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Phytochemical composition, antifungal activity, *in vitro* and *in vivo* toxicity of *Syzygium cumini* (L.) Skeels leaves extract

[Composición fitoquímica, actividad antifúngica, toxicidad in vitro e in vivo del extracto de hojas de *Syzygium cumini* (L.) Skeels]

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Section **Biological activity**

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Abstract: This study determined phytochemical composition, antifungal activity and toxicity *in vitro* and *in vivo* of *Syzygium cumini* leaves extract (Sc). Thus, was characterized by gas chromatography coupled to mass spectrometry and submitted to determination of Minimum Inhibitory (MIC) and Fungicidal concentrations (MFC) on reference and clinical strains of *Candida* spp. and by growth kinetics assays. Toxicity was verified using *in vitro* assays of hemolysis, osmotic fragility, oxidant and antioxidant activity in human erythrocytes and by *in vivo* acute systemic toxicity in *Galleria mellonella* larvae. Fourteen different compounds were identified in Sc, which showed antifungal activity (MIC between 31.25-125 µg/mL) with fungistatic effect on *Candida*. At antifungal concentrations, it demonstrated low cytotoxicity, antioxidant activity and neglible *in vivo* toxicity. Thus, Sc demonstrated a promising antifungal potential, with low toxicity, indicating that this extract can be a safe and effective alternative antifungal agent.

Keywords: Dentistry; Plants extracts; *Syzygium cumini*; Antifungal agents; Toxicity tests.

Resumen: Este estudio determinó la composición fitoquímica, la actividad antifúngica y la toxicidad *in vitro* e *in vivo* del extracto de hojas de *Syzygium cumini* (Sc). Así, se caracterizó mediante cromatografía de gases acoplada a espectrometría de masas y se sometió a determinación de Concentraciones Mínimas Inhibitorias (CMI) y Fungicidas (MFC) sobre cepas de referencia y clínicas de *Candida* spp. y mediante ensayos de cinética de crecimiento. La toxicidad se verificó mediante ensayos *in vitro* de hemólisis, fragilidad osmótica, actividad oxidante y antioxidante en eritrocitos humanos y por toxicidad sistémica aguda *in vivo* en larvas de *Galleria mellonella*. Se identificaron catorce compuestos diferentes en Sc, que mostraron actividad antifúngica (CMI entre 31.25-125 µg/mL) con efecto fungistático sobre *Candida*. En concentraciones antifúngicas, demostró baja citotoxicidad, actividad antioxidante y toxicidad *in vivo* insignificante. Por lo tanto, Sc demostró un potencial antifúngico prometedor, con baja toxicidad, lo que indica que este extracto puede ser un agente antifúngico alternativo seguro y eficaz.

Palabras clave: Odontología; Extractos de plantas; *Syzygium cumini*; Agentes antifúngicos; Ensayos de toxicidad.

INTRODUCTION

Medicinal plants have been long considered promising resources for the discovery of new therapeutic agents to treat human diseases (Pereira *et al*., 2016; De Araújo *et al*., 2018) such as oral candidiasis caused by *Candida* spp. (Peleg *et al.*, 2010; Rodrigues *et al.*, 2014; Muadcheingka & Tantivitayakul, 2015; Silva-Rocha *et al.*, 2015).

Oral candidiasis is a superficial fungal infection which may present clinically as pseudomembranous candidiasis, erythematous candidiasis, hyperplastic candidiasis, denture stomatitis or angular cheilitis. *C. albicans* is the predominant species in oral candidiasis, with rates of prevalence of 61.6% followed by non albicans species that has increased significantly such as *C. glabrata* (15.2%), *C. tropicalis* (10.4%), C. *krusei* (1.6%), among others (Muadcheingka & Tantivitayakul, 2015), with reports of high azole resistance rates in this species, specially to fluconazole, with resistance rates from 9 to 14% in *C. glabrata* and 11.6% in *C. tropicalis* (Perlin *et al.*, 2017).

Fungal infections range from superficial mucosal (of importance in Dentistry), dermal infections, and hematogenously disseminated infections with sizable mortality rates, next to 50% (Gulati & Nobile, 2016) to 72.2% (Doi *et al.*, 2016), especialy in immunocompromised individuals or in critically ill patients (Doi *et al.*, 2016; Gulati & Nobile, 2016; Savastano *et al*., 2016). Thus, oral candidiasis and disseminated infections caused by *Candida* remains a public health issue, with high prevalence rates worldwide (Doi *et al.*, 2016; Pereira *et al*., 2016; Savastano *et al.*, 2016). The study of naturally-occurring molecules has gained relevance particularly because of the increasingly rates of microbial resistance and substantial toxicity caused by administration of current antifungal drugs (Coleman *et al.*, 2010; Doi *et al.*, 2016; Gulati & Nobile, 2016; Pereira *et al*., 2016; Savastano *et al*., 2016), such as fluconazole, itraconazole, miconazole and amphotericin B (Doi *et al.*, 2016; Gulati & Nobile, 2016; Savastano *et al*., 2016), which can cause adverse effects such as nausea and vomiting, headach, hepatotoxicity, gastrointestinal disturbance, skin irritation, burning sensation, deppending of class or type of antifungal agent (Millsop & Fazel, 2016).

Therefore, there is a need to investigate the effectiveness of natural products such as plant extracts as potential complementary therapeutic agents for dental use (De Araújo *et al*., 2018). *Syzygium cumini* (L.) Skeels (synonyms: *Syzygium jambolanum, Syzygium jambolana, Eugenia cumini*, *Eugenia jambolana*, *Eugenia Caryophyllifolia*), Myrtaceae, popularly known as jambolan, olive or purple olive, among other names, can be found in subtropical regions, including Brazil (Migliato *et al*., 2006; Migliato *et al*., 2007; Ayyanar & Subash-Babu, 2012; Baliga *et al*., 2013; Srivastava & Chandra, 2013; Chagas *et al*., 2015). This plant has been used in folk medicine for the treatment of various diseases especially diabetes for its hypoglycemic effects (Migliato *et al*., 2006; Migliato *et al*., 2007; Ayyanar & Subash-Babu, 2012; Baliga *et al*., 2013; Chagas *et al*., 2015). However, there are also reports of pharmacological studies that includes biological activities such as anti-inflammatory, cardioprotective, antioxidant, antimicrobial/antibacterial and antifungal effects (Migliato *et al*., 2007; Ayyanar & Subash-Babu, 2012; Baliga *et al*., 2013; Chagas *et al*., 2015). Especially, some studies highlight its antifungal activity against *Candida* spp. (Oliveira *et al*., 2007; Höfling *et al*., 2010; Pereira *et al*., 2016), such as *C. albicans* (Oliveira *et al*., 2007; Höfling *et al*., 2010; Pereira *et al*., 2016), *C. krusei* (Oliveira *et al*., 2007; Höfling *et al*.,2010), *C. dubliniensis, C. glabrata, C. guilliermondii, C. lusitaniae, C. parapsilosis, C. rugosa, C. tropicalis* and *C. utilis* (Höfling *et al*., 2010).

The leaves are specially used in dermopathies, gastropathies, constipation, leucorrhea, diabetes (Chagas *et al*., 2015) and there are evidences showing its antifungal activity (Oliveira *et al*., 2007; Pereira *et al*., 2016). Besides that, reports in the literature of different compounds such as flavonoids, terpenoids and phenolics, and specifically sitosterol, betulinic acid, crategolic acid, quercetin, myricetin, methylgallate, kaempferol (Srivastava & Chandra, 2013), acylated flavonol glycosides, quercetin, myricetin, myricitin, myricetin 3-O-4-acetyl-L-rhamnopyranoside, triterpenoids, esterase, galloyl carboxylase and tannin (Ayyanar & Subash-Babu, 2012) have been reported to be present in the leaves of *S. cumini*.

Thus, since previous evidence showed a potential antimicrobial effect of *S. cumini* leaves extract (Sc) (Pereira *et al*., 2016) and has encouraged further investigation on the antifungal activity of the extract against additional yeast strains. The present

study was designed to investigate the phytochemical composition of the extract and determine its antifungal activity against different reference strains and clinical isolates of *Candida* spp. Further toxicological assessment of the extract was carried out in the present study *in vitro* and *in vivo*.

Three hypotheses were tested during the evaluation of the biological properties of *S. cumini* extract, namely: (1) the extract has inhibitory activity against *Candida* spp.; (2) the extract is biologically compatible with human erythrocytes, and (3) the extract is not toxic in an *in vivo* model of *Galleria mellonella* larvae.

MATERIAL AND METHODS

Plant material

The plant material used in this study consisted of *Syzygium cumini* leaves, which were collected in August 2013 in Campina Grande, Paraíba State, Brazil (7º 22' 25" S, 35º 59' 32"W), botanically identified in herbarium Lauro Pires Xavier at the Department of Molecular Biology, Federal University of Paraíba, João Pessoa, Paraíba, with voucher number JPB 58.543. The present research was registered in the National System of Genetic Heritage Management and Association Traditional Knowledge of the Brazilian Ministry of the Environment unde number A3223F1. To obtain a hydroalcoholic extract, plant material was processed through cleaning, drying in an air-circulating oven at 40ºC until stabilization of final weight and grinding in a Wiley mill (SL 30 Solab, Piracicaba, SP, Brazil) of 10 mesh. Finally, hydroalcoholic extracts was prepared by maceration following the proportion 200 g of ground plant to 1000 mL of 70% hydroalcoholic solution (purity grade PA 99,5%) and after that the extract was subjected to evaporation under reduced pressure and lyophilized (LS 3000 Terroni®) at -20º to -40ºC (Pereira *et al*., 2016).

Phytochemical analysis: Extraction method

The plant material was extracted as previously described (Rigobello *et al*., 2015), with modifications. Briefly, Sc leaves were submitted to liquid-liquid extraction and solid-phase extraction. The extract obtained was weighed, dissolved into 40% ethanol solution (purity grade PA 99,5%), diluted into ultrapure water and filtered. The resulting extract (pH = 7.0) was placed in a separatory funnel, followed by addition of 10 g of NaCl. The organic compounds were extracted by the addition of ethyl acetate (purity grade PA). The mixture was allowed to stand, then the organic phase was partitioned and then transferred to a beaker to dry, followed by addition of 20 g of anhydrous $NaSO_4$ (purity grade 99%). The supernatant was filtered, and the extracted organic phase was placed into a desiccator until reduced to a volume of 3 mL.

Cromatographic analysis

The organic compounds present in the extract were identified by gas chromatography coupled to mass spectrometry (GC-MS) using a gas chromatograph (Thermo Scientific TRACE 1300) coupled to a mass spectrometer with quadrupole analyzer (Thermo Scientific ISQ-QD). The analysis was performed by means of chromatographic separation on an HP-5MS fused silica capillary column (30 m x 0.25 mm x 0.50 μm) (Varian Technlogies, EZ-Guard Columns) and on an SPB-624 column (30 m x 0.25 mm x 0.50 μm).

The GC oven temperature setting for both columns was 40°C for 2 min, 5°C/min to 70 °C maintained for 10 min, and 10°C/min to 200°C for 30 min. Helium was used as the carrier gas with a flow rate of 1 mL/min. The injector temperature used was 250°C in splitless mode for a ratio of 33.3.

The volume of the injected material was 1 μL and the detection was performed by a selective mass detector equipped with an electron impact source at 70 eV. Data acquisition was obtained in full scan mode. The temperature of the ion source and the transfer line of the mass spectrometer were 250°C and 275°C, respectively. The scanning range of *m/z* was from 50 to 650 with a solvent cut-off time of 5 min.

The chemical compounds were characterized by comparison between the mass spectra obtained in the GC-MS analysis and those found in the NIST library (National Institute of Standards and Technology) (The NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral library version 2.0 g. build May 19, 2011) as described (Rigobello *et al*., 2015), with modifications.

Microorganisms, growth conditions, and antifungal activity of Sc extract

Reference strains of *Candida albicans* (ATCC 10231), *C. glabrata* (ATCC 90030), *C. krusei* (ATCC 6258), *C. tropicalis* (ATCC 750) and clinical

strains of *C. albicans* (LM1 and LM3) were used. The inocula were prepared according to the protocol M27-A3 for yeasts (CLSI, 2008), with modifications, using Sabouraud Dextrose broth (Difco, Detroit, USA) and standardized in a spectrophotometer (Model GT 7220 BioPet Technologies, Monte Alto, Brazil) at 530 nm (absorbance values between 0.08-0.1) and was successively diluted to a final concentration of $2.5x10^3$ CFU/mL for microdilution assays.

The experiments were performed using 96-well microplates (Cralpast, Cotia, Brazil). The extract was tested at concentrations ranging from 1000 to 7.8 μg/mL, and the standard drugs nystatin and fluconazole (Sigma-Aldrich, St. Louis, MO) were tested at concentrations from 64 to 0.5 μg/mL. A yeast growth control, culture media sterility control and vehicle control (negative control) were also included in susceptibility assays.

The microplates were incubated at 37 °C for 24-48h. The Minimum Inhibitory Concentration (MIC) of the Sc extract and standard drugs was defined as the lowest concentration capable of inhibiting visible microbial growth (CLSI, 2008). The visual reading was further confirmed with the addition of resazurin (0.01%) to the wells of the microplate (Sigma-Aldrich, St. Louis, MO).

The Minimal Fungicidal Concentration (MFC) was determined by subculturing 10 μL of the wells corresponding to concentrations ≥ the MIC onto Sabouraud Dextrose Agar plates (Difco, Detroit, USA). The plates were incubated at 37 °C for 24-48 h, and the MFC was considered as the lowest concentration of the drug capable of inhibiting the subculture growth. The tests were carried out in triplicate of three independent experiments (n = 9) and the results were expressed as the mode of the replicates.

Effects of Sc extract on C. albicans growth kinetics

The effects of Sc extract on *C. albicans* (ATCC 10231) growth kinetics were determined as described (Klepser *et al*., 1997; Cantón *et al*., 2009; Dias De Castro *et al*., 2013), with modifications. The inocula and microdilution assays were prepared according to the step described above. After steps of microdilution, the microplates were incubated at 37 °C for 24h and during this period, the extract, nystatin (Sigma-Aldrich, St. Louis, MO) and control groups with the dilution vehicle and only inocula (yeast viability) were kept in contact with *C. albicans* cell suspension in the microplates.

At selected time intervals (0, 1, 2, 3, 4, 6, 8, 12 and 24 h), 10 μL of the samples of Sc extract and nystatin at concentrations equivalent to the 1xMIC, 2xMIC and 4xMIC and control groups were collected and plated onto Sabouraud Dextrose Agar (Difco, Detroit, USA) and incubated at 37 °C for 24-48h for count of CFUs. The experiment was carried out in triplicate (n = 3), and the results were expressed as mean CFU/mL.

Cytotoxicity analysis in human erythrocytes

The potential deleterious effects of Sc extract on human erythrocytes were determined *in vitro*. Human erythrocytes types A, B, AB, O were used, which were obtained from the Transfusion Unit of the Lauro Wanderley University Hospital at the Federal University of Paraíba. Samples were obtained from blood unusable for blood transfusions (blood to be discarded) (De Araújo *et al*., 2018; Lira *et al*., 2018).

The experiments followed the guidelines of the Resolution 466/2012 of the National Health Council and followed the principles outlined in the Helsinki Declaration for experimental research envolving humans. Therefore, the study was submitted and approved by the Research Ethics Committee at the State University of Paraíba, under protocol CAAE: 62508416.8.0000.5187. An informed consent of the hematological donor unit was obtained prior to material collection.

Hemolytic potential of Sc extract on human erythrocytes

Blood samples (types A, B, AB and O) were collected and added to 0.9% NaCl at a ratio of 1:30 and centrifuged (1540 g for 5 min) in triplicate. The final sediment was resuspended in 0.9% NaCl (purity grade PA) to obtain a suspension of 0.5% blood. After that, samples were used for the analysis of the hemolytic activity. In the group test, five hundred microliters of Sc extract at different concentrations (31.25, 62.5, 125, 250 and 500 μg/mL) were added to 2 mL suspensions of erythrocytes. The samples were incubated (1 h at 22 ± 2°C) under agitation (0,25 g). After that were centrifuged (1540 g for 5 min) and hemolysis was quantified by spectrophotometry at a wavelength of 540 nm (De Araújo *et al*., 2018; Lira *et al*., 2018).

Erythrocytes suspensions (0% hemolysis)

were used as a negative control and suspensions of erythrocytes plus Triton X-100 at 1% (purity grade PA) (100% hemolysis) were used as a positive control (De Araújo *et al*., 2018; Lira *et al*., 2018). The experiments were performed in triplicate (n = 3), and the results were expressed as mean ± standard error of the mean (SEM).

Analysis of osmotic fragility in human erythrocytes

Aliquots of 0.5 mL of the extract at different concentrations (31.25, 62.5, 125, 250 and 500 μg/mL) were incubated (1 h at 22 ± 2°C) with 2 mL of erythrocytes suspensions. The samples were centrifuged (1540 g for 5 min) and the supernatant was discarded. The red cells were resuspended in hypotonic sodium chloride solution (0.24%) (purity grade PA) and agitated at 0,25 g during 20 min at 22 ± 2°C. Afterwards, the samples were centrifuged (1540 g for 5 min) and hemolysis was quantified by spectrophotometry at a wavelength of 540 nm. Suspension of erythrocytes (0% hemolysis) was used as a negative control, and a solution of erythrocytes plus 0.24% sodium chloride solution was used as a positive control (100% hemolysis) (Lira *et al*., 2018). Three independent experiments were performed, and the results were expressed as percentage (mean ± standard error of the mean) of hemolysis as compared to the positive control group.

Oxidant and antioxidant potential of Sc extract on human erythrocytes in the presence of phenylhydrazine

A 30% suspension of erythrocytes in PBS (11.35 g NaH_2PO_4 .$2H_2O$; 24.36 g Na_2HPO_4; and 7.18 g NaCl for 1 L; pH 7.4) (purity grade PA), supplemented with glucose (200 mg/dL) at pH 7.6 was previously prepared (De Araújo *et al*., 2018). To investigate the oxidant potential 0.5 mL of the extract at different concentrations (31.25, 62.5, 125, 250 and 500 μg/mL) were incubated (1 h at 22 ± 2°C) under steady agitation (0,25 g) with 2 mL of this erythrocytes suspensions. Next, the samples were centrifuged (1540 g for 5 min) and the percentage of methemoglobin (MetHb) formation in relation to total hemoglobin (Hb) was quantified by spectrophotometry at a wavelength of 630 nm and 540 nm, respectively. Phenylhidrazine (purity grade PA), an oxidizing agent was used as a positive control. Three independent experiments were performed, and the results were expressed as percentage of methemoglobin (mHb) formation in relation to hemoglobin (Hb) - mHb (% Hb) as compared to the positive control group (De Araújo *et al*., 2018).

To investigate the antioxidant potential, after an incubation period of 1 h in the step described above, 1 mmol/L of phenylhydrazine (purity grade PA) was added. The suspensions were aerated and maintained under steady agitation (0,25 g) for 20 min at 22 ± 2°C. After this period, the samples were centrifuged (1540 g for 5 min), diluted in phosphate buffer solution (9 g $Na_2HPO_4.12H_2O$, 5.7g KH_2PO_4 to 1 L) (purity grade PA), and the percentage of metHb in relation to Hb was quantified by spectrophotometry at 630 nm and 540 nm respectively. The percentage of metHb formed was compared with the values obtained for vitamin C (20 mmol/L) (purity grade PA), an antioxidant agent. The results expressed as the methemoglobin formation percentage, in function of hemoglobin–MetHb (% Hb). All experiments were performed in triplicate (De Araújo *et al*., 2018).

Antioxidant potential of Sc extract on human erythrocytes in the presence of reactive oxygen species

This assay was performed according to the experiments described (Lira *et al*., 2018), with modifications. 0.5 mL of the extract at concentrations of 31.25, 62.5 and 125 μg/mL was incubated with 2 ml of a 0.5% erythrocytes suspension in 0.9% NaCl (purity grade PA) and in the presence of a 40 mM hydrogen peroxide solution (purity grade PA). Samples were incubated (4 h at 25 ± 2°C) and after that, were centrifuged (1540 g for 5 min) and hemolysis was quantified by spectrophotometry at a wavelength of 540 nm (Lira *et al*., 2018).

An erythrocyte suspension (0% hemolysis) was used as a negative control, and a solution of erythrocytes plus H_2O_2 (40 mM) was used as a positive control (100% hemolysis). A group containing an erythrocyte suspension plus H_2O_2 and Vitamin C (1000 μg/mL) (purity grade PA) was also tested as the reference antioxidant (Lira *et al*., 2018).

The experiments were performed in triplicate, and the results were expressed as percentage (mean ± standard error of the mean) of hemolysis as compared to the positive control group.

In vivo acute toxicity in *Galleria mellonella* systemic model

The acute systemic toxicity of Sc extract was determined as previously described (Megaw *et al*., 2015; Rochelle *et al*., 2016; Sardi *et al*., 2017), with modifications. Increasing doses of the extract with values above those with antimicrobial activity were tested to determine its LD_{50} (minimum dose able to kill 50% of the larvae over time). Ten larvae weighing between 0.2 and 0.3 g with no signs of melanization were randomly selected for each group. A total of 5 µL of the extract at doses of 1.0, 3.0, 7.0, 10.0 and 12.5 g/kg or the control (10% EtOH, v/v) (purity grade PA 99,5%), were injected into the hemocoel of each larva via the last left proleg using a 25-µL Hamilton syringe (Hamilton, Reno, NV). The larvae were incubated at 30°C and their survival was recorded at selected intervals for up to 72 h, observing if there is a finding of changes such as the development of signs of melanization and a presence of touch immobilization. After this evaluation period, the larvae displaying no movements upon touch and with high levels of melanization were counted as dead. The dose that killed 50% of the sample was considered to be the LD_{50} and the dose capable of killing 100% of the larvae was considered the lethal dose.

Statistical analysis

The growth kinetics data were analyzed by one-way ANOVA followed by Tukey's post-hoc test in SPSS Statistical Program for Windows®, version 20.0 (IBM, Chicago, USA). The cytotoxicity data were analyzed by one-way ANOVA followed by Dunnett's post-test. The values were expressed as mean ± standard error of the mean (SEM) or standard deviation of the mean (SD). The *in vivo* toxicity data were analyzed using the Kaplan-Meier survival curve and estimates of differences in survival were compared using the log-rank test. The data were analyzed in GraphPad Prism 6.0 software (San Diego, CA, USA), with a 5% significance level.

RESULTS

Phytochemical analysis

The chemical characterization of Sc extract considered compounds with at least ≥40% probability as compared to the reference mass spectra present in the NIST library. As shown in Table No. 1 and Table No. 2, a total of 14 different compounds were identified, with polar and nonpolar characteristics.

Antifungal activity

S. cumini extract was tested for its inhibitory (MIC) and lethal (MFC) activity against *Candida* spp. The fungicidal or fungistatic capacity of the extract was characterized based on the MFC/MIC ratio, as shown in Table No. 3. The extract showed antifungal activity on all *Candida* spp. strains tested, with MIC values between 31.25 and 125 µg/mL, and MFC values between 250 µg/mL and ≥ 1000 µg/mL. Based on the MFC/MIC ratio (Siddiqui *et al*., 2013), Sc extract can be classified as fungistatic.

Effects of Sc extract on C. albicans growth kinetics

As shown in Figure No. 1, Sc extract significantly affected the growth kinetics of *C. albicans* after 8 h of treatment at different concentrations as compared to nystatin (p=0.001). After 12 h and 24 h, the extract no longer demonstrated growth inhibitory effects, with results similar to those observed in the negative control group (p>0.05). The findings indicated that Sc extract has a fungistatic effect, particularly when in contact with *C. albicans* cells for at least 8 h.

Hemolytic potential of Sc extract on human erythrocytes

As shown in Figure No. 2, the extract was able to cause concentration-dependent hemolysis of human erythrocytes. Similar results were also found when compared to the positive control (Table No. 4). Type O erythrocytes showed greater hemolytic susceptibility even at the lowest concentrations of the extract, whereas types AB and O erythrocytes were the most sensitive blood types at the highest tested concentrations of Sc extract, with higher percentages of cytotoxicity.

Analysis of osmotic fragility in human erythrocytes

As shown in Figure No. 3, Sc extract was able to decrease hemolysis levels in human erythrocytes at lower concentrations, with statistically significant difference for most of the blood types and concentrations tested. Better results were found mainly on blood type B erythrocytes.

Antioxidant potential of Sc extract on human erythrocytes in the presence of reactive oxygen species

S. cumini extract was able to reduce the hemolytic

percentage of erythrocytes exposed to H_2O_2, with a statistically significant difference (p>0.0001) at the lowest concentrations (31.25 µg/mL and 62.5 µg/mL), as shown in Figure No. 4. These findings indicate that Sc extract had an antioxidant effect on oxidative stress induced by H_2O_2. Interestingly, the results were relatively similar to those obtained with a standard antioxidant (Vitamin C) used as control in the assay.

Table No. 1
Identification of the compounds present in *Syzygium cumini* leaves extract by gas chromatography coupled to mass spectrometry (HP-5MS capillary column)

PEAK	RT (min)	COMPOUND	CAS NUMBER	P (%)	MF	MW
1	9.36	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	10230-62-3	87.74	$C_6H_8O_4$	144
2	17.86	Thymine	65-71-4	65.32	$C_5H_6N_2O_2$	126
3	19.41	3,3-Dimethylthietane	13188-85-7	67.50	$C_5H_{10}S$	102
4	19.47	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	28564-83-2	93.09	$C_6H_8O_4$	144
5	20.73	Alpha-Terpineol	98-55-5	51.47	$C_{10}H_{18}O$	154
6	21.98	5-Hydroxymethylfurfural	67-47-0	40.63	$C_6H_6O_3$	126
7	26.19	Phenol, 2,6-bis(1,1-dimethylethyl)-	128-39-2	40.17	$C_{14}H_{22}O$	206
8	26.42	Benzoic acid,4-ethoxy-,ethyl ester	23676-09-7	72.01	$C_{11}H_{14}O_3$	194
9	28.13	Ethyl α-D-glucopyranoside	19467-01-7	53.59	$C_8H_{16}O_6$	208
10	31.17	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	82304-66-3	64.36	$C_{17}H_{24}O_3$	276
11	32.33	Hexadecanoic acid,ethyl ester	628-97-7	47.02	$C_{18}H_{36}O_2$	284
12	35.11	Phytol	150-86-7	43.94	$C_{20}H_{40}O$	296

Legend: R: Retention time; P: percentage of probability based on comparison with the mass spectra library; MF: molecular formula; MW: molecular weight)

Table No. 2
Identification of the compounds present in *Syzygium cumini* leaves extract by gas chromatography coupled to mass spectrometry (SPB – 624 column)

PEAK	RT (min)	COMPOUND	CAS NUMBER	P (%)	MF	MW
1	24.59	2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	10230-62-3	62.60	$C_6H_8O_4$	144
2	24.92	2-(5H)-furanone	497-23-4	60.25	$C_4H_4O_2$	84
3	29.63	4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	28564-83-2	94.17	$C_6H_8O_4$	144
4	32.53	5-Hydroxymethylfurfural	67-47-0	89.15	$C_6H_6O_3$	126
5	34.68	Hydroquinone	123-31-9	40.73	$C_6H_6O_2$	110

(Legend: R: Retention time; P: percentage of probability based on comparison with the mass spectra library; MF: molecular formula; MW: molecular weight)

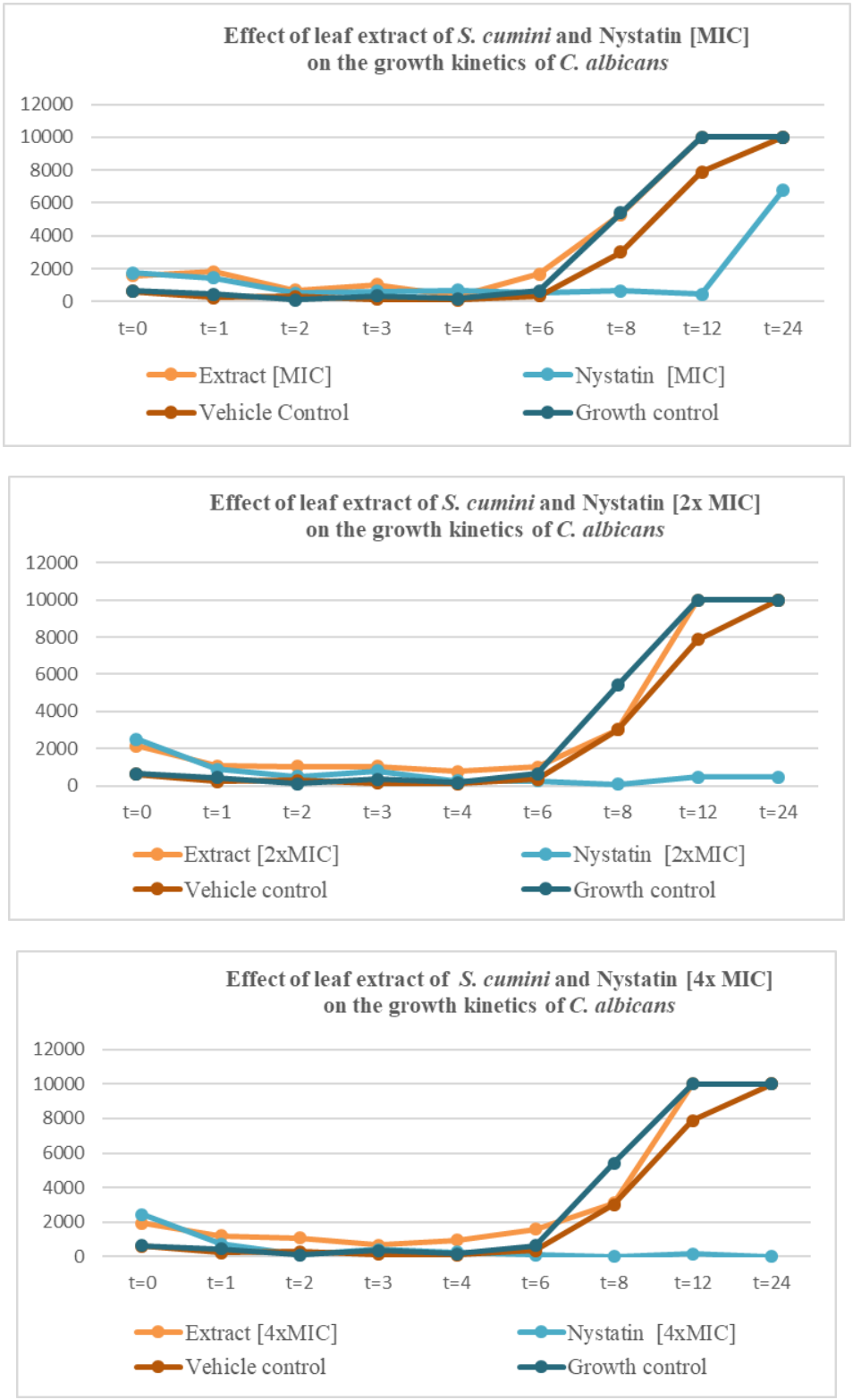

Figure No. 1
Effect of *Syzygium cumini* leaves extract and Nystatin on the growth kinetics of *Candida albicans* (ATCC 10231). The results are expressed as mean CFU/mL

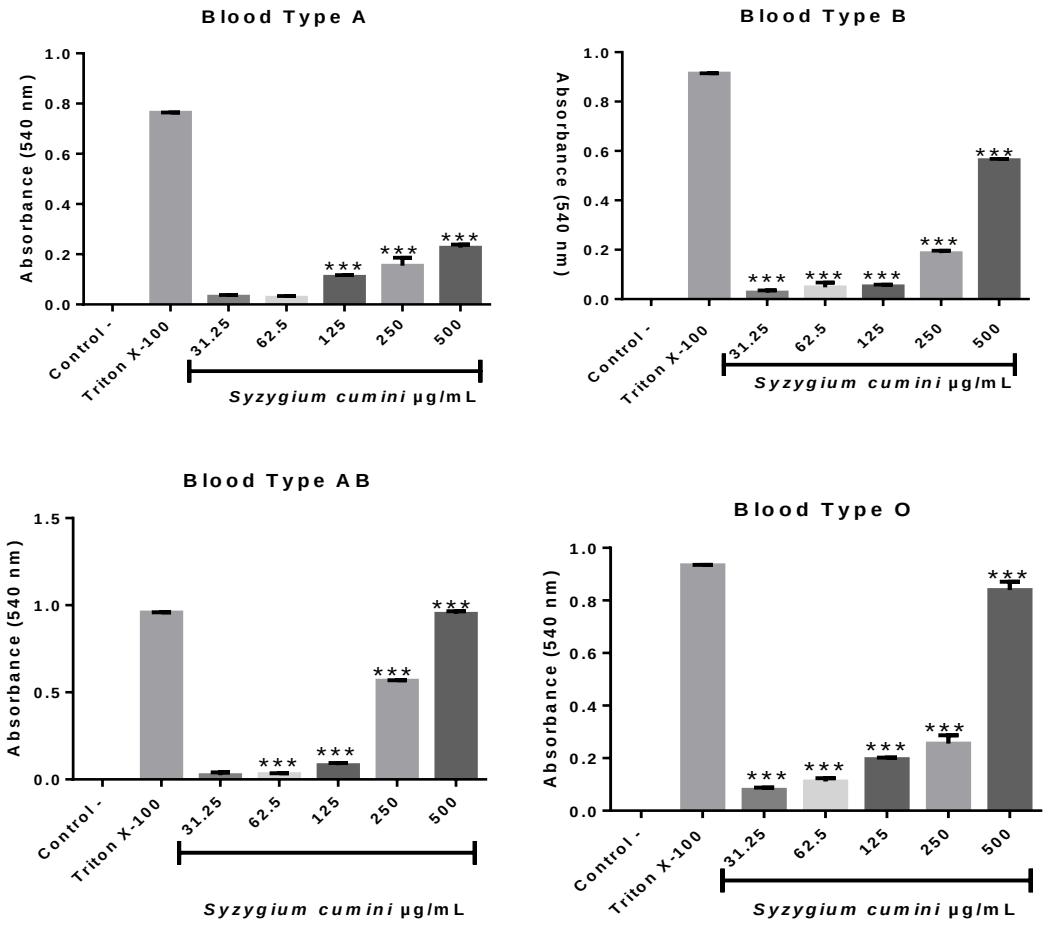

Figure No. 2

Hemolytic effects of *Syzygium cumini* leaves extract on human erythrocytes of blood types A, B, AB and O as compared to the negative control. The results are expressed as mean ± SEM. Analysis by one-way ANOVA followed by Dunnett's post-test. (*p<0.05; **p<0.001; ***p<0.0001)

Table No. 3
Antifungal activity of the *Syzygium cumini* leaves extract and standard drugs on *Candida* spp. strains

Strain	Sc			NYS			FLU		
	MIC (µg/ml)	MFC (µg/ml)	MFC/MIC	MIC (µg/ml)	MFC (µg/ml)	MFC/MIC	MIC (µg/ml)	MFC (µg/ml)	MFC/MIC
***C. albicans* (ATCC 10231)**	125	1000	8	4	64	16	32	>64	>2
***C. glabrata* (ATCC 90030)**	62.5	>1000	>16	2	64	32	>64	>64	>1
***C. albicans* (ATCC 10231) + *C. glabrata* (ATCC 90030)**	125	1000	8	4	32	8	>64	>64	>1
***C. krusei* (ATCC 6258)**	31.25	250	8	>64	>64	>1	>64	>64	>1
***C. tropicalis* (ATCC 750)**	62.5	>1000	>16	4	>64	>16	8	>64	>8
***C. albicans* (LM 01)**	31.25	1000	32	4	16	4	64	>64	>1
***C. albicans* (LM 03)**	31.25	>1000	>32	2	>64	>32	32	>64	>2

(Legend: Sc: *Syzygium cumini* extract; NYS: Nystatin; FLU: Fluconazole; MIC: Minimum inhibitory concentration; MFC: Minimum Fungicidal Concentration; MFC/MIC: MFC/MIC ratio)

Table No. 4
Percentage of hemolysis caused by *Syzygium cumini* leaves extract on human erythrocytes of blood types A, B, AB and O, as compared to the positive control (1% Triton X-100)

Sc extract concentration / Blood type	% hemolysis				
	31.25 µg/mL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
A	**4.0**	**3.5**	**14.4**	**20.1**	**29.5**
B	**2.8**	**5.2**	**5.7**	**20.0**	**61.0**
AB	**2.5**	**3.1**	**8.4**	**59.0**	**99.1**
O	**8.0**	**11.0**	**23.0**	**27.0**	**89.0**

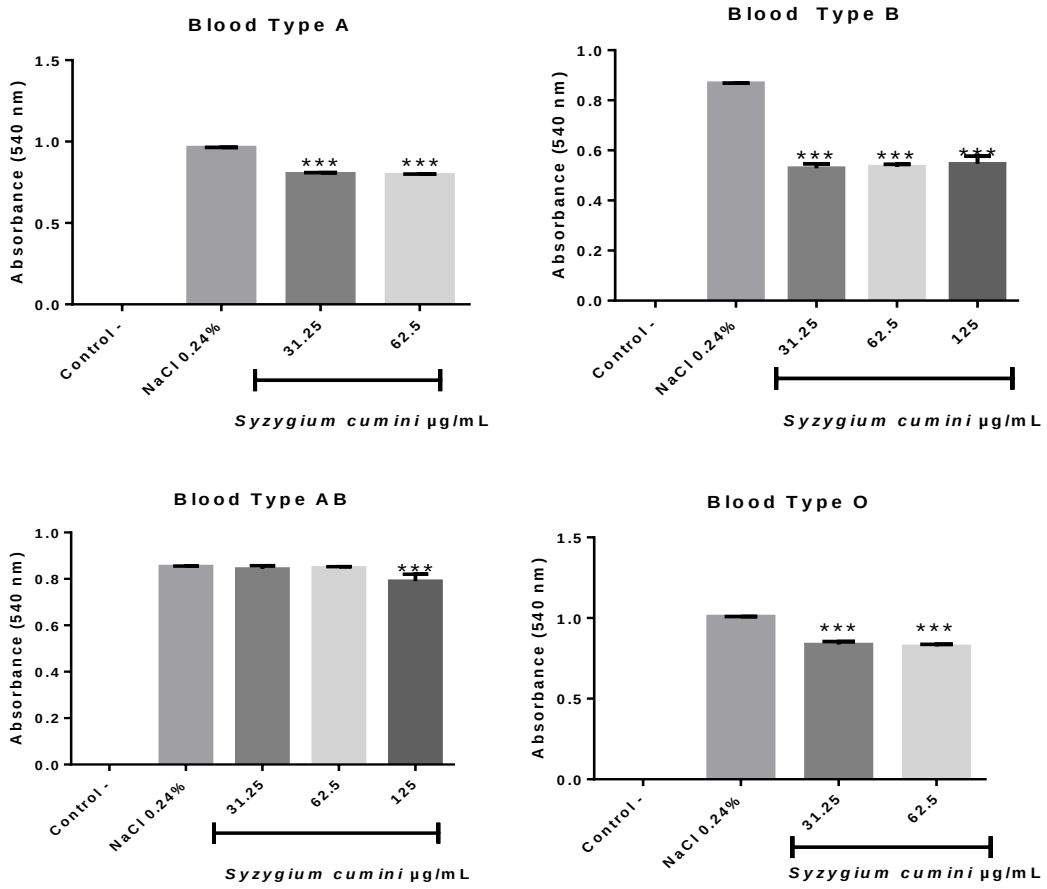

Figure No. 3
Anti-hemolytic effects of *Syzygium cumini* leaves extract on human erythrocytes of blood types A, B, AB and O in hypotonic solution (0.24% NaCl). The results were compared to the positive control and are expressed as mean ± SEM. Analysis by one-way ANOVA followed by Dunnett's post-test (*p<0.05; **p<0.001; ***p<0.0001)

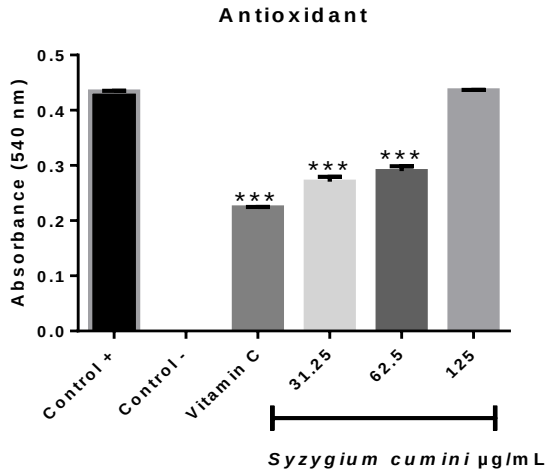

Figure No. 4
Antioxidant effects of *Syzygium cumini* leaves extract on hemolysis induced by hydrogen peroxide in human erythrocytes of blood type AB. The results were compared to the positive control and are expressed as mean ± SEM. Analysis by one-way ANOVA followed by Dunnett's post-test (p*<0.05; ***p*<0.001; ****p*<0.0001)**

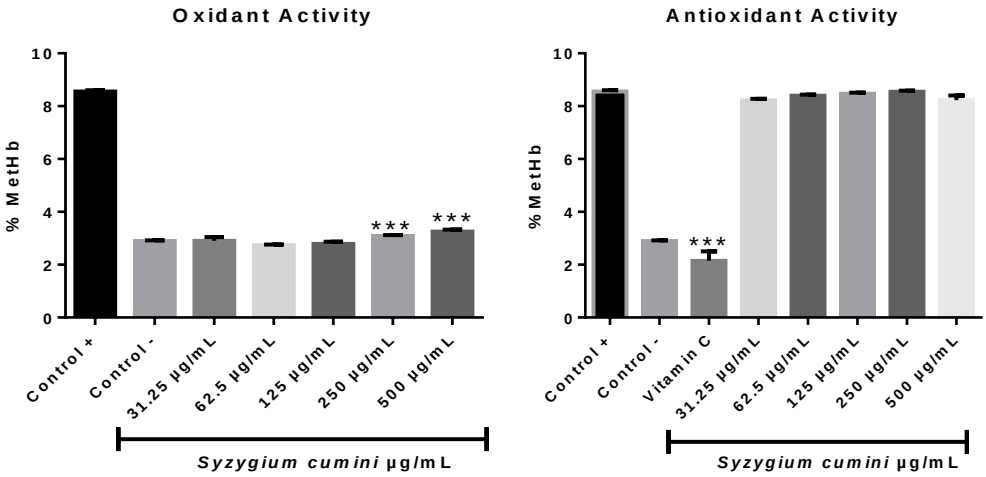

Figure No. 5
Oxidant and antioxidant effects of *Syzygium cumini* leaves extract on on human erythrocytes of blood type AB. The results were expressed as the mean percentage of metHb formation as compared to the negative (oxidant assay) and positive (antioxidant assay) controls. Analysis by one-way ANOVA followed by Dunnett's post-test. (p*<0.05; ***p*<0.001; ****p*<0.0001)**

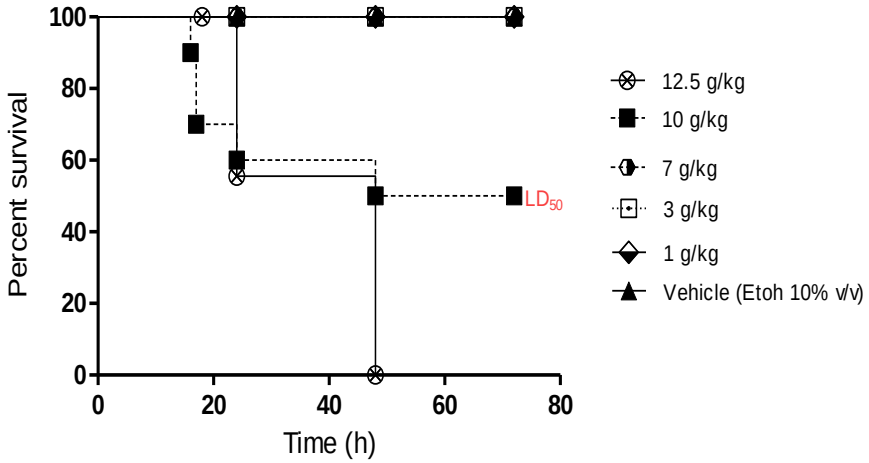

Figure No. 6
***In vivo* acute systemic toxicity of *S. cumini* leaves extract in *Galleria mellonella* larvae. The graph shows a survival curve of larvae treated with the extract at different concentrations, or dilution vehicle, over time (p>0.05, Log-rank test)**

Oxidant and antioxidant potential of Sc extract on human erythrocytes in the presence of phenylhydrazine

The percentages of methemoglobin (MetHb) and hemoglobin (Hb) were quantitated upon erythrocyte exposure to Sc extract and phenylhydrazine. As shown in Figure No. 5, Sc extract was capable to promote oxidation of Hb into metHb only at the highest concentrations tested as compared to the negative control. At concentrations equal to, or lower than, 125 µg/mL, Sc extract did not induce the occurrence of Hb oxidation in human erythrocytes.

On the other hand, the antioxidant activity test (Figure No. 5) showed that, as compared to the results obtained with phenylhydrazine, the metHb/Hb formation levels induced by the erythrocytes exposed to the positive control and treated later with Sc extract were not significantly reduced. These findings suggest that Sc extract does not have a protective effect in cells previously exposed to phenylhydrazine-induced oxidative reactions.

In vivo toxicity in Galleria mellonella systemic model

S. cumini extract was tested for its *in vivo* toxicity in a systemic model of *G. mellonella* larvae. The extract showed low toxicity on the larvae (Figure No. 6) when administered at doses up to 7 g/kg (p>0.05), with no significant difference when compared to the dilution vehicle (10% EtOH, v/v) (p>0.05). At the doses of 10 g/kg (LD_{50}) and 12.5 g/kg, Sc extract affected the viability of the larvae, killing 50% and 100% of the larvae, respectively (p<0.0001).

DISCUSSION

The alarmingly high microbial resistance rates to current antifungals (Doi *et al*., 2016; Gulati & Nobile, 2016; Savastano *et al*., 2016) has reinforced the need for discovery of new substances with pharmacological potential (Coleman *et al*., 2010; Pereira *et a*l., 2016), which includes those from medicinal plants, such as *S. cumini.*

Different secondary metabolites are responsible for the biological effects of medicinal plants (De Araújo *et al*., 2018). Consistent with this, the literature has related the antimicrobial activity of extracts with the presence of phenolic compounds (Harsha & Anilakumar, 2014; Cartaxo-Furtado *et al*., 2015), flavonoids (Cartaxo-Furtado *et al*., 2015) and tannins (Gowri & Vasantha, 2010; Cartaxo-Furtado *et al*., 2015). Moreover, phenolic compounds (Evensen & Braun, 2009), flavonoids (Pereira *et al*., 2016) and saponins (Coleman *et al*., 2010; Gowri & Vasantha, 2010; Pereira *et al*., 2016) been reported as having antifungal activity.

Thus, the antimicrobial activity of *S.*

cumini leaves extract can be attributed to bioactive compounds present in its composition, including saponins, phenolic compounds, tannins and flavonoids, since they were preliminarily identified in its composition (Pereira *et al*., 2016). However, the chromatographic identification carried out in the present study provided additional details on the chemical composition of Sc extract, including the determination of the probable major compounds responsible for the pharmacological activity of the extract. Among the compounds detected in Sc extract and their respective biological activity are: antimicrobial and antifungal activity of 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, a flavonoid compound (Gopalakrishnan & Udayakumar, 2014; Sunita & Manju, 2017; Teoh & Don, 2014; Teoh *et al*., 2011; Peng & Don, 2013); antimicrobial activity of Benzoic acid, 4-ethoxy-, ethyl ester, an aromatic acid ester (Daffodil *et al*., 2012); 7,9-Di-*tert*-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione, a flavonoid compound (Sharif *et al*., 2015; Sunita & Manju, 2017); 2-(5H)-furanone, a terpene compound (Trizna *et al*., 2015; Sharafutdinov *et al*., 2017); alpha-terpineol (Mahdavi *et al*., 2017), a monoterpenoid compound (Satyal *et al*, 2017); hexadecanoic acid, ethyl ester, a polyphenolic compound (Sharma *et al*., 2018) and hydroquinone, a phenolic compound (Jyoti *et al*., 2016; Gutiérrez *et al*., 2017; Żbikowska *et al*., 2017); and antifungal activity of alpha-terpineol (Satyal *et al*., 2017). The presence of these substances in *S. cumini* extract suggest that the biological effects attributed to these compounds may be responsible for the potentially promising antimicrobial activity of the extract.

Based on its MIC values, Sc extract showed an active antimicrobial potential (MIC < 1000 μg/mL) (Holetz *et al*., 2002; Morales *et al*., 2008) on all tested *Candida* spp. strains, more specifically a moderate (MIC: 100-500 μg/mL) to high (MIC < 100 μg/mL) activity (Holetz *et al*., 2002; Morales *et al*., 2008), depending on the strain. The MFC/MIC ratio indicated a fungistatic activity of Sc extract (MFC/MIC ≥ 4) (Siddiqui *et al*., 2013) on all *Candida* ssp strains.

Remarkably, some *Candida* strains tested in our study were found to be less susceptible to fluconazole treatment, which is consistent with current microbial resistance reports (Doi *et al*., 2016; Gulati & Nobile, 2016; Savastano *et al*., 2016).In addition, the combination of *C. albicans* (ATCC 10231) and *C. glabrata* (ATCC 90030) cultures was less susceptible to the action of Sc extract and fluconazole, which suggests that coinfection plays a significant role in microbial resistance to antimicrobials such as fluconazole (Gomes *et al*., 2011; Savastano *et al*., 2016), miconazole, itraconazole and amphotericin B (Savastano *et al*., 2016).

S. cumini extract had inhibitory activity against *Candida* spp., which confirmed the first hypothesis of this study. The extract was also tested for its *in vitro* cytotoxicity in human erythrocytes and *in vivo* toxicity in *Galleria mellonella* larvae. The hemolytic activity test used herein has been widely reported in the literature to investigate the cytotoxic potential of chemical substances (Tupe *et al*., 2015; Mehreen *et al*., 2016; Vo *et al*., 2017; De Araújo *et al*., 2018; Lira *et al*., 2018; Figueirêdo Júnior *et al*., 2019). To complement the analysis of hemolysis, we further carried out an osmotic fragility assay (He *et al*., 2009; Waczuk *et al*., 2015; Duarte *et al*., 2016; De Araújo *et al*., 2018; Lira *et al*., 2018; Figueirêdo Júnior *et al*., 2019). To the best of our knowledge, this is the first study using these *in vitro* and *in vivo* toxicological models to determine the toxicity of Sc extract.

The results of these tests suggest that Sc extract had a low hemolytic activity (< 40% hemolysis) as compared to the positive control for most of the concentrations tested. At the highest concentrations, however, the hemolytic activity of Sc extract ranged from moderate (40-80 % hemolysis) to high (> 80 % hemolysis) depending on the blood type (Rangel *et al*., 1997). At antifungal concentrations (MIC ≤ 125 μg/mL), Sc extract demonstrated low hemolytic activity on human erythrocytes of all blood types. Hence, Sc extract cytotoxicity seems to be negligible at these concentrations, which is desirable for future clinical use.

Hemolysis caused by Sc extract at higher concentrations may be a result of the presence of phytochemical compounds such as saponins, which are known to display hemolyzing properties (Coleman *et al.*, 2010). In this context, as erythrocytes contain high concentrations of polyunsaturated fatty acids, molecular oxygen and ions linked in the ferrous state (Lira *et al*., 2018), these cells are affected by reactive oxygen species due to high cell concentration of oxygen and hemoglobin (Hb) and the absence of cellular

components to synthesize new proteins (Carl *et al*., 2016). So, since erythrocytes undergo structural and functional changes causing dysfunction of the cationic pumps due to oxidative modifications in lipids and membrane proteins, which can destabilize them (Carl *et al*., 2016), the occurrence of hemolysis can be justified by reactions involving free radicals which making the cells susceptible to lipid peroxidation in cell membranes (Lira *et al*., 2018). The differences in the hemolytic levels indicated blood-type related cytotoxicity of Sc extract. This fact could be justified by differences in carbohydrate structures present on some membrane glycoproteins and glycolipids of erythrocytes from hosts with different blood types in the ABO system. (Batissoco, Novaretti, 2003; Daniels, 2009; Paiva *et al*., 2009; Carl *et al*., 2016). These diferences are attributed to antigenic variability denominated antigens A and B synthesized by glycosyltransferases that catalyze the addition of specific monosaccharides to the precursor, denominated H antigen (Batissoco, Novaretti, 2003; Daniels, 2009; Paiva *et al*., 2009; Carl *et al*., 2016). Thus, the pertinent considerations regarding the chemical composition of the extract and the inherent differences in the erythrocytes of the different blood groups may justify the variations observed in the hemolytic profiles induced by Sc extract.

The evaluation of the anti-hemolytic activity of Sc extract allowed to estimate that at certain concentrations and depending on the blood type, it exerts a protective effect on the cell membrane of erythrocytes exposed to osmotic stress (He *et al*., 2009; Waczuk *et al*., 2015; Duarte *et al*., 2016; Figueirêdo Júnior *et al*., 2019). This effect could be evidenced by the reduced percentage of cellular damage in cells previously treated with Sc extract. Taken altogether, the results of the hemolysis and osmotic fragility tests revealed that Sc extract may have a dual role either inducing or protecting from cell membrane damage depending on the concentration used.

The need to investigate new substances with antioxidant activity (Mohamed *et al*., 2013; Lira *et al*., 2018; Figueirêdo Júnior *et al*., 2019) encouraged us to determine the antioxidant potential of Sc extract. The findings of our study suggest that, at low concentrations, Sc extract may be beneficial by acting as an antioxidant agent against hydrogen peroxide-induced oxidative challenge (Mohamed *et al*., 2013; Harsha & Anilakumar, 2014; Figueirêdo Júnior *et al*., 2019).

Phenolic compounds (Mohamed *et al*., 2013; Harsha & Anilakumar, 2014; Lira *et al*., 2018), flavonoids (Mohamed *et al*., 2013; Harsha & Anilakumar, 2014), terpenes (Santos *et al*., 2013) and saponins (Gowri & Vasantha, 2010) have been reported to have antioxidant activity and may explain the antioxidant properties of Sc extract observed in our study. However, the chemical compounds identified in the chromatographic analysis may also play a role in the antioxidant activity of Sc extract, including 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (Čechovská *et al*., 2011; Teoh *et al*., 2011; Peng & Don, 2013; Gopalakrishnan & Udayakumar, 2014), a flavonoid compound (Gopalakrishnan & Udayakumar, 2014); 5-Hydroxymethylfurfural (Kim *et al*., 2011; Zhao *et al*., 2013; Li *et al*., 2015; Mopuri *et al*., 2018), an intermediate produced through the degradation of hexoses and the Maillard reaction (Zhao *et al*, 2013); 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one, a furan ketone (Gopalakrishnan & Udayakumar, 2014); phytol (Santos *et al*., 2013; Jorge *et al*., 2017), a diterpene compound (Santos *et al*., 2013; Gopalakrishnan & Udayakumar, 2014; Jorge *et al*, 2017) and hydroquinone (Erenler *et al*., 2016) a phenolic compound (Gutiérrez *et al*., 2017). Thus, the presence of these molecules of different families can direct and justify the antioxidant properties of Sc extract.

The toxicological findings obtained in our study partially confirmed the second hypothesis that the extract is biologically compatible with human erythrocytes. While a thorough toxicological assessment in other relevant *in vivo* models is pending, the results obtained in the present study support the evidence that Sc extract has low toxicity against other cell lines, such as human keratinocytes and murine macrophages (Pereira *et al*., 2016). Thus, these results suggest that at antifungal concentrations, *S. cumini* extract demonstrated low cytotoxicity in different cell lines.

We further determined the toxicity of Sc extract using a well-known, validated *in vivo* model (Megaw *et al*., 2015; Rochelle *et al*., 2016; Sardi *et al*., 2017; Allegra *et al*., 2018), which indicated a negligible toxicity of the extract upon systemic administration in *G. mellonella* larvae. In this model, the LD_{50} of the extract was extremely high values

(500.000 µg/mL), i.e. 4.000 – 160.000 times greater than its MIC values against planktonic cells (depending on the fungal strains), as verified in the microbiological tests. In addition, it is important to consider that such concentration is 1.000x greater than that capable of effectively disrupting *Candida albicans* biofilms (500 µg/mL), as previously described (Pereira *et al*., 2016). Thus, the *in vivo* analysis in *G. mellonella* suggests a large margin of safety for the use of the extract at antifungal concentrations against planktonic or biofilm *Candida spp*. These findings confirm the third hypothesis tested in this study that under these conditions Sc extract does not promote toxicity in *G. mellonella* larvae.

Taken altogether, the findings of this study support the view that further research should focus on other toxicological assays like acute and chronic toxicological screening and mutagenic activity of Sc leaves extract using other relevant *in vivo* and *in silico* (if applicable) models.

CONCLUSION

The present study demonstrated that the leaf extract of *S. cumini* has various substances associated with antimicrobial effects. *S. cumini* extract has promising antifungal activity on *Candida* spp. and showed a fungistatic activity, with significant inhibitory effects on *C. albicans* growth kinetics. In terms of toxicity, the extract showed low cytotoxicity against human erythrocytes when used at antifungal concentrations, as well as low hemolytic activity and protective effects against osmotic stress-induced hemolysis. The extract did not oxidize hemoglobin in erythrocytes and displayed antioxidant activity at low concentrations upon hydrogen peroxide-induced stress. Lastly, the extract showed a very large margin of safety in an *in vivo* invertebrate model of systemic toxicity. Taken altogether, the findings of our study provide evidence on the effects of *S. cumini* leaves extract as an alternative antifungal agent. Further research should investigate whether the biological activity of the extract is due to the presence of specific compounds or to the mixed, complex combination of phytochemicals present therein. Moreover, further studies should focus on the toxicological parameters of the extract for future clinical use in relevant mammal and *in silico* (if applicable) models.

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Complementary material

Effect of *Syzygium cumini* leaves extract and Nystatin on the growth kinetics of *Candida albicans* (ATCC 10231). The results are expressed as mean CFU/mL)

Different letters indicate statistically significant diferences

T	Gr	Conc.	Mean	SD	*	*p*
0h	**SC**	MIC	1566,67	152,75	a	0,697
		2xMIC	2133,33	896,29	a	
		4xMIC	1933,33	577,35	a	
	Nys	MIC	1733,33	776,75	a	
		2xMIC	2500,0	1135,78	a	
		4xMIC	2433,33	115,47	a	
	VC		611,11	280,38	a	
	CC		655,0	72,78	a	
1h	**SC**	MIC	1800,0	458,26	a	0,059
		2xMIC	1066,67	115,47	a	
		4xMIC	1200,0	871,78	a	
	Nys	MIC	1433,33	650,64	a	
		2xMIC	900,0	624,5	a	
		4xMIC	733,33	305,51	a	
	VC		233,33	150,0	a	
	CC		444,33	120,3	a	
2h	**SC**	MIC	700,0	458,26	a	0,100
		2xMIC	1033,33	665,83	a	
		4xMIC	1100,0	458,26	a	
	Nys	MIC	533,33	321,46	a	
		2xMIC	500,0	264,58	a	
		4xMIC	133,33	152,75	a	
	VC		300,0	223,61	a	
	CC		100,0	86,6	a	
3h	**SC**	MIC	1033,33	901,85	a	0,095
		2xMIC	1033,33	1040,83	a	
		4xMIC	666,67	152,75	a	
	Nys	MIC	633,33	208,17	a	
		2xMIC	800,0	624,5	a	
		4xMIC	433,33	450,92	a	
	VC		133,33	141,42	a	
	CC		355,33	344,56	a	
4h	**SC**	MIC	333,33	57,74	a	0,135
		2xMIC	766,67	57,74	a	
		4xMIC	966,67	351,19	a	
	Nys	MIC	700,0	721,11	a	
		2xMIC	266,67	115,47	a	
		4xMIC	266,67	152,75	a	
	VC		100,0	158,11	a	
	CC		166,33	152,53	a	
6h	**SC**	MIC	1666,67	1858,31	a	0,096
		2xMIC	1000,0	360,56	a	
		4xMIC	1600,0	1228,82	a	
	Nys	MIC	566,67	814,45	a	
		2xMIC	266,67	288,68	a	
		4xMIC	100,0	173,21	a	
	VC		344,44	412,65	a	
	CC		655,0	692,96	a	
8h	**SC**	MIC	5300,0	4256,76	a	0,001
		2xMIC	3000,0	2457,64	a	

		4xMIC	3100,0	1539,48	a	
	Nys	MIC	666,67	305,51	b	
		2xMIC	66,67	57,74	bc	
		4xMIC	0,0	0,0	c	
	VC		3022,22	3592,28	a	
	CC		5400,0	4267,32	a	
12h	**SC**	MIC	10000,0	0,0	b	0,001
		2xMIC	10000,0	0,0	b	
		4xMIC	10000,0	0,0	c	
	Nys	MIC	466,67	115,47	a	
		2xMIC	466,67	461,88	a	
		4xMIC	166,67	288,68	a	
	VC		7877,78	3281,68	a	
	CC		10000,0	0,0	a	
24h	**SC**	MIC	10000,0	0,0	b	0,001
		2xMIC	10000,0	0,0	b	
		4xMIC	10000,0	0,0	b	
	Nys	MIC	6800,0	5542,56	a	
		2xMIC	466,67	723,42	a	
		4xMIC	0,0	0,0	a	
	VC		10000,0	0,0	a	
	CC		10000,0	0,0	a	

(Legend: T= Time; Gr =Groups (Sc: *S. cumini* extract; Nyst: nystatin; VC: vehicle control; GC: growth control); Mean: mean CFU/mL; SD: standard deviation; *p*: *p*-value of the statistical analysis