2.3 Minimum inhibitory concentration, Mycelial growth, and Fungal biomass

For the microbiological assessments were used strains of *Penicillium panemun, Penicillium citrinum, Cladosporium oxysporum, Cladosporium subliforme*, and *Aspergillus chevalieri* isolated from bread and identified through molecular technique.
Genomic DNA of the fungi was extracted using the extraction kit “Power Soil DNA Isolation kit”(MoBio Laboratories, Inc.) following the manufacturer’s instructions, except for the initial phase, during which the fungi were grown in broth BD (Potato-dextrose) and using about 200 mg of mycelium for the extraction. DNA integrity was verified with agarose gel 1%.
In order to enlarge the region of rRNA ITS1-5.8S-ITS2 we performed a polymerase chain reaction (PCR) by using the primers ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCCCGCTTATTGATATGC-3’) (White et al., 1990) according to the methodology described by Rhoden et al. (2012).
The CPR products were purified using the enzymes shrimp alkaline phosphatase and exonuclease I (Sigma-aldrich). The sequencing was carried out using the sequencing platform ABI-Prism 3500 Genetic Analyzer (Applied Biosystems) and results were analyzed through software BioEdit 7.2.5. Nucleotid sequences were compared with others available on the database of the “National Center for Biotechnology Information” (NCBI) using the nBLAST tool with filtering for “type strains.”

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined according to methodology a proposed by Dalben-Dota et al. (2010) as well as the document by CLSI, regulation M38 A established by the National Committee for Clinical Laboratory Standart – NCCLS (2002) with modifications for natural products suggested by Capoci et al. (2015). 500µL of fungal suspension were added to sterile test tubes standardized at $5x10^4$ UFC/mL in medium RPMI and 500 µL of curcuma aqueous extract with serial microdilution reaching the final concentrations of 100; 50; 25; 12.5; 6.25; 3.12; 1.56; 0.78, and 0.4 mg/ml of extract and a 48-hour incubation at 35 °C.

Mycelial growth and Fungal biomass

According to the methodology by Marques et al. (2004), with modifications, mycelium disks of 5 mm in diameter were removed from each of the fungal species developed in MEA inoculated in the center of petri dishes containing medium BDA and incubated for days at 25 °C. From these colonies we removed plugs of

5mm in diameter inoculated in Petri dishes with the mediums BDA control (without extract addition) and mediums BDA tests containing 1, 2, 5, and 6% of rhizome powder obtained both through direct and sterile via added directly to the culture medium and incubated at 25°C for seven days. The diameter of the colony was measured in millimeters on the seventh day of incubation. The inhibition percentage was obtained through equation Eq (1) by Albuquerque et al. (2006) and modified by Tian et al. (2011). Dc corresponding diameter value of the control group and Dt is the diameter of tests groups.

Dry weight was determined seven days after the incubation of isolates at 25ºC in mediums BDA control and tests, for which the medium with the cultivation was liquefied through autoclave at 100 ºC, and the mycelium removed using a clamp. Subsequently, the mycelium was transferred to filter paper previously weighted and taken to the greenhouse at 70 ºC. After 72 h of drying, the filter papers were weighted daily until reaching the constant weight and obtaining the value for fungal biomass gravimetrically. Each microbiological tests was performed in three replicates. The mean values were used for drawing the tables and significant difference ($p<5\%$) through the t-Student test.

$$\frac{\mathbf{Dc - Dt}}{\mathbf{Dc}} \mathbf{x\ 100} \quad (1)$$

3. Results and Discussion

3.1 Quantification of curcumin

The quantification of curcumin in the rhizome powder presented the values of 2.95% for the powder which had not been subjected to thermal treatment and 2.28% for the sterilized powder, indicating a significant difference in the content of this substance with the material autoclave.

Wakte et al. (2011) conducted research to optimize the extraction of curcumin from dried rhizomes of *Curcuma Longa* using microwave extraction techniques (water and alcohol as solvents), ultrasonic, Soxhlet with acetone and supercritical carbon dioxide assisted techniques, obtaining 90.47% of curcumin recovery with microwave technique, followed by ultrasound, supercritical and soxhlet technique, with rates of 71.42; 69.36 and 2.1%, respectively. Enzyme-assisted ionic liquid extraction of curcumin was also used by Sahne et al. (2017), achieving an increase in curcumin recovery from 3.58% to 5.73% using enzymatic pre-treatment with a mixture of amylase and amyloglucosidase enzymes to destroy the curcuma cell wall before the extraction process.

Raja et al. (2016) reported ar-turmerone (32.73%), curlone (13.36%), beta-cedrene (5.98%), bergamottin (5.39%), and 1-Isobutyl-2.5-dimethyl benzene (4.64%), respectively, as active compounds in the curcuma alcoholic extract. Chen et al. (2018) found 37.19% of curcumin in curcuma alcoholic extracts its main compound. Lim et al. (2011) used curcuma powder to produce bread and reported the percentage of 2.65 mg/g of curcumin in the rhizome powder with the significant content loss (32-54%) during the baking of the bread.

Prathapan et al. (2009) reported that the concentration of curcumin in the saffron powder thermally treated presented no significant difference in none of the treatments at temperatures from 60 ºC to 100 ºC during the periods from 10 to 60 min. Park et al. (2011) added curcuma powder to the formulation of cakes and pasta and verified relative thermal stability thermal during the baking process, and curcumin remained present at high concentrations in the cakes.

According to Cecilio et al. (2000), the chemical composition found in the curcuma rhizome is influenced by several factors, such as cultivation, type of plantation, type of soil, climate, fertilization, water availability, harvest period, among others. Other important factors for the composition variability of the bioactive are the techniques applied to extract substances of interest as well as the solvents used.

3.2 Antifungal Assessment

The minimum inhibitory concentration (MIC) test revealed that the inhibition of the fungal development occurred for all the strains in our study. Increased inhibition level occurred by adding the concentration of the aqueous extract, in which *Cladosporium oxysporum* and *Cladosporium subliforme* were the most sensitive strains, with inhibitory concentrations of 3.12 mg/ml and 6.25 mg/ml, respectively. For the fungi, *Penicillium panemum*, *Penicillium citrinum*, and *Aspergillus chevalieri* the MIC appeared at 25 mg/ml.

Research by Kumar et al. (2016) used curcuma essential oil at 2.45 mg/ml with activity inhibitory on the growth of *Fusarium graminearum*. The authors also found an inhibition of the zearalenone production at 30 mg/ml, a concentration which also showed an increase in the production of fungal biomass.

Aspergillus niger, Botrytis cinerea, Fusarium moniliforme, Glomerella cingulata, and *Phyllosticta* caricae were assessed regarding their development in the presence of different plant extracts obtained using hot water, methanol at 80%, and acetone. Extracts of *Curcuma Longa* presented significant inhibitory action at 6.7 mg/ml of the extract containing acetone, *Phyllosticta* caricae, followed by the inhibition of *Aspergillus niger, Botrytis cinerea, Glomerella cingulata,* and *Phyllosticta* caricae with rhizome methanolic extract. *F.moniliforme* did not suffer any inhibition in the presence of none of the three curcuma extracts (Lee et al., 2007).
The results found when verifying the mycelium diameter and the biomass inhibition are described in Tables 1 and 2, respectively. It is possible to observe that all isolates inoculated in test medium showed different mycelium size in comparison with the isolates from the control medium control. *Cladosporium subliforme* had the highest value of decrease against the remaining fungi, while *Aspergillus chevalieri* presented no significant decline in the mycelial growth.

Table 1: Percentage of mycelial inhibition at the concentrations of 1%, 2%, 5%, and 6% of Curcuma Longa L. aqueous extract.

Fungi	D*	%D1%	%D 2%	%D 5%	%D 6%
Pp	5.6[a]	69.6	82.1	78.5	80.3
Pc	1.4[a]	35.7	57.1	71.4	78.5
Co	2.7[a]	7.40	3.70	25.9	25.9
Cs	4.1[a]	78.0	82.9	85.3	85.3
Ac	1.3[a]	23.0	23.0	23.0	23.0

D: mean diameter of the colony in mm after a seven-day incubation in BDA control %D: mycelium inhibition percentage. Pp: *Penicillium panemun*, Pc: *Penicillium citrinum*, Co: *Cladosporium oxysporum*, Cs: *Cladosporium subliforme*, and Ac: *Aspergillus chevalieri*. a,b Different letters in the same line indicate significant difference ($p<5\%$) through t-Student test.

Tanveer et al. (2015) suggest that the antimicrobial activity associated with phenolic compounds present in plant materials are related to the capacity of interaction between its chemical structure (hydrophobic and hydrophilic parts) and the cell membranes of microorganisms leading to alterations in permeability, reduced cytoplasmic content, and precipitation of proteins, which, in turn, generate cellular damage and development inhibition. According to Shelef (1984), the presence of plant spices (aromatic herbs) affects all the phases of microbial growth: phase lag is prolonged, growth speed during the logarithmic phase is reduced, and the total number of cells is decreased.

Table 2: Percentage of fungal biomass inhibition at the concentrations of 1%, 2%, 5%, and 6% of Curcuma Longa L. aqueous extract.

Fungi	P*	%P1%	%P 2%	%P 5%	%P 6%
Pp	0.42[a]	92.8	95.2	90.4	90.4
Pc	0.21[a]	93.8	88.0	88.5	89.0
Co	0.11[a]	10.9	38.1	50.9	54.5
Cs	0.47[a]	94.4	96.1	98.5	98.7
Ac	0.013[a]	69.9	77.4	77.4	69.9

P: dry biomass weight in grams after a seven-day incubation at BDA control; %P: percentage of biomass inhibition; Pp: *Penicillium panemun*, Pc: *Penicillium citrinum*, Co: *Cladosporium oxysporum*, Cs: *Cladosporium subliforme*, and Ac: *Aspergillus chevalieri*. a,b Different letters in the same line indicate a significant difference ($p<5\%$) through the t-Student test.

Even though neither relevant or specific research has been reported for the strains approached in our study, microorganisms belonging to the same genera were assessed in the presence of *Curcuma longa* L. extract. Chen et al. (2018) assessed the alcoholic extract activity of curcuma against eleven fungal species: *Fusarium graminearum*, *Fusarium chlamydosporum, Alternaria alternate, Fusarium tricinctum, Sclerotinia sclerotiorum, Botrytis cinerea, Fusarium culmorum, Rhizopus oryzae, Cladosporium cladosporioides, Fusarium oxysporum*, and *Colletotrichum higginsianum* and obtained 63.80%; 67.97%; 61.17%; 65.40%; 63.80%; 53.50%; 63.50%; 46.27%; 29.90%; 41.20%, and 34.50% of mycelium inhibition, respectively, at the concentration of 0.5 mg/ml of curcuma ethanolic extract. The authors also assessed the synergetic effects of the curdione compound on the other seven compounds. Separately, curdione had inhibition of 52.9% *Fusarium graminearum* and in association with isocurcumenol and β-elemene inhibited 100% of fungal growth, while the synergism with curcumin resulted in 93.6% of inhibition with the same microorganism.

The strains of *C. subliforme* and *P. panemum* proved more susceptible to biomass inhibition, showing reductions of 98.5% and 95.2%, respectively, at the concentration of 5% of the extract. *P. citrinum* had the highest sensitivity at the minimum extract concentration with 93.8% of inhibition, while *C. oxysporum* had the highest inhibitory effect at 6% of the extract. Lee et al. (2007) reported a sharp decrease in the biomass percentage of *A. niger*; *B. cinerea*, *F. moniliforme*, *G. cingulate*, and *P. caricae* in the presence of 20 mg/ml of acetone extract of *C. longa*.

Ferreira et al. 2013b found reduced mycelium growth and lower viability of spores of *Aspergillus flavus* from the *Curcuma longa* L. essential oil at the concentration of 0.10%. The concentration of 0.5% of *C. longa* essential oil reached 99.9% and 99.6% of inhibition and 0.5% of standard curcumin 96.0% as well as 98.6% of inhibition in the production of aflatoxin B1 and aflatoxin B2, respectively, in *Aspergillus flavus* (Ferreira et al., 2013a).

Relevant data on the antifungal capacity of other plant extracts have been reported by Castro et al. (2017), who showed the action of cinnamon essential oils (*Cinnamomum zeylanicum*), lemongrass (*Cymbopogon flexuosus*), eucalyptus (*Eucalyptus globulus*), clove (*Eugenia caryophyllus*), and rosemary at inhibiting the development of *Alternaria alternata* at the concentrations of 0.1%, 0.1% 0.05%, 0.1%, 0.025%, and 0.025%, respectively. Eldaysty et al. (2014) obtained 70% of mycelium inhibition for *Penicillium* sp and 31% for *Aspergillus niger* in the presence of 10% and 20% of alcoholic extract from pomegranate peel, respectively. The concentration of 30% of extract promoted the total inhibition of the development of both strains. Idris & Nadzir (2017) tested the aqueous, alcoholic, and methanolic extracts of *Centella asiatica* for the inhibition of *Aspergillus niger* and *Bacillus subtilis* and reached the highest inhibition percentage by applying 100% of the ethanolic extract during the disk diffusion analysis, in which *B. subtilis* was the most sensitive microorganism to the test.

4. Conclusions

The raw aqueous extract of *Curcuma longa* L. proved promising results as a potential inhibitor agent of the filamentous strains analysed in this study for indicating significant inhibition of the mycelial growth of *Cladosporium subliforme, Penicillium panemun,* and *Penicillium citrinum*. In addition to decline the fungal biomass development from 10.8 to 98.7%, except for *A. chevalieri*, which proved resistant at all concentrations applied in both tests, being sensible only for CMI containing 25 mg/ml of extract. Further, *in vitro* and *in vivo* studies are crucial to potentialize the bioactive extraction with antifungal action of curcuma as well as establish its stability in the food matrix.

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