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Research Article

Purification, Characterization, and Inhibition *Cryptococcus neoformans* Succinate Dehydrogenase

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Abstract

Introduction: Succinate dehydrogenase (SDH) catalyzes the oxidation of succinate to fumarate in the Krebs cycle and transfers the electrons from succinate to ubiquinol. SDH, are important enzymes in the biosynthesis of ATP. The aim of this study was to purify, characterized and inhibit SDH from *Cryptococcus neoformans* the human pathogenic basidiomycetic yeast.

Materials and Methods: *Cryptococcus neoformans* was grown and its cells were harvested and subjected to breakage with glass beads. SDH was precipitated with 80% of ammonium sulfate and purified using Sephacryl S-300 gel filtration. Different natural products were tested against yeast cell growth inhibition. Furthermore, transmission electron microscopy analysis was used to examine the inhibited *C. neoformans* cells

Results: SDH was 65-fold purified from *Cryptococcus neoformans* with an overall yield of ~ 33% and specific activity of 13.7 unit/mg. The native SDH was a multi-enzyme system with total molecular weight = 141 kDa. Analysis of the purified SDH on SDS-PAGE showed that it is composing of four subunits of molecular weights: 66, 30, 26, and 12.8 kDa. Malonate is a competitive inhibitor against succinate with Kis value of 4.3 mM, while di-methyl-malonate recorded mixed type of inhibition to succinate dehydrogenase, with Kis value of 25 mM and Kii value of 7 ml/unite. Also, diethylmalonate inhibitor exhibited competitive inhibition type, Kis value was 28 mM. SDH was inhibited by bee propolis and its effect on wild *Cryptococcus neoformans* capsules under electron microscope was monitored. The cell wall of the treated cells showed pores and their numbers increased when 265 mg/ml (MIC) propolis concentrations was used.

Conclusion: The obtained data indicated that *Cryptococcus neoformans* succinate dehydrogenase kinetic mechanism is ordered sequential enzyme mechanism. Cells treated by 256 mg/ml propolis ethanolic extract showed no capsule and the organelles inside cell appeared to be destroyed.

Keywords: Cryptococcus neoformans; Succinate Dehydrogenase; Bee Propolis; Plant Extract; Transmission Electron Microscope

Introduction

Cryptococcus neoformans is one of the opportunistic fungi attacking immunocompromised individuals and causes cryptocosis. The current used anticryptococcal drugs are of limited effectiveness and rather are toxic to the patients. In that respect, we have purified and inhibit several enzymes from pathogenic yeast *Cryptococcus neoformans* like malate dehydrogenase [1], fatty acid synthetase [2] and NAD (H)-dependent specific isocitrate dehydrogenase [3]. Succinate dehydrogenase is a multisubunit mitochondrial enzyme that is part of both Krebs cycle and electron transfer chain. SDH catalyzes the oxidation of succinate to fumarate and donates the reducing equivalents to ubiquinone [4]. Most SDH and fumarate reductase enzymes are composed of four nonidentical subunits with a flavoprotein (Fp) of about 70 kDa, an iron-sulfur protein (Ip) of about 30 kDa, and two hydrophobic anchoring subunits of 7 - 17 kDa. The Fp contains the active site and the unusual cofactor [5,6].

A model of the quaternary structure of the tetrameric *Saccharomyces cerevisiae* succinate dehydrogenase was constructed based on the crystal structures of the *Escherichia coli* succinate dehydrogenase [7]. SDH of *Saccharomyces cerevisiae* is composed of four nonidentical subunits encoded by the nuclear genes SDH1, SDH2, SDH3, and SDH4. The hydrophilic subunits, SDH1p and SDH2p, comprise the catalytic domain involved in succinate oxidation.

They are anchored to the inner mitochondrial membrane by two small, hydrophobic -subunits, whereas SDH3p and SDH4p, are required for electron transfer and ubiquinone reduction [8].

The dicarboxylate carrier has been characterized and purified from mitochondria of wild strain Saccharomyces cerevisiae [9]. Saccharomyces cerevisiae SDH SDS-PAGE of the flow-through hydroxylapatite column illustrated five protein bands with M(r) ranging from 28,000 to 35,000, by silver nitrate staining. The purified protein presented the same properties as the dicarboxylate carrier in native mitochondria and displayed a single protein band with an M(r) of 28,000 as determined by SDS-PAGE. The specific activity of the purified carrier showed a 53-fold increase compared to that of the initial material. The current study was focusing on the purification and characterization of SDH enzyme. In addition, the purified enzyme will have subjected to different chemical and natural inhibitors which may stop the respiration cycle of the pathogen and thus preventing its pathogenicity.

Materials and Methods

Experimental microorganism

In this study, two *Cryptococcus neoformans* strains were used. A local strain of Cryptococcus neoformans isolated from Egyptian pigeon dropping [10]. Also, *Cryptococcus neoformans* ATCC 52817 strain, acapsular mutant strain (Acap), that was kindly provided by W. Niehaus, (Virginia Tech., USA).

Culture media

Growth enrichment medium (M1)

Medium components were ammonium hydrogen phosphate [(NH_4) HPO_4] 2.4g; potassium mono-hydrogen phosphate (K_2HPO4) 1.92g; sodium chloride (NaCl) 4.35g; magnesium sulfate ($MgSO_4$) 0.12g; glucose 27g and, 1 ml mineral salts. All components were dissolved in 1 liter distilled water and autoclaved as cited in [10].

Purification and characterization of Succinate dehydrogenase from *Cryptococcus neoformans* a capsular mutant strain ATCC 52817

Due to the difficulty of preparation of succinate dehydrogenase enzyme (SDH) from the capsulated *C. neoformans* isolate, it has been purified and study from A capsular mutant strain (ATCC 52817).

Enzyme Assay

Succinate dehydrogenase was assayed spectrophotometrically at 25°C, 600 nm in a mixture containing 50 mM phosphate buffer pH 7.2; 1 mM potassium cyanide (KCN), 45 µM 2-6 di-chlorophenol indophenol (DCPIP), 17 mM di-sodium succinate, and 100 µL of purified enzyme (final mixture volume 1 ml). An extinction coefficient of 21 x 10^4 M^{-1} cm^{-1} for DCPIP reduction was used to calculate SDH activity.

SDH activity = Δ A0/ extinction coefficient for DCPIP x assay volume where Δ A0 is the deference between absorbance at 600 nm for 60 seconds [11].

Purification steps of succinate dehydrogenase from C. neoformans a capsular mutant strain ATCC 52817

Preparation of SDH enzyme crude extract

Cells of *C. neoformans* (ATCC 52817 strain) were grown at 37°C in growth medium (M1) with final pH 7.5. Cells of 1 day old culture were collected by centrifugation at 5000 rpm, washed with sterile distilled water, and stored in glycerol at -20°C till further work. All steps for purification were performed at 4°C. Thirty grams of prepared cells were suspended in buffer containing 25 mM sucrose; 5 mM Ethylene diamine tetra-acetic acid (EDTA); and 1.5g bovine serum albumin (BSA), all completed to one liter of distilled water; pH was adjusted at 7.0 and glass beads (0.5 mm) were added at ratio of 1 - 3g of chilled glass beads per gram of cell wet weight, then ultra-sonication (Ultrasonic processor, Cole Parmer, USA) was conducted to cell suspension for 15 minutes, at 60 - 70W, 50% duty cycle. The homogenate was centrifuged (Hettich, Germany) for 15 minutes at 5000 rpm, the pellets were neglected and the supernatant was re-centrifuged at 10000 rpm for 20 minutes the resulting pellet was again re-suspended in BSA-free sucrose/EDTA buffer containing sucrose (25M) and (EDTA) 5 mM; pH 7.0 and re-centrifuged at 13.000 rpm for 10 minutes. The pellet was again re-suspended in 40 ml BSA-free sucrose/EDTA buffer and the resulting suspension was defined as the SDH enzyme crude extract. The temperature of the suspension preparation was kept at 4°C during all procedures [12].

Ammonium sulfate precipitation

The crude extract (protein) was fractionated between 20% - 80% ammonium sulphate saturation at neutral pH and 4°C. Protein that was precipitated by 80% ammonium sulfate was re-suspended in 50 ml phosphate buffer pH 7.5 containing 1 mM EDTA, 2 mM $MgCl_2$, 1 mM mercaptoethanol and 1 mM phenyl methyl sulphonyl fluoride (PMSF) [13].

Sephadex G-25 gel filtration

Ammonium sulfate saturation (80%) was desalted by applying to 1.5 x 12 cm column of sephadex G-25 which was equilibrated with 0.1M phosphate buffer (pH 7.2). The column was eluted with 0.1M phosphate buffer at flow rate 15 ml/hr, after elution the fractions were collected and the enzyme activity was determined. Fractions with higher SDH activity were applied to sephacryle S-300 column.

Sephacryle S-300 gel filtration

A column of dimensions 1.5 x 15 cm packed with sephacryle S-300 was equilibrated with phosphate buffer (pH 7.2). The sephadex G-25 eluted fractions were applied to Sephacryle S-300 column. Succinate dehydrogenase was then eluted with phosphate buffer pH 7.2. Active peak fractions were pooled and defined as the Sephacryle S-300 fractions. The obtained SDH fractions were analyzed by SDS-PAGE and native PAGE to determine the degree of purity.

Determination of total protein concentration

Lowery., *et al.* [14] method was used to determine the total protein concentration of all steps in the purification procedure.

Enzyme subunit Molecular weight determination

The molecular weight of tested enzyme (SDH) was determined based on the mobility through (10%) poly-acrylamide gel as compared with the mobility of protein markers [15].

Determination of SDH molecular mass by native-gradient PAGE of SDH purified from *C. neoformans*

The molecular weight of the native SDH (whole enzyme) was determined from a standard curve of molecular weight of known protein markers using the method of Laemmli [15] with 10% poly-acrylamide gel electrophoresis (PAGE) at 4°C.

Determination of succinate dehydrogenase kinetic mechanism

Kinetic mechanism of SDH was explained in the present study by analyzing parameters for the enzyme reaction. These parameters were; substrate reaction including, initial velocity, and product inhibition. Succinate dehydrogenase is going on as indicated on the following equation:

$$\text{Succinate + FAD+} \xrightarrow{\text{Succinate dehydrogenase}} \text{Fumarate + } FADH_2$$

Initial velocity studies

Then the effect of different substrate concentrations on purified SDH activity in the absence of products (forward direction) was determined then the reaction velocity calculated using linear least squares.

Product inhibition

Amount of produced fumarate product was measured in the presence of different concentrations of Na-succinate, FAD, and/or fumarate itself in the reaction mixture.

Data processing

Kinetic data were recorded, and fit by non-linear least squares using Microsoft Office Excel 2003 software program.

Inhibition of the purified succinate dehydrogenase from *C. neoformans* by chemical inhibitors (malonate and malonate derivatives)

Malonate and malonate derivatives (dimethylmalonate and diethylmalonate) were tested against different concentrations of Na-succinate as substrate. SDH activity in the direction of succinate oxidation was carried out to avoid interference of fumarate as product inhibition [16].

Malonate

Effect of different malonate concentrations against different concentration of di-sodium succinate was tested. The activity of SDH enzyme was calculated and the type of inhibition was determined.

Dimethyl-malonate

Dimethyl-malonate concentrations ranging from 10, 20, 30, 40, and 50 mM were tested against different concentrations of di-sodium succinate (5, 10, 15, 20, 25, and 40 mM) was tested. The type of inhibition was determined.

Diethyl-malonate, $(C_2H_5)2(C_3H_2O_4)$

Di-ethyl malonate concentrations ranging from 10 to 50 mM were tested against different concentrations of di-sodium succinate ranging from 5 to 40 mM, and the activity of SDH was determined spectrophotometrically at 600 nm. and the type of inhibition was determined.

Bee propolis alcoholic extract preparation [17]

Bee propolis (gum powder) produced by honeybee was obtained. The propolis extract was obtained by extracting 400g of natural propolis in 1600 ml of 70% ethanol for a period of 30 days, the extract was filtrated using Whatman No.1 filter paper, the pellet was neglected and filtrate was stored at -20ºC.

Evaluation of antifungal activity of some plant extracts and bee propolis against the growth of wild-type *C. neoformans* (tested isolate)

Selected plants and bee propolis

Thirteen plant species were chosen (Clove, Thyme, Garlic, Fennel, Berry, Onion, Aloe, Green tea, Ginger, Rocket salad, Cabbage, Pomegranate and Bee gum); to be tested against the efficiency of *C. neoformans* growth. The basis of selection of these plant species was adjusted according to their reported antimicrobial properties.

Plants were observed to be free from insect damage and brought to the laboratory for extraction. Selected plants were extracted by water and examined for their activity against *C. neoformans*. Bee propolis was used as natural compound in a powder state.

Preparation of plant aqueous extracts:

All plants were cleaned by running tap water and then with distilled water and left in air to dry at room temperature. Dried plant material is ground in a grinder. The aqueous extraction of water soluble ingredient of the plant parts was carried out as described by Asuzu [18]. From each ground sample 15g were extracted by shaking for 3 days with 35 ml of deionized water in separate container. The extracts were filtrated using Whatman No.1 filter paper, after which the filtrates were separately concentrated by evaporation at 30°C using water bath. The concentrated extracts were stored at 4°C for further work, the working solutions were Millipore filtrated (0.22 µm).

Garlic alcoholic extract preparation

Garlic extract was prepared according to method described Bakri and Douglas [19]. The peeled fresh garlic (80g) was chopped and homogenized in 100 ml of 70% methanol, centrifuged, filtered through Whatman No.1 filter paper. By subtracting the weight of insoluble material from the weight of original garlic, the final concentration of garlic extract in solution was determined which was Millipore filtrated (pore size 0.22 µm). The filtrate was then kept at -20°C for further work.

Effect of plant extracts using agar disc diffusion method:

Petri-dishes containing equal aliquots of M1 medium were inoculated with 100 µl freshly prepared wild *C. neoformans* strain, and then 6 mm of Whatman No.1 filter paper disks were separately saturated with 40 µl of each plant extracts, dried and placed onto the center of M1 medium plates. The plates were then incubated at 37°C for 48h. Diameters of inhibition zones, if present were then measured for each tested material.

Effect of alcoholic propolis and garlic extracts on growth of *C. neoformans* [20]

Disc diffusion assay was carried out using M1 broth medium. Sterilized Whatman filter paper of 6 mm diameter was impregnated with 40 µl of different concentration of propolis (6, 8, 16, 32, 64, 128, 256 and 512 mg/ml) and garlic extracts (200, 400, 600, or 800 mg/ml) on the center of plates seeded with *C. neoformans* cells. Plates were incubated at 37°C for 48h and observed for appearance of clear zones around paper discs.

Determination of MIC of bee propolis and garlic alcoholic extracts against wild *C. neoformans*

Overnight culture of *C. neoformans* isolate was prepared by inoculating 50 ml M4 broth medium with *C. neoformans* cell suspension, concentration of inoculum cell suspension was adjusted to be 106 cells/ml. From M4 broth medium; 800 µl were dispensed into each well of 24-well of microtitre plates (Sigma, USA). Then each well was inoculated with 100 µl of inoculum suspension. Bee propolis ethanolic extract (100 µl) from each concentration (4, 6, 12, 16, 32, 64, 128, 256 and 512 mg/ml) was added to each well separately. One control sample was carried out using 100 µl of, ethanol 70%, 100 µl of *C. neoformans* suspension and 800 µl of unseeded M4 broth medium, another control sample was conducted by adding 100 µl of *C. neoformans* suspension only to 800 µl malt extract broth. Plates were incubated at 37°C for 48h. Inhibition of growth was determined by counting cell number. The MIC was defined as the concentration at which the lowest growth was observed after incubation. For measurement of Minimal lethal concentration (MLC), 50 µl from each well were taken and sub-cultured onto M4 medium without propolis for 72h. The MLC was defined as the concentration at which no growth was observed after subculture. Similarly, garlic methanolic extract (100 µl) from each concentration (50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 mg/ml) was added to each well separately, a control sample was carried out using 100 µl of, methanol 70%, 100 µl of *C. neoformans* suspension and 800 µl of M4 broth medium. Plates were incubated at 37°C for 48h. Inhibition of growth was determined by counting cells numbers.

The effect of bee propolis and garlic extracts on respiration of *Cryptococcus neoformans*

Preparation of yeast suspension

Overnight culture of *C. neoformans* isolate was centrifuged at 5000 rpm and 0.5g of the cell pellet was added to 5 ml buffer saline solution (pH 7.0), and let to stand for 15 minutes until yeast became fully hydrated.

Respiration measurement

Sample bottle of oxygen meter containing 1.8 ml of buffer saline, 100 µl of (either propolis, garlic or green tea extracts) at conc. (100 mg/ml) were added. Each of these three treatments was then inoculated with 100 µL of the above prepared yeast suspension then the data were recorded for 15 minutes. Control sample was prepared using distilled water instead of extracts. A curve was plotted between time and O_2 consumed (ppm); the slope of curve considered as oxygen consumption rate (m.mol O_2/mg of yeast/h).

Effect of propolis ethanolic extract on capsule of wild *C. neoformans* cells [17]

Ethanolic propolis extract was selected to investigate its effect on the capsule structure of *C. neoformans*, where it was observed to possess relatively inhibitory higher activity.

Preparation of yeast suspension

Overnight culture of *C. neoformans* isolate was centrifuged at 5000 rpm and 0.2g of cell pellet was treated with 0.2 ml (w/v) of 128 or 256 mg/ml ethanolic propolis extract (MIC and MLC propolis extract conc.) and incubated for 48h at 37°C.

Scanning electron microscope (SEM) of *C. neoformans* treated by bee propolis

Samples which prepared above were fixed in 1.5% glutaraldehyde in potassium phosphate buffer (pH 6.0) for 2h, and washed with the phosphate buffer. Samples were dried in critical point dryer and coated with fine gold particle vapor. Samples (control and treated cells) were examined and photographed with scanning electron microscope at (Alexandria University. Faculty of Science. Electron microscopic laboratory).

Results

Purification of Succinate dehydrogenase from *Cryptococcus neoformans* (ATCC 52817 strain)

SDH activity was estimated in acapsulated *C. neoformans* (ATCC 52817 strain) grown on M1 broth medium that showed maximum SDH activity (0.7unit/ml) with specific activity (7.7units/mg) on the second day of its growth. However, increasing the incubating period lead to proportional decrease in SDH activity.

From table 1 succinate dehydrogenase was purified to 65-fold with an overall yield of ~33% and specific activity of 13.7 unit/mg. The eluted SDH enzyme fractions obtained from sephacryle S-300 column, were stored at -20 C in (phosphate buffer pH 7.2, 1mM EDTA, 1mM $MgCL_2$, and 1 mM mercaptoethanol) without appreciable loss of the activity. The enzyme at this stage was used for the subsequent analysis.

Steps	Total Volume (ml)	Activity (U/ml)	Total unit (U)	Total protein (mg)	Specific activity (units/mg)	Purification fold (n-fold)	Yield (%)
Crude extract	150	0.55	83.38	400	0.21	1	100
Amm. Sulfate saturation (80%)	70	1.15	80.35	148	0.54	2.57	96.4
Sephadex G-25	100	0.39	38.8	13	2.98	14.16	46.6
Sephacryle S-300	40	0.69	27.4	2	13.7	65.1	32.9

Table 1: Purification of Succinate dehydrogenase from Cryptococcus neoformans.

One unit of enzyme is defined as the amount of enzyme which catalyzes the oxidation of 1 µmole of FADH per minute at pH 7.2 and 25°C.with assay mix.

30 gm of frozen cells were processed.

Determination of SDH molecular weight by SDS-PAGE

As shown in figure 1a and 1b, it was found that the native SDH has a molecular weight of 141 kDa; however, analysis of the purified SDH subunit on SDS-PAGE showed that SDH enzyme composed of four subunits of molecular weights: 66, 30, 26, and 12.8 kDa

Determination of succinate dehydrogenase kinetic mechanism

Analyses of SDH kinetics depend on the study of different parameters, such as substrate reaction, initial velocity, and product inhibition.

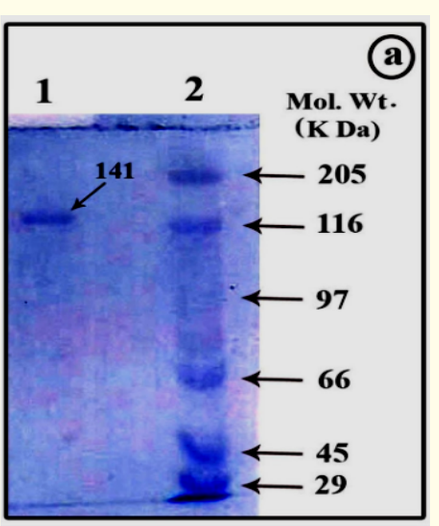


Figure 1a: Native polyacrylamide gel electrophoresis (10%) of succinate dehydrogenase purified from C. neoformans, lane 1: purified native SDH, lane 2: high mol. wt. protein markers: α-Macroglobulin (205 KDa), β-galactosidase from E. coli, Phosphorylase-B from rabbit muscles, Bovine Serum Albumin (BSA), Ovalbumin, and Carbonic anhydrase from bovine erythrocyte.

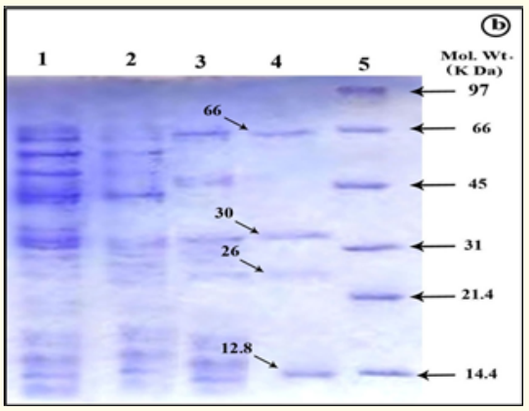


Figure 1b: NSDS-polyacrylamide gel electrophoresis (10%) of succinate dehydrogenase subunits purified from C. neoformans, lane 1: crude extract, lane 2: 80% amm. sulphate, lane 3: sephadex G-25, lane 4: sephacryle S-300, lane 5: low mol. wt. protein markers: phosphorylase B (97 KDa) from rabbit muscles, Bovine Serum Albumin, Ovalbumin, Carbonic anhydrase from bovine erythrocyte, Soybean Trypsin inhibitor, and Lysozyme.

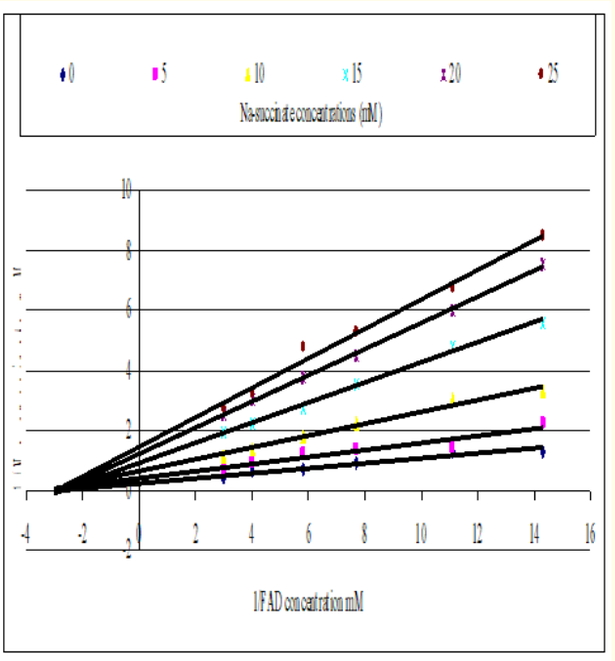


Figure 2: Initial velocity pattern.

Substrate reaction

As shown in figure 2, substrate(Na-succinate) reaction recorded Km value of 0.047 mM with enzyme Vmax equal to 0.44 ml/unit.

Initial velocity studies

The regular increase in substrate concentrations either Na-succinate or FAD in the absence of product fumarate or FADH led to high increase in reaction initial velocity in the forward direction (oxidation of succinate) (Figure 2). Double reciprocal plots of the initial velocity showed a series of intersecting lines. Na-succinate concentrations ranging from 0, 5, 10, 15, 20, 25, and 30 mM and FAD concentration 0.05, 0.1, 0.2, 0.3 and 0.4 mM indicated a series of intersecting lines that indicating a sequential enzyme mechanism. The initial velocity of reaction in the absence of product increased calculated kinetic constants were: Vmax, 1.7 ml/unit; Km succinate 3 mM.

Product inhibition

Since the products of the reaction are the substrates in the reverse direction, it would be expected to tie up the enzyme in a non-useful form and act as inhibitors [21]. The types of inhibition pattern obtained could define or could be crucial in defining the type of kinetic mechanism elicited by the enzyme.

Product inhibition studies were focused in the direction of oxidation of succinate and reduction of fumarate (Table 2).

The products of succinate dehydrogenase in the direction of fumarate reduction were FAD and succinate. However, fumarate and FADH are the products of the enzymatic reaction in the succinate oxidation direction. One of the substrates of the enzyme varied at fixed saturating levels of the other and the data were plotted in double reciprocal fashion.

The increase of FAD concentration inhibited the forward SDH reaction and accelerate the reverse reaction, which produced a competitive inhibition pattern with Kis = 0.1 mM (Table 2), where FAD varied from 0 to 0.4 mM against FADH (0.02 to 0.1 mM), at high concentration of fumarate 100 mM.

The variation of fumarate (0 to 25 mM) versus succinate (5 to 40 mM) at saturating concentration of FAD (0.7 mM) produced uncompetitive inhibition with Kii value 4 ml/unite (Table 2).

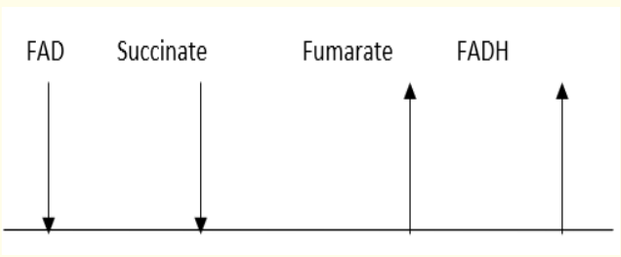
Also, FADH (0 to 0.1 mM) was varied against succinate (5 to 40 mM) at saturating level of FAD (0.7 mM). FADH exhibited Competitive inhibition versus succinate with Kis = 0.027 mM (Table 2).

Varied substrate conc. (mM)	Fixed substrate conc. (mM)	Inhibitors conc. (mM)	Pattern	Kis (mM)	Kii (mM)
FADH (0.02 - 0.1)	Fumarate (100)	FAD (0 - 0.4)	Competitive	0.1	0.0
Succinate (5 - 40)	FAD (0.7)	Fumarate (0 - 25)	Un-Competitive	0.0	4
Succinate (5 - 40)	FAD (0.7)	FADH (0 - 0.1)	Competitive	0.027	0.0
FAD (5 - 35)	Succinate (70)	Fumarate (0 - 25)	Competitive	5	0.0

Table 2: Product inhibition of purified SDH kinetic mechanism.

The effect of fumarate (0 to 25 mM) was investigated against FAD (0.02 to 0.3 mM) at 70 mM succinate. The result indicated competitive inhibition also with Kis = 5 mM (Table 2).

The previous inhibition pattern indicating that the mechanism of SDH activity is ordered sequential enzyme mechanism as follows:



Figure

Inhibition of SDH from Cryptococcus neoformans (ATCC 52817 strain) by chemical inhibitors (malonate and malonate derivatives)

Malonate and malonate derivatives were chosen to be tested against SDH activity as substrate analogs.

The effect of malonate and malonate derivatives (dimethyl and diethyl malonate) on SDH activity (unit/ml) was recorded in table 3. The regular increase in malonate concentration (0 to 50 mM) against different concentrations of succinate ranging from 5 to 40 mM at fixed concentration of another parameters indicated a competitive inhibition and the Kis value of malonate was 4.3 mM. Di-methylmalonate recorded mixed inhibition type to succinate dehydrogenase, where Kis value was 25 mM while Kii value was 7 ml/unite Diethylmalonate inhibitor exhibited competitive inhibition type, Kis value was 28 mM.

Evaluation of anticryptococcal activity of some selected plant extracts

Clove, Thyme, Garlic, Fennel, Berry, Pomegranate, Aloe, Green tea, Ginger, Rocket salad, Cabbage, Anise, and Bee gum); were chosen to be tested against the *C. neoformans* growth inhibition. Bee propolis recorded the highest anticryptococcal activity followed with garlic cloves and green tea (Figure 3).

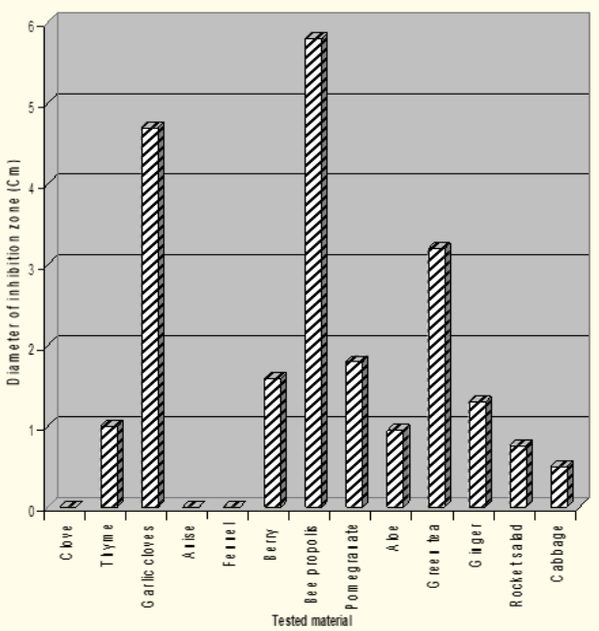


Figure 3: Evaluation of antifungal activity of plant extracts and bee propolis against wild Cryptococcus neoformans.

Evaluation of antifungal activity of alcoholic extracts of bee propolis and garlic against the growth of wild *Cryptococcus neoformans*

Cryptococcus neoformans isolated and identified from Egyptian field, was tested against different concentrations (6, 8, 16, 32, 64, 128, and 256 mg/ml) of ethanolic propolis extract and different concentrations of garlic methanolic extracts (200, 400, 600, and 800 mg/ml). It was observed that increasing extract concentration leads to increasing the resulting inhibition zone as shown in tables 4 and 5.

Propolis extract (mg/ml)	Inhibition zone diameter (Cm)
Control (ethanol)	0.2
6	1.2
8	1.66
16	2.13
32	2.78
64	3.36
128	4.52
256	6.73

Table 4: Effect of different concentrations of bee propolis ethanolic extract on growth of *C. neoformans.*

Garlic extract (mg\ml)	Inhibition zone diameter (Cm)
Control (methanol)	0.22
200	0.92
400	1.5
600	2.2
800	3.78

Table 5: Effect of different concentrations of garlic methanolic extract on *C. neoformans.*

Evaluation of the time course of the inactivation of SDH purified from *Cryptococcus neoformans* (ATCC 52817 strain)

Determination of MIC of bee propolis and garlic alcoholic extracts against wild *C. neoformans*

As shown in figure 4, it was found that, MIC value of propolis against *C. neoformans* was 128 mg/ml. MIC value recorded to be 256 mg/ml. These results demonstrated that propolis possess a considerable fungicidal activity against growth of *C. neoformans.* Similarly, from figure 5 MIC of garlic methanolic extract calculated to be 400 mg/ml.

Effect of bee propolis, garlic and green tea extracts on respiration of *Cryptococcus neoformans:*

As shown in table 6 the respiration rate of *C. neoformans* in presence of water and alcoholic extracts of garlic and green tea increase which observed from high oxygen consumption; oxygen consumption rate illustrated garlic methanolic extract was 0.875 m.mol/mg/h and garlic aqueous extract was 0.488 m.mol/mg/h while green tea aqueous and methanolic extracts were recorded to be respectively 0.285 and 0.585 m.mol/mg/h by comparing with control.

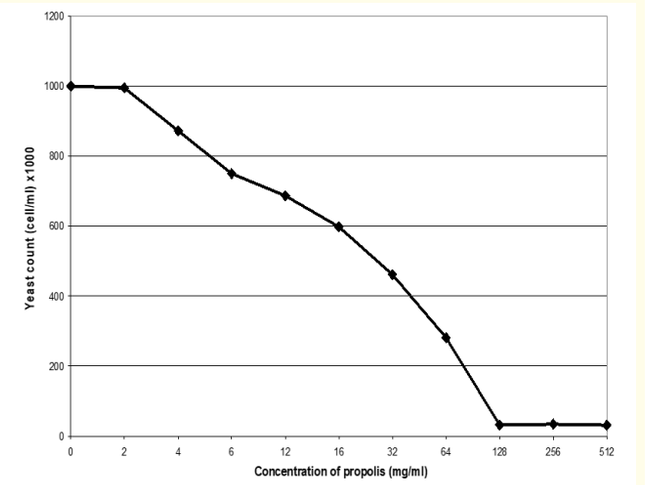


Figure 4: Determination of MIC of bee propolis against *C. neoformans.*

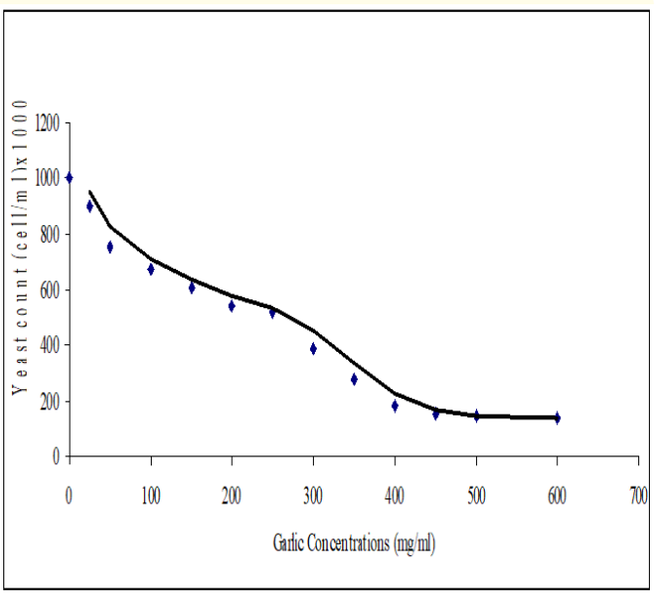


Figure 5: Determination of MIC of garlic against *C. neoformans.*

On the other hand, oxygen consumption rate of propolis ethanolic extract was 0.135 m.mol/mg/h and propolis aqueous extract was recorded 0.21 m.mol/mg/h. this indicating that garlic and green tea water and methanolic extracts increased *C. neoformans* respiration while propolis water and alcoholic extract decrease respiration rate of *C. neoformans* all compared with control.

Inhibitor	Oxygen consumption rate (m.mol/mg/h)
Water	0.295
Methanol	0.240
Ethanol	0.170
Propolis ethanolic extract	0.210
Propolis aqueous extract	0.135
Garlic Methanolic extract	0.875
Garlic aqueous extract	0.488
Green Tea methanolic extract	0.585
Green tea Aqueous extract	0.285

Table 6: The effect of bee propolis, garlic and green tea extracts on respiration of *Cryptococcus neoformans.*

Effect of bee propolis ethanolic extract on *C. neoformans* capsule as the most important virulence factors

Scanning electron microscope

As shown in figure 6a-6c the size of propolis ethanolic extract treated cells were markedly reduced compared to the control cell treatment with 128 mg/ml (MIC). Moreover, the cell wall of the treated cells showed pores and their numbers increased when 265 mg/ml (MIC) propolis concentrations was used compared to that at 128 mg/ml.

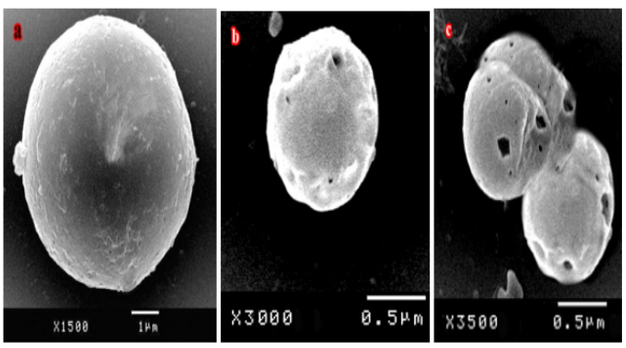


Figure 6: Scanning electron micrograph of *C. neoformans*

a: Control yeast cell at 1500X.

b: Cell treated with 128 mg/ml (MIC) Propolis extract at 3000X.

c: Cells treated with 256 mg/ml (MLC) Propolis extract at 3500X.

Discussion

Purification of Succinate dehydrogenase from *Cryptococcus neoformans*

In the present work succinate dehydrogenase (SDH) was purified to 65-fold with an overall yield of 32.9% and SDH specific activity of 13.7 unit/mg from *Cryptococcus neoformans* (Acapsule mutant strain). The native SDH was multi-enzyme system of total molecular weight = 141 kDa; however, analysis of the purified SDH on SDS-PAGE showed that SDH enzyme composed of four subunits of molecular weights: 66, 30, 26, and 12.8 kDa.

The mitochondrial succinate dehydrogenases of eukaryotic cells are essential for oxidative metabolism via the citric acid cycle and for efficient exchange of metabolites and reducing equivalents across the mitochondrial membrane. Fungi such as Saccharomyces cerevisiae that can function either in a respiratory or a fermentative manner contain four succinate dehydrogenase isozymes, a mitochondrial SDH1, SDH2, SDH3 and SDH4 forms [22].

The succinate dehydrogenase that has been purified appeared to be of mitochondrial form, based on; the physiology of the organism, the high specific activity of succinate dehydrogenase in crude extracts and the substrate inhibition by fumarate.

Oyedotun and Lemire [23] reported that the yeast succinate dehydrogenase (SDH) is a tetramer of non-equivalent subunits and their data suggest that heme does not play an essential role in quinone reduction. For the same consideration Bullis and Lemire [24] reported that succinate dehydrogenase is an intrinsic or inner mitochondrial membrane protein that catalyses the oxidation of succinate and donates electrons to the respiratory chain via quinone acceptors. It is a heterotetramer composed of a flavoprotein, an iron-sulfur, and two hydrophobic subunits. The same investigators purified succinate dehydrogenase by blue native gel electrophoresis, determined the amino-terminal sequence of the Sdh4p subunit and used this information to clone the SDH4 gene.

Determination of (SDH) kinetic mechanism

Kinetic mechanisms are divided into two major groups, sequential or non-sequential [21]. One of the substrates of bi-substrate reaction is varied at fixed levels from the other and the data are plotted in double reciprocal fashion. If the families of lines produced are parallel then a ping-pong mechanism is implicated [25]. If the families of lines are intersecting then a sequential mechanism is implicated [26]. The kinetic pattern obtained in this study from initial rate studies in both the forward and reverse directions are consistent with an ordered sequential mechanism. In the present investigation the reduced and oxidized flavin nucleotides (FADH and FAD) were competitive product inhibitors at concentration (100mM) of the substrate. These data indicated that FADH and FAD compete for the same form of the enzyme.

Succinate was competitive inhibitor with different concentrations of FAD which also indicated that FAD and succinate bind to the same enzyme form. Also, the result obtained from product inhibition patterns indicated competitive inhibition, except the variation of succinate versus fumarate which gave uncompetitive inhibition. The sequential mechanisms included an ordered bi-bi reaction. These results are in almost agreement with that reported for succinate dehydrogenase by Tomita., *et al.* [27] who constructed kinetic models of various mitochondrial metabolisms including gene expression, electron transport (respiratory chain), TCA cycle, fatty acid metabolism oxidation, inner-membrane metabolite carriers, and protein carriers.

Inhibition of the purified *C. neoformans* succinate dehydrogenase by chemical inhibitors

Malonate is an inhibitor of cellular respiration, because it binds to the active site of succinate dehydrogenase in the citric acid cycle but does not react, thereby competing with succinate. For the oxidative phosphorylation reaction, malonate is an inhibitor for complex II which, again, contains succinate dehydrogenase [28].

The present work indicated the inhibition of succinate dehydrogenase with malonate and malonate derivatives; dimethyl and diethyl-malonate as example of dicarboxylates. The regular increase in malonate concentration against different concentrations of succinate at fixed concentration of other parameters indicated a competitive inhibition and the Kis value of malonate was 4.3 mM, while di-methyl-malonate recorded mixed type of inhibition to succinate dehydrogenase, where Kis value was 25 mM while Kii value was 7 ml/unite. Also, diethylmalonate inhibitor exhibited competitive inhibition type, Kis value was 28 mM. It has been observed that succinate dehydrogenase was inhibited by diverse classes of compounds such as dicarboxylates, pyrophosphate, quinols and nitrophenols [29].

Inhibition of succinate dehydrogenase (SDH) by dicarboxylates with oxaloacetate and ketomalonate and without malonate, succinate, and glutarate was studied. Ketodicarboxylates at low concentrations inhibit SDH in competitive manner. Increase in their concentrations results in appearance of the noncompetitive component. The extent of SDH inhibition by keto-dicarboxylates increases with structural similarity of the inhibitor and the substrate, irrespective of preliminary incubation of the enzyme with the inhibitor [30].

Evaluation of the time course of the inactivation of SDH purified from *Cryptococcus neoformans* by bee propolis and garlic extracts

Succinate dehydrogenase is a regulator enzyme for respiration in *Mycobacterium tuberculosis* [31]. The present study evaluated the effect of bee propolis and garlic alcoholic extracts on purified SDH activity percentage. As shown in figure 6 the size of propolis ethanolic extract treated cells were markedly reduced compared to the control cell treatment with 128 mg/ml (MIC). Moreover, the cell wall of the treated cells showed pores and their numbers increased when 265 mg/ml (MIC) propolis concentrations was used compared to that at 128 mg/ml. Both bee propolis and garlic extract might be used for *Cryptococcus neoformans* treatment, but it need further studies to be verified. Minimum inhibitory concentrations (MICs) of *Thymus vulgaris* (Thyme) essential oil was from 32 to 128 µg/ml. *In vitro* interaction tests between this oil and fluconazole (FCZ) showed no potentiation of the antifungal action of this drug. Its effect on mitochondrial metabolism of fungal cells was also evaluated and results demonstrated alterations on the mitochondrial enzyme activity of fungal cells only at concentrations > 1,024 µg/mL [32]. This investigation describes the antifungal action of *T. vulgaris*, showing its potential in the development of alternatives in the treatment of *C. neoformans.*

The basic finding of this is manuscript is the purification scheme of SDH, kinetics enzyme mechanism elucidation and enzyme inhibition by bee glue or propolis. The nature of SDH inhibition by bee propolis from kinetics point of view and its effect on wild *Cryptococcus neoformans* cells under electron microscope was monitored.

Conclusion

The obtained data indicated that *Cryptococcus neoformans* succinate dehydrogenase kinetic mechanism is ordered sequential enzyme mechanism. Cells treated by 256 mg/ml propolis ethanolic extract showed no capsule and the organelles inside cell appeared to be destroyed.

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