A Chitin-binding Protein Purified from *Moringa oleifera* Seeds Presents Anticandidal Activity by Increasing Cell Membrane Permeability and Reactive Oxygen Species Production

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Candida species are opportunistic pathogens that infect immunocompromised and/or immunosuppressed patients, particularly in hospital facilities, that besides representing a significant threat to health increase the risk of mortality. Apart from echinocandins and triazoles, which are well tolerated, most of the antifungal drugs used for candidiasis treatment can cause side effects and lead to the development of resistant strains. A promising alternative to the conventional treatments is the use of plant proteins. *M. oleifera* Lam. is a plant with valuable medicinal properties, including antimicrobial activity. This work aimed to purify a chitin-binding protein from *M. oleifera* seeds and to evaluate its antifungal properties against *Candida* species. The purified protein, named *Mo-CBP*₂, represented about 0.2% of the total seed protein and appeared as a single band on native PAGE. By mass spectrometry, *Mo-CBP*₂ presented 13,309 Da. However, by SDS-PAGE, *Mo-CBP*₂ migrated as a single band with an apparent molecular mass of 23,400 Da. Tricine-SDS-PAGE of *Mo-CBP*₂ under reduced conditions revealed two protein bands with apparent molecular masses of 7,900 and 4,600 Da. Altogether, these results suggest that *Mo-CBP*₂ exists in different oligomeric forms. Moreover, *Mo-CBP*₂ is a basic glycoprotein (pI 10.9) with 4.1% (m/m) sugar and it did not display hemagglutinating and hemolytic activities upon rabbit and human erythrocytes. A comparative analysis of the sequence of triptic peptides from *Mo-CBP*₂ in solution, after LC-ESI-MS/MS, revealed similarity with other *M. oleifera* proteins, as the 2S albumin *Mo-CBP*₃ and flocculating proteins, and 2S albumins from different species. *Mo-CBP*₂ possesses in vitro antifungal activity against *Candida albicans*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis*, with MIC₅₀ and MIC₉₀ values ranging between 9.45–37.90 and 155.84–260.29 µM, respectively. In addition, *Mo-CBP*₂ (18.90 µM) increased the cell membrane permeabilization and reactive oxygen species production in *C. albicans* and promoted degradation of circular plasmid DNA.
INTRODUCTION

Candida species encompass a group of yeast that normally lives on the skin and mucous surfaces of healthy individuals (Mavor et al., 2005). Fortunately, just around 10% of approximately 200 Candida species identified to date are considered pathogenic to man. Most medically important Candida species include *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, which colonize the human body surface (Miceli et al., 2011). Candidiasis is the name given to the infections caused by the yeasts that belong to the genus *Candida* and avoidance of this disease constitutes a big challenge for modern medicine, with a significant impact on morbidity and overall mortality, especially among immunocompromised patients (Gil-Alonso et al., 2016). Incidentally, *C. albicans* is the principal causative agent of candidiasis (Pierce et al., 2015). The indiscriminate use of antibiotics, the incidence of diabetes mellitus type 1 and 2, and diseases or conditions which debilitating the immune system (chemotherapy, seropositive individuals, etc.) are within the factors associated with the increasing risk of developing candidiasis. In addition, patients subjected to surgery associated with the use of probes are also routinely affected by this infection (Schelenz, 2008; Anchak and Fries, 2010; Sadine et al., 2014; Zhang et al., 2014; Alnuaimi et al., 2015).

Antifungal drugs currently available on the market for candidiasis treatment are often effective, but they can lead to the development of resistant strains, which could compromise the treatment efficiency. Drug resistance is related to three mechanisms: (1) decrease of intracellular drug concentration, (2) drug target alterations, and (3) metabolic bypasses (Sanglard, 2016). Furthermore, there are studies showing that these compounds may promote nephrotoxic (Niemirovicz et al., 2016), teratogenic (Dimopoulou et al., 2017), and cardiotoxic effects (Koch et al., 2015). Thus, it is very important to search for new compounds with antifungal activity, allowing the development of drugs and/or alternative treatments (Vandeputte et al., 2012; Salas et al., 2015). In this context, plants emerge as promising sources of bioactive compounds against *Candida* spp., since they have a large variety of primary (especially proteins) and secondary metabolites that are effective in defending plants against herbivores and/or pathogens (Wong et al., 2010). For instance, several plant proteins are known to possess harmful against herbivores and/or pathogens (Wong et al., 2010). Secondary metabolites that are effective in defending plants against phytopathogenic fungi (Wang et al., 2012; Batista et al., 2014; Freitas C. D. et al., 2016), as well as against *Candida* spp. (Bertini et al., 2012; Gomes et al., 2012; Berthelot et al., 2016). Therefore, CBP are promising agents that have the potential to be used as antifungal compounds.

*Moringa oleifera* Lamarck (Moringaceae) is a fast-growing perennial species native to northeastern India that has a small to medium stature and is adapted to a variety of climates (Anwar et al., 2007). This species is well known in the world, particularly in tropical and subtropical regions, for its flocculation, nutritional, and pharmacological properties (Ndabigengesere et al., 1995; Santos et al., 2015; Freitas J. H. et al., 2016). Recently, our research group purified two CBP from *M. oleifera* seeds, eluted from a cation-exchange matrix with 0.5 M and 0.6 M NaCl, respectively, named Mo-CBP2 and Mo-CBP4 (Mo: *M. oleifera*; CBP: "Chitin-Binding Protein"), both of glycoprotein nature (Pereira et al., 2011; Gifoni et al., 2012). *Mo-CBP3* displayed in vitro antifungal activity against *Fusarium solani*, *F. oxysporum*, *Colletotrichum musae*, and *C. gloesporioides*, phytopathogenic fungi to economically and nutritionally important crops (Batista et al., 2014; Freire et al., 2015), whereas *Mo-CBP4* showed anti-inflammatory and antinociceptive activities in rats (Pereira et al., 2011). In our present investigation on *M. oleifera* seed proteins, we found an additional CBP eluted at a lower NaCl concentration (0.4 M) from the cation-exchange matrix, named *Mo-CBP5*, which displayed antifungal activity against *Candida* species. In this study, a purification protocol of *Mo-CBP3* and its biochemical properties are presented. In addition, to evaluate the antifungal mode of action of *Mo-CBP2*, its ability to increase the cell membrane permeability and to induce endogenous production of reactive oxygen species in *C. albicans*, used as a model, were analyzed, as well as its DNase activity. In view of the potential biotechnological applications of *Mo-CBP2* as an antifungal agent, the hemolytic activity on red blood cells (cytotoxicity) was also investigated.

**MATERIALS AND METHODS**

**Biological Material and Chemical Reagents**

*Moringa oleifera* seeds were collected from trees at the Campus do Pici of the Federal University of Ceará – UFC (Ceará, Brazil) under authorization (number: 47766) of the Chico Mendes Institute for Conservation of Biodiversity – ICMBio. A voucher specimen (N EAC34591) was deposited in the Prisco Bezerra Herbarium (UF). The yeasts *C. albicans* (ATCC 10231),
**C. parapsilosis** (ATCC 22019), **C. krusei** (ATCC 6258), and **C. tropicalis** (clinical isolate) were obtained from the culture collection of the Laboratory of Emergent and Reemergent Pathogens – LAPERE, Department of Pathology and Legal Medicine, UFC. Rabbit blood was obtained from animals of colonies maintained at the UFC. Human blood samples (ABO system) were obtained from healthy donors in the blood bank HEMOCE (Hemotherapy Center of Ceará). Experimental protocols were approved by the Ethics Committee of UFC, Brazil (protocol number: 77/2016).

Molecular mass markers, chromatographic matrices, IPG buffer, and immobilized pH gradient gel strips were obtained from GE Healthcare Life Sciences (New York, NY, United States). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, United States).

**Protein Quantification**

Quantification of soluble protein was determined following the method described by Bradford (1976), using bovine serum albumin (BSA) as a protein standard. Absorbance at 280 nm was also used to detect the presence of protein in the chromatographic eluates.

**Purification of a Chitin-binding Protein from M. oleifera Seeds (Mo-CBP2)**

Purification of Mo-CBP2 followed the procedure described by Gifoni et al. (2012), with modifications. Defatted seed flour was extracted with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl (1:10, m/v), for 3 h at 4°C. The resulting suspension was filtered and centrifuged at 15,000 × g for 4 h. Extracted with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl (1:10, m/v), for 3 h at 4°C, Mo-CBP2 (5.0 µg) was loaded on a chitin column (3.5 cm × 20.0 cm) previously equilibrated with the above buffer. The M. oleifera chitin-binding proteins (Mo-CBPs) were eluted with 0.05 M acetic acid, pooled, dialyzed against distilled water at 4°C, and lyophilized. Mo-CBPs (400 mg) were dissolved in 20 mL of 0.05 M sodium acetate buffer, pH 5.2, and applied to a CM-Sepharose™ Fast Flow column, pre-equilibrated with the same buffer. The adsorbed proteins were recovered by stepwise elution with increasing NaCl concentrations. Mo-CBP2 (Mo: M. oleifera; CBP: Chitin-Binding Protein) was dialyzed against distilled water at 4°C and subject to further analysis.

**Characterization of Mo-CBP2**

Polyacrylamide Gel Electrophoresis Analysis

The purity of Mo-CBP2 was observed after polyacrylamide gel electrophoresis (PAGE) in the absence (native-PAGE) and presence of sodium dodecyl sulfate (SDS-PAGE). Native-PAGE was performed according to Reisfeld et al. (1962). Mo-CBP2 (5.0 µg) was loaded on 15% (m/v) polyacrylamide gel (10.0 cm × 8.0 cm) prepared in 1.5 M potassium acetate, pH 4.3. SDS-PAGE was performed as previously described (Laemmli, 1970). Mo-CBP2 samples (5.0 µg) were prepared in the sample buffer, in the presence or absence of 8% (v/v) 2-mercaptoethanol (2-ME) or 0.1 M dithiothreitol (DTT), and boiled at 98°C for 4 h. The samples were loaded on 15% (m/v) polyacrylamide gel prepared in 0.025 M Tris-HCl buffer, pH 8.9, containing 1% (m/v) SDS. The protein bands were detected by staining with 0.025% (m/v) Coomassie Brilliant Blue R-250 in methanol, acetic acid, and water (1.0:3.5:8.0, v/v/v). Molecular mass standards (GE Healthcare) were also loaded on the gel. The electrophoretic profile of reduced Mo-CBP2 was analyzed by tricine-SDS-PAGE (Schägger and von Jagow, 1987). The samples (10.0 µg) were incubated in 0.1 M DTT for 4 h at 98°C and alkylated with 0.2 M iodoacetamide (IAA) for 45 min at room temperature, followed by additional treatment with 4% (v/v) 2-ME for 10 min at 98°C. The samples were loaded and the electrophoretic run was carried out. Proteins were stained as above. Low molecular mass markers (Sigma–Aldrich) were also used.

**Mass Spectrometric Analysis**

Electrospray ionization mass spectrometry (ESI-MS) was performed using a Synapt G1 HDMS mass spectrometer (Waters) which was coupled to a nanoUPLC system. The intact mass of Mo-CBP2 (1 mg/mL in 0.1% [v/v] formic acid) was fractioned by reverse-phase chromatography using a gradient from 3 to 70% (v/v) acetonitrile with 0.1% (v/v) formic acid on HSS T3 C18 column (1.8 µm, 75 µm × 20 mm). Data were collected using MassLynx 4.1 (Waters). The acquired MS data were processed using a maximum-entropy technique (MaxEnt) to obtain a deconvoluted spectrum (Ferrige et al., 1991).

**Isoelectric Point Determination**

Isoelectric focusing (IEF) of Mo-CBP2 was performed according to Görg et al. (2000). Mo-CBP2 (150.0 µg) was solubilized in 250 µL of rehydration buffer, incubated with an immobilized pH gradient gel strip (11 cm, pH 6.0–11.0), and left standing for 12 h at room temperature. IEF was performed on a Multiphor II Electrophoresis system (GE Healthcare) using a stepwise protocol: 200 V/60 min, 500 V/90 min, 5,000 V/2.5 h, and 10,000 V/5.5 h. After IEF, the gel strip was brought in contact with the equilibration buffer containing 2.5% (m/v) DTT, for 15 min, followed by incubation with 7.5% (m/v) IAA, for 15 min, and submitted to SDS-PAGE (15%) on a vertical system (16.0 cm × 18.0 cm, Hoefer SE 600 Ruby, GE Healthcare). Molecular mass markers were run in the same gel. Proteins were stained with colloidal Coomassie (Candiano et al., 2004) and visualized using the Image Master TM 2D Platinum v.7.0 (GE Healthcare).

**Carbohydrate Determination**

The presence of covalently bound carbohydrate in the Mo-CBP2 structure was evaluated by periodic acid-Schiff staining (Zacharius et al. 1969). Briefly, Mo-CBP2 (15.0 µg) electrophoresis was performed as described above, the gel was fixed in 7.5% (v/v) acetic acid for 2 h and immersed in 0.2% (v/v) periodic acid for 45 min at 4°C, followed by staining in the periodic acid-Schiff reagent for 45 min at 4°C. Next, the gel was submerged in a 0.5% (m/v) potassium metabisulfite solution prepared in 0.05 M HCl. Fetalin (30.0 µg) was used as positive control. The neutral sugar content of Mo-CBP2 was estimated.
spectrophotometrically by the phenol-sulfuric acid method with reference to D-glucose (DuBois et al., 1956).

**Amino Acid Sequencing**

The N-terminal sequence analysis was done using an Automated Protein Sequencer (Shimadzu PPSQ-23A) via Edman degradation. The phenylthiohydantoin amino acid derivatives were detected at 269 nm after separation on an RP-HPLC C18 column (4.6 mm × 2.5 mm) under isocratic conditions, according to the supplier's instructions. To obtain internal protein sequences, Mo-CBP2 (50.0 µg) was digested with 1.0 µg trypsin (Promega®) at 37°C for 16 h. The tryptic peptides were fractionated by reverse-phase chromatography using a gradient from 3 to 40% (v/v) acetonitrile with 0.1% (v/v) formic acid on HSS T3 C18 column (1.8 µm, 75 µm × 20 mm). After elution, data-dependent analysis (DDA) of peptides was performed using a Synapt HDMS mass spectrometer (nanoESI-Qq-TOF; Waters), where the three top peaks were submitted to MS/MS. The data were processed using ProteinLynx Global Server v.2.4 software (PLGS) and subjected to database search using the Mascot search engine (Perkins et al., 1999). MS/MS ion searches were performed against the NCBI non-redundant database (last accessed on January 14, 2017) using a significance threshold of \( p < 0.05 \). Searches for similar proteins in public sequence databases were performed using BLASTp (Altschul et al., 1990).

**Evaluation of Hemagglutinating Activity**

Hemagglutinating activity was assessed using untreated or trypsin-treated rabbit and human (ABO system) erythrocytes (Lis and Sharon, 1972). A serial 2-fold dilution (100 µL) of Mo-CBP2 (5.0 mg/mL in 0.15 M NaCl) in a 96-well plate was mixed with 100 µL of a 4% (v/v) erythrocyte suspension in 0.15 M NaCl from 1 h up to 24 h at 37°C (Raja et al., 2011). Hemagglutination was checked in comparison to a blank in which Mo-CBP2 had been omitted. The minimal protein concentration after serial dilution still promoting visible agglutination with naked eyes was taken to calculate the hemagglutinating activity.

**Evaluation of Antifungal Activity and Mode of Action of Mo-CBP2**

**In Vitro Antifungal Activity Test**

The antifungal activity of Mo-CBP2 was tested against the yeasts *C. albicans, C. krusei, C. parapsilosis*, and *C. tropicalis*. The susceptibility of *Candida* spp. to *Mo-CBP2* was evaluated using the broth microdilution method, as described by the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2012), with modifications. Potato dextrose broth (PDB) medium and nystatin (EMS) were used instead of Roswell Park Memorial Institute (RPMI) medium and amphotericin B. Inocula (final concentration of 0.5 – 2.5 × 10³ CFU/mL) were prepared from 1-day-old cultures grown on potato dextrose agar (PDA) at 35°C in PDB medium. *Mo-CBP2, Mo-CBP3* (Gifoni et al., 2012), *Mo-CBP4* (Pereira et al., 2011) (0.1–1,346.46 µM), and nystatin (0.085–1,067.04 µM) were prepared in ultrapure water and sterilized using a 0.22 µm membrane filter. Aliquots (100 µL) of each yeast suspension (0.5–2.5 × 10³ CFU/mL) were incubated in 96-well flat plates with 50 µL of Mo-CBP2 and additional 50 µL of PDB medium (4-fold concentrated). Plates were incubated at 37°C for 24 h and the yeast growth was monitored at 620 nm using an automated microplate reader (Epoch, BioTek). Nystatin and 0.15 M NaCl were used as positive and negative controls, respectively. *Mo-CBP3* and *Mo-CBP4* were used as reference proteins, as they are also CBP from *M. oleifera* seeds. Yeast-free control was also included. The Minimum Inhibitory Concentrations (MIC50 and MIC90) were defined as the lowest protein or nystatin concentration capable of inhibiting 50% and 90% fungal growth, respectively.

**Evaluation of Cell Membrane Integrity of *C. albicans* Cells after Mo-CBP2 Treatment**

Plasma membrane permeabilization was measured by propidium iodide uptake (Regente et al., 2014). *C. albicans* cells (0.5–2.5 × 10³ CFU/mL) were cultured in the presence of 18.9 µM Mo-CBP2 or 11.11 µM nystatin (positive control), whose concentrations corresponding to their respective MIC50 calculated in this study, or 0.15 M NaCl (negative control), for 24 h at 37°C. The *Mo-CBP2* and nystatin-treated cell suspensions (100 µL) were incubated with 0.001 M propidium iodide in 96-well microplates for 30 min at 30°C, under constant and moderate agitation. Next, the cells were observed under a fluorescence microscope (Olympus System BX 60; excitation wavelength, 400–500 nm; emission wavelength, 600–700 nm).

**Evaluation of Reactive Oxygen Species (ROS) Production by *C. albicans* Cells after Mo-CBP2 Treatment**

This was done according to Thordal-Christensen et al. (1997), with modifications (Batista et al., 2014). Aliquots (100 µL) of the cell suspensions (0.5–2.5 × 10³ CFU/mL) previously treated with *Mo-CBP2*, nystatin or NaCl, as described above, were incubated with 100 µL of 3,3′-diaminobenzidine (DAB, 1.0 mg/mL in H2O) for 1 h at 30°C. Next, the cells were observed under a light microscope (Olympus System Microscope BX 60).

**Assessment of DNase Activity of Mo-CBP2**

The effect of *Mo-CBP2* on DNA degradation was assessed as previously described (Tomar et al., 2014a), using the pUC18 plasmid of *E. coli*. The plasmid (500.0 ng) was incubated with *Mo-CBP2* (500.0 ng) in 0.05 M Tris-HCl buffer, pH 7.4, in a total reaction volume of 20 µL, for 1 h at 37°C. Recombinant DNase I (2 units, RNase-free, Roche), BSA (500.0 ng), and 0.05 M Tris-HCl buffer, pH 7.4, all equally incubated with the plasmid, were used as controls. The samples were loaded on 1% (m/v) agarose gel (10.0 cm × 10.0 cm), prepared in the TAE buffer (0.05 M Tris-HCl, 0.02 M sodium acetate, and 0.001 M EDTA), mounted on a horizontal system (MultiSUB Midi10, Cleaver Scientific), and run at 6 V/cm. The DNA bands were visualized under UV light after staining the gel with 0.5 µg/mL ethidium bromide solution for 30 min.
Evaluation of Hemolytic Activity of Mo-CBP2

The hemolytic assay was performed using rabbit and human (ABO system) erythrocytes collected in heparinized tubes (Choi and Lee, 2014). Erythrocytes were separated from plasma by centrifugation (3,000 \times g, 10 min, 25°C) and washed three times with 0.15 M NaCl. An aliquot (100 µL) of a 4% (v/v) suspension was incubated with an equal volume of Mo-CBP2 (1.9–1000 µg/mL), both prepared in 0.15 M NaCl, for 1 h at 37°C. After incubation, the mixtures were centrifuged at 3,000 \times g for 10 min at 25°C and aliquots of the supernatants transferred to Eppendorf tubes. Absorbance readings were taken at 414 nm (spectrophotometer Novaspec II, Pharmacia) to monitor the release of hemoglobin. Triton X-100 (0.1%, v/v) and 0.15 M NaCl were used as positive and negative controls, respectively. The hemolysis index was calculated using the following equation: Hemolysis (%) = ([Aprotein \ - \ ANaCl]/[ATriton \ - \ ANaCl]) \times 100, where A means absorbance at 414 nm.

Statistical Analysis
Data were obtained from three independent experiments, each one done in triplicate. The results are expressed as the mean ± standard deviation (SD). Tukey’s test was used to compare means and the results were considered to be significant at \( p < 0.05 \). GraphPad Prism 5.02 software was used for statistical analysis.

RESULTS

Purification of Mo-CBP2

Mo-CBP2 was purified by a combination of albumin separation from the crude extract and two chromatographic steps. The crude extract obtained from M. oleifera seeds presented 205.41 mg protein/g of defatted seed. Exhaustive dialysis of the crude extract against distilled water produced the albumin fraction that concentrated approximately 55% of the soluble proteins. Chromatography of albumin on a chitin column produced a chitin-binding protein peak (Mo-CBPs) (Supplementary Figure 1A), which represented 23.2% of the seed extract soluble proteins. Mo-CBPs chromatographed on a CM-Sepharose™ Fast Flow column emerged as a through fraction and three adsorbed protein peaks (Supplementary Figure 1B). Mo-CBP2 was recovered after elution of the adsorbed proteins from the column with 0.4 M NaCl included in 0.05 M sodium acetate buffer, pH 5.2, yielding 0.32 mg protein/g of the defatted flour, or 0.2% of the soluble proteins of the seed crude extract (Table 1).

Molecular and Physicochemical Properties of Mo-CBP2

Purity, Molecular Mass, Isoelectric Point, and Carbohydrate Content

A single polypeptide band appeared after native electrophoresis of Mo-CBP2 (Supplementary Figure 2, inset), suggesting that the purification procedure employed yielded a contaminant-free, homogenous protein. ESI-MS of the intact protein revealed a major peak at 13,309 Da (Supplementary Figure 2). However, SDS-PAGE of Mo-CBP2 under non-reducing conditions showed a 23,400 Da protein band (Supplementary Figure 3, lane 1). Mo-CBP2 treated with 8% (v/v) 2-ME or 0.1 M DTT presented the same prominent band of 23,400 Da and additional faint bands (Supplementary Figure 3, lanes 4 and 7). In tricine-SDS-PAGE, Mo-CBP2 treated with 0.1 M DTT, alkylated with 0.2 M IAA followed by treatment with 4% (v/v) 2-ME revealed the generation of two protein bands with apparent molecular masses of 7,900 and 4,600 Da (Supplementary Figure 4, lanes 2, 4, and 6). Two-dimensional electrophoresis analysis of Mo-CBP2 revealed the presence of a protein spot of 22,300 Da apparent molecular mass and isoelectric point (pI) of 10.9 (data not shown). Moreover, Mo-CBP2 was stained by periodic acid-Schiff’s reagent on SDS-PAGE, suggesting it is a glycoprotein (Supplementary Figure 5), confirmed by quantitative analysis that showed 4.1% (m/m) covalently linked neutral carbohydrates to the Mo-CBP2 structure.

Amino Acid Sequence

Edman degradation of Mo-CBP2 did not give any sequence, suggesting that its N-terminal residue was blocked. However, after tryptic hydrolysis of Mo-CBP2 followed by LC-ESI-MS/MS analysis of the resulting fragments, six peptides were identified (Table 2) and their sequences deposited in the Swiss-Prot database (access number: C0HKC5). By aligning the sequences of these fragments against the non-redundant protein sequence database of NCBI, limited to Brassicales for the first three peptides and Viridiplantae for the last three peptides, these Mo-CBP2 sequences were more closely related (75–100% identity) to other proteins purified from M. oleifera, as 2S albumin precursors (Mo-CBP3 isoforms: AHG99684.1; AHG99683.1; AHG99682.1), a flocculating protein (prf | 2111235A), and MO 2.1 (AAB34890.1). Similarities (63–83%) were also found with 2S albumins of other plant species (Capparis masaikai [BAA12204.1, P80351.1], Bertholletia excelsa [ACI70207.1], Ziziphus jujuba [XP_015899022.1]).

Assessment of Hemagglutination Activity of Mo-CBP2

Mo-CBP2 did not agglutinate rabbit and human erythrocytes, even at 5.0 mg/mL. These results did not differ regardless of whether the erythrocytes were trypsin-treated or untreated.

| TABLE 1 | Purification of Mo-CBP2. |
|------------------------------------------|--------------------------|
| Purification steps | Total protein (mg) \(^a\) | Yield (% \(^b\)) |
| Crude extract | 205.41 ± 2.55 | 100 |
| Albumin | 112.45 ± 3.00 | 54.7 |
| Chitin chromatography | 47.57 ± 5.46 | 23.2 |
| CM-Sepharose chromatography (Mo-CBP2) | 0.32 ± 0.02 | 0.2 |

Results are the mean ± standard deviation of six similar runs. \(^a\)Purification steps are described in Materials and Methods. \(^b\)Total amount of protein recovered from 1 g of defatted flour from M. oleifera seeds. Protein recovery at each purification step (crude extract, 100%).
TABLE 2 | Amino acid sequences of tryptic peptides from Mo-CBP2 identified by LC-ESI-MS/MS.

| Peptide sequence | Mass (Da) | Proteins (species) | NCBI accession number | Identity (%) |
|------------------|-----------|--------------------|-----------------------|--------------|
| CPSLR            | 863.3508  | 2S albumin precursor (Moringa oleifera) | AHC99684.1 | 100 |
| QPFDQR           | 789.3526  | 2S albumin precursor (Moringa oleifera) | AHC99684.1 | 100 |
| CCQQLR           | 1229.5258 | 2S albumin precursor (Moringa oleifera) | AHC99684.1 | 100 |
| QGFOQTHQR        | 1071.4878 | 2S albumin precursor (Moringa oleifera) | AHC99684.1 | 100 |
| IPACNLGPMR       | 1311.5858 | 2S albumin precursor (Bertholletia excelsa) | ACI70207.1 | 73 |
| QAVOLTHQGGOVPQOWVR | 2129.0552 | Mabinlin-1 (Capparis masaikai) | PB0351.1 | 63 |

Antifungal Activity and Mode of Action of Mo-CBP2

Mo-CBP2 inhibited the development of C. krusei, C. albicans, C. tropicalis, and C. parapsilosis with MIC50 values varying from 9.45 to 37.90 µM, whereas Mo-CBP3 and Mo-CBP4, also CBP, had MIC50 between 261.67 and 310.06 µM. To cause 90% fungal growth inhibition, the MIC50 for Mo-CBP3, Mo-CBP4, and Mo-CBP5 were in the ranges of 155.84–260.29 µM, 560.32–600.23 µM, and 564.89–598.10 µM, respectively. MIC50 for nystatin varied from 11.11 to 22.23 µM and MIC90 from 55.55 to 133.38 µM (Table 3).

Treatment of C. albicans, used as a representative model, with 11.11 µM nystatin or 18.90 µM Mo-CBP2 for 24 h altered the cell membrane permeability as revealed by propidium iodide uptake (Figure 1).

In addition, ROS overproduction was observed, recognized as internal dark staining, after incubation of C. albicans cells with 11.11 µM nystatin or 18.90 µM Mo-CBP2 (Figure 2). Similar to the recombinant DNase I, Mo-CBP2 (500.0 ng) also promoted DNA degradation of the E. coli plasmid (pUC18), whereas both BSA and 0.05 M Tris-HCl buffer, pH 7.4, were inactive (Figure 3).

Assessment of the Cytotoxicity Effect of Mo-CBP2

Bovine serum albumin and Mo-CBP2 did not exhibit any hemolytic activity on rabbit and human erythrocytes in all concentrations analyzed, contrary to the positive control, Triton X-100, which caused strong hemolysis on the tested erythrocytes (data not shown).

DISCUSSION

The World Health Organization (WHO) has long been concerned with the rational use of antibiotics and the emergence of super-resistant microorganisms. World Health Organization [WHO] (2015) launched the first “World Antibiotics Awareness Week” with the theme “Antibiotics: Handle with care,” from which a list with recommendations to

TABLE 3 | Antifungal activitya of chitin-binding proteins from M. oleifera seeds and nystatin against Candida species.

| Fungal strains | Mo-CBP2 | Mo-CBP3 | Mo-CBP4 | Nystatin |
|----------------|---------|---------|---------|----------|
| C. albicans ATCC 10231, MIC50, µM | 18.90 ± 169.50 | 299.30 ± 600.23 | 290.27 ± 598.10 | 11.11 ± 65.55 |
| C. krusei ATCC 6258, MIC50, µM | 9.45 ± 158.84 | 270.60 ± 560.32 | 261.67 ± 564.89 | 11.11 ± 65.55 |
| C. parapsilosis ATCC 22019, MIC50, µM | 37.90 ± 260.29 | 310.07 ± 570.65 | 309.65 ± 573.16 | 22.23 ± 133.38 |
| C. tropicalis, MIC50, µM | 18.90 ± 180.98 | 303.98 ± 588.26 | 300.12 ± 585.45 | 22.23 ± 133.38 |

aAntifungal activity was evaluated after 24 h incubation at 37°C. b Minimum concentrations that inhibited 50 and 90% of fungal growth, respectively. c Clinically isolated strain. Different capital letters in the row mean significant differences (p < 0.05, Tukey’s test) between data.
prevent antibiotic resistance was produced. In consonance with this concern, several research groups have been searching for new antimicrobial agents to efficiently overcome the development of microbial resistance, devoid of side effects, and also not expensive. It has long been known that plants are rich sources of new compounds with potential for the treatment of various diseases, including infectious diseases. Studies exploring the mechanism of action and structure-activity aspects of such natural compounds are important toward the discovery and development of novel antimicrobial agents (Hayashi et al., 2013). In connection with this global antimicrobial resistance dilemma the current study reported on the purification and characterization of Mo-CBP₂, and evaluated its antifungal effect.

**FIGURE 1** | Fluorescence microscopy of C. albicans cells treated with 0.15 M NaCl (A), 11.11 µM nystatin (B), or 18.90 µM Mo-CBP₂ (C), followed by incubation with 0.001 M propidium iodide. Bars = 200 µm.

**FIGURE 2** | Induction of ROS generation in C. albicans. Light micrography of cells previously treated with 0.15 M NaCl (A), 11.11 µM nystatin (B), or 18.90 µM Mo-CBP₂ for 24 h at 37°C (C), followed by incubation with DAB. The presence of ROS was observed by dark staining (reddish-brown) reaction inside cells. Bars = 10 µm.
Mo-CBP$_2$ analyzed by PAGE under native condition appeared as a single band protein. After tricine-SDS-PAGE under reducing conditions, Mo-CBP$_2$ dissociated in two protein bands with apparent molecular masses of 7,900 and 4,600 Da, values that summed up (12,500 Da) result in a molecular mass near to that determined by mass spectrometry analysis (13,309 Da). However, Mo-CBP$_2$ analyzed by SDS-PAGE under non-reducing conditions migrated as a 23,400 Da protein. These findings suggest the formation of dimer species of Mo-CBP$_2$, with each monomer composed of the 7,900 and 4,600 Da subunits probably linked by disulfide bond. The other CBP previously purified by our research group, Mo-CBP$_3$ (Gifoni et al., 2011) and Mo-CBP$_4$ (Pereira et al., 2011), also behaved as oligomeric proteins. Actually, several other similarities between Mo-CBP$_2$ and Mo-CBP$_3$ and Mo-CBP$_4$ exist, like the molecular mass of the intact proteins (~13,300, 12,200, and 11,800 Da, respectively) and subunits (~4,600/7,900, 4,100/8,100, and 3,900/8,400 Da, respectively) (Pereira, 2014; Freire et al., 2015), which suggest that they are part of the same family of CBP. Similar oligomeric behavior was also observed for other proteins from M. oleifera seeds. Indeed, both the hemagglutinin MoL and the flocculating lectin cMoL presented molecular profiles similar to Mo-CBP$_2$, appearing in dimer or trimer conformations (Katre et al., 2008; Luz et al., 2013). The in silico analysis of the recombinant protein MO 2.1 showed that the dimer form was the most stable structural arrangement (Pavankumar et al., 2014). Thus, it is plausible to suggest that the M. oleifera proteins aggregate into oligomers to attain a stable and lower energy structural state.

Mo-CBP$_2$ is a glycoprotein as are other CBP from M. oleifera purified by our research group (Pereira et al., 2011; Gifoni et al., 2012). However, a difference in relation to Mo-CBP$_3$ and Mo-CBP$_4$ is that the carbohydrate content was approximately two times higher in Mo-CBP$_2$. Another similar characteristic to Mo-CBP$_3$ and Mo-CBP$_4$ is that Mo-CBP$_2$ does not present any hemagglutinating activity upon human or rabbit erythrocytes, contrary to Mol and cMoL proteins (Katre et al., 2008; Luz et al., 2013). Therefore, Mo-CBP$_2$ is a merolecitin as it apparently possesses a unique carbohydrate binding site (Peumans and Van Damme, 1995) which binds to N-acetyl-D-glucosamine and its derived polysaccharide chitin, thus incapable of establishing bridges between two of more blood cells toward agglutinating them (Gifoni et al., 2012).

The MS/MS analysis done with Mo-CBP$_2$ after tryptic digestion, as an attempt to disclose its primary structure, allowed identification of 6 peptide fragments that together comprise 55 amino acid residues, or approximately 45% of the total amino acid residues of the studied protein. Four cysteine (C) residues were present, which might form inter or intrachain disulphide bonds. Glutamine (Q) was the most abundant amino acid residue (about 31%), which could be related to dimer formation tendency of Mo-CBP$_2$. It was previously reported that glutamine-rich regions may cause aggregation by formation of β-pleated sheets held together by hydrogen bonds (Perutz et al., 1994; Michelitsch and Weissman, 2000; Broin et al., 2002). In addition, the presence of glutamine at the N-terminus of Mo-CBP$_2$ could also explain the failure to identify the protein sequence by Automated Edman degradation. Indeed, cyclization of N-terminal glutamine to pyroglutamate leads to a blocked chain, and this event has been described for several 2S albums (Moreno et al., 2005). Particularly noteworthy is that among the identified amino acid residues of Mo-CBP$_2$, 14.5% corresponded to the positively charged arginine (R) and histidine (H), whereas negatively charged, polar uncharged, and non-polar amino acid residues correspond to 1.8, 45.5, and 38.2%, respectively. This is compatible with the cationic property of Mo-CBP$_2$ (pI = 10.9) and its adsorption to the cation exchange chromatography column equilibrated at pH 5.2, during the purification process.

Searches against the non-redundant protein sequence database of NCBI using BLASTp revealed that Mo-CBP$_2$ is closely related to Mo-CBP$_3$ and flocculating proteins from M. oleifera, and 2S albums from different plant sources. Sequence alignment of the six identified Mo-CBP$_2$ peptides with the sequences of four Mo-CBP$_3$ isoforms showed 63 to 100% of similarity, except the QPDFQR peptide that aligned exclusively with the isoform 2 (Mo-CBP$_3$-2), with 83% of similarity. Among the Mo-CBP$_3$ isoforms, the mean percentage sequence identity was found to Mo-CBP$_3$-3 (NCBI accession number AHG99684.1), the most abundant of them in the seeds of M. oleifera (Freire et al., 2015). With the M. oleifera genome recently available (Tian et al., 2015), we tried to localize the nucleotide sequence that corresponds to Mo-CBP$_2$ using the peptides generated after its tryptic digestion. However, as the primary structures of all Mo-CBP$_n$ proteins are very similar,
it was not possible to predict the Mo-CBP$_2$ sequence. For instance, it was reported that the flocculent peptides MOCP$_{2.1}$ and MOCP$_{2.2}$ are distinguished by a single amino acid residue (Shebek et al., 2015). Similar situation might occur with Mo-CBP$_2$ and other M. oleifera proteins. Besides of being a CBP, Mo-CBP$_3$ is also a member of the 2S albumin family, on the base of their structural similarities (Freire et al., 2015). Therefore, it is reasonable to suggest that Mo-CBP$_2$ is also a member of the 2S albumin family. The similarities between the Mo-CBP$_2$ peptide sequences and 2S albuamins from C. masaikai (63–83%), B. excelsa (73%), and Z. jujuba (63%) reinforce this hypothesis, although these plant species belong to other botany families different from Moringaceae. 2S albuamins are reserve proteins found in mono- or dicotyledonous plants (Youle and Huang, 1981), which apparently are also related to plant defense and some have antifungal activity (Agizio et al., 2006; Moreno and Clemente, 2008; Cândido et al., 2011), including against Candida species (Ribeiro et al., 2012).

The antimicrobial properties of M. oleifera have been previously reported. For instance, the aqueous extracts from seeds and other plant parts exhibited antimicrobial activity (Saadabi and Zaïd, 2011; Osource et al., 2013), particularly for Gram positive and Gram negative bacteria rather than for Candida spp. Recently, flavonoids extracted from M. oleifera seed coat were successfully tested as they exhibited antibiofilm potential against Staphylococcus aureus (Gram positive), Pseudomonas aeruginosa (Gram negative) and the yeast C. albicans (Osource and Arora, 2015). Moreover, organic extracts from several M. oleifera parts also showed antibacterial activity (Brilhante et al., 2015).

Although the antibacterial and anti-Candida activities of M. oleifera are well known, to the best of our knowledge, this is the first report of a protein from M. oleifera seeds with antifungal activity. Interestingly, all chitin binding proteins (Mo-CBP$_2$, Mo-CBP$_3$, and Mo-CBP$_4$) evaluated in this work exhibited inhibitory activity through Candida spp. Comparative analyses between antifungal effects displayed by Mo-CBPs evidenced Mo-CBP$_2$ as the most potent protein amongst them. Mo-CBP$_2$ inhibited C. albicans, C. parapsilosis, C. krusei, and C. tropicalis growth with MIC$_{50}$ much lower (9.45–37.90 μM) than the other proteins (261.67–310.06 μM). However, much higher concentrations of Mo-CBPs (155.84–600.23 μM) were needed to inhibit 90% of fungal growth. C. krusei cells were the most sensitive to Mo-CBP$_2$ (MIC$_{50}$ 9.45 μM and MIC$_{90}$ 155.84 μM). This is an important finding since C. krusei is a potentially multi-drug resistant pathogenic yeast due to its intrinsic resistance to fluconazole and tendency to develop reduced echinocandin susceptibility during prolonged therapy and under selection pressure (Schorzoni et al., 2013; Tavernier et al., 2015). The antifungal mode of action of Mo-CBP$_2$ is probably linked to its ability to disrupt the cell membrane integrity of C. albicans, as propidium iodide, which is membrane impermeable, was taken up by cells exposed to Mo-CBP$_2$, and interacted with nucleic acids as revealed by the appearance of red fluorescence (Wang et al., 2015). Increasing permeability of cell membrane can result from depolarization, disruption of lipid domain organization, pore formation, and unbalance of intracellular electrochemical gradients, leading to loss of membrane functions and cell death (Lee and Lee, 2015; Lee et al., 2015). We hypothesize that the increased permeability of C. albicans cell membrane by Mo-CBP$_2$ exposure might be due to interaction of this protein with chitin, which is one of the major components of fungal cell walls. As suggested for some antifungal peptides, chitin binding ability could help Mo-CBP$_2$ targets fungal cells efficiently and kill them by disruption of the plasma membrane integrity that increases permeabilization, or by forming pores directly (Bahar and Ren, 2013). Mo-CBP$_2$ could also gain access to the cell membrane by crossing the cell wall during the exponential growth phase of the yeast cells, when they exhibit increased porosity allowing the passage of compounds up to 70,000 Da (Klis et al., 2014). Once there, Mo-CBP$_2$, as a basic protein rich in positively charged amino acid residues, could establish electrostatic interaction with the negatively charged cell membrane leading to its disarrangement and cell lysis. Alternatively, Mo-CBP$_2$ could form transient pores through which it could gain access to the cell interior and enters into contact with intracellular targets (Li et al., 2012; Taniguchi et al., 2013; Choi and Lee, 2014). Regardless whether Mo-CBP$_2$ gained access or not to the Candida cell interior it promoted ROS generation, which are toxic to microorganisms (Mello et al., 2011; Wang et al., 2015). Like for other various antifungal proteins the mechanism of ROS generation after Mo-CBP$_2$ treatment of Candida cells is yet unknown. Nevertheless, ROS are natural compounds produced during the cell metabolism and play important roles in cell signaling and homeostasis, and high levels of ROS generated under environmental stress can result in significant damage to cell structures (Wang et al., 2015). Additionally, excessive ROS damages proteins, lipids, and DNA (Deavall et al., 2012) that besides increasing cell membrane permeability can lead ultimately to cell death. For instance, nystatin, an antifungal, disrupted the cell membrane integrity and induced increased ROS levels in C. albicans cells.

Moreover, in the case of internalization into the cell interior, Mo-CBP$_2$ could interact directly with DNA and exert DNase activity as it broken down in vitro the E. coli plasmid pUC18, like the commercial recombinant DNase. This finding corroborates with previous studies on the DNase activity of other CBP (Guevara-Morato et al., 2010; Menezes et al., 2014) and 2S albuamins from different plant sources (Odintsova et al., 2010; Tomar et al., 2014a,b). Thus, besides its low molecular mass, positive net charge, ability to disrupt cell membrane, and DNase activity, Mo-CBP$_2$ may also exhibit antifungal effect by interacting with the genetic material of C. albicans cells, leading to its degradation.

In addition to broad spectrum of action on pathogenic microorganisms, low toxicity is a desirable feature of new candidates as antifungal molecules. Hemolytic effect is often considered when antimicrobial safety of new compounds is tested as drugs for human and animal use (Kannan et al., 2013; Sellami et al., 2013; Christoffersen et al., 2015). Although Mo-CBP$_2$ is toxic to Candida spp., this protein did not cause hemolysis in human and rabbit erythrocytes, even in the highest concentration tested (1000 μg/mL). Despite the nystatin effectiveness as antifungal agent and prescription in superficial candidiasis treatment, its clinical use is limited due to its
toxicity to human erythrocytes (Bassi and Kaur, 2015). Melittin, other potent antimicrobial peptide against Candida spp., causes hemolysis in human blood cells even in low concentrations, which limits its use in antifungal therapy (Lee and Lee, 2015; Lee et al., 2015). Thus, discovery of potent antimicrobial agents with high selectivity and reduced or no toxicity to mammalian cells is still a challenge for the scientific community.

In summary, the data presented in this study highlight the potential use of Mo-CBP2 as an anticanical agent, based on its ability to inhibit Candida spp. growth with apparently low toxicity on mammalian cells.

AUTHOR CONTRIBUTIONS

Conceived and designed the study and experiments: JN, MP, LR-B, BR, TG, AM-M, RB, and IV. Performed the experiments: JN, MP, LR-B, TL, HC, JF, ML, and IV. Analyzed the data: JN, MP, JO, LR-B, HC, DS, BR, TG, ML, IV. Contributed reagents/materials/analysis tools: JO, DS, BR, TG, AM-M, RB, and IV. Wrote the paper: JN, MP, JO, LR-B, and IV. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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\textbf{Conflict of Interest Statement:} The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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