Vol.65: e22210539, 2022
https://doi.org/10.1590/1678-4324-2022210539
ISSN 1678-4324 Online Edition

Article – Human and Animal Health

Anticandidal Activity of Hydroalcoholic Extract of *Phyllanthus niruri* L. (Stone-Breaker)

Flávia Camila Maia[1]
https://orcid.org/0000-0003-1141-6661

Felipe Jóia [1]
https://orcid.org/0000-0003-3842-889X

Gayan Kanchana Wijesinghe[1,2]
https://orcid.org/0000-0003-3304-9110

Vanessa da Silva Cardoso[1]
https://orcid.org/0000-0001-6950-6841

Janaína Priscila Barbosa[1]
https://orcid.org/0000-0001-5356-0325

Valéria Alessandra Prado Defávari Franco[1]
https://orcid.org/0000-0002-3258-769X

Simone Nataly Busato de Feiria [1]
https://orcid.org/0000-0001-6200-9893

Paula Cristina Anibal[1]
https://orcid.org/0000-0001-8587-4636

Thais Rossini Oliveira[1]
https://orcid.org/0000-0001-5675-6067

José Francisco Höfling[1]
https://orcid.org/0000-0002-6054-0688

Giovana Claudia Boni [1]
https://orcid.org/0000-0002-6900-1162

[1]Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba, Departamento de Diagnóstico Oral, Área de Microbiologia e Imunologia, Piracicaba, São Paulo, Brasil; [2]University of Sri Jayewardenepura, Faculty of Allied Health Sciences, Department of Medical Laboratory Sciences, Nugegoda, Sri Lanka.

Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Jane Manfron Budel

Received: 17-Aug-2021; Accepted: 17-Mar-2022.

*Correspondence: flcmaia@hotmail.com; Tel.: +55-19-981447959 (F.C.M.).

HIGHLIGHTS

- Hydroalcoholic extract of *Phyllanthus niruri* (HE-Pn) demonstrates anti-*Candida* activity.
- HE-Pn reduces the germ-tube formation and adhesion of *Candida albicans*.
- HE-Pn causes cell wall detachment, cytoplasmic granulation, vacuolation and chromatin condensation.
- HE-Pn shows no toxicity on human keratinocyte cell line.

Abstract: *Candida* is becoming more resistant to conventional treatments, and causes persistent and severe infections. This study evaluates the antifungal and virulence activities of the hydroalcoholic extract of *Phyllanthus niruri* (HE-Pn) on *Candida*. HE-Pn was prepared by maceration technique. Chemical composition of HE-Pn was determined using Gas Chromatography-Mass Spectrometry (GS-MS). Antifungal screening was done using agar well diffusion. CLSI M27-A3 was used to determine the Minimum Inhibitory (MIC) and Fungicidal Concentrations (MFC). Effects of HE-Pn on adhesion and germ tube of *C. albicans* (ATCC MYA-2876) were determined using XTT assay and germ tube formation assay, respectively. Transmission Electron

Microscopy (TEM) was performed to visualize the post-exposure cellular changes. HE-Pn cytotoxicity was determined using human keratinocyte cell line (HaCaT). Chlorhexidine digluconate (2 mg/mL) was used as the positive control. Linolenic acid ethyl ester was the most abundant chemical component of HE-Pn. All strains tested were sensitive to HE-Pn. MIC were 0.03 - 8 mg/mL and MFC were 0.5 - 64 mg/mL for all test strains. *C. albicans* (ATCC MYA-2876) showed 50% of adhesion reduction with > 4 mg/mL of HE-Pn and germ-tube formation was inhibited with 0.25 and 2 mg/mL. TEM exhibited cytoplasmic granulation, intracellular vacuoles, detachment of cell wall and plasma membrane and chromatin condensation of *Candida*. No toxicity of HE-Pn was noted on HaCaT cells. HE-Pn shows an anti-*Candida* activity and can be used as an inhibitory agent against adhesion and germ tube formation of *Candida albicans* (ATCC MYA-2876) without causing any toxicity to human cells.

Keywords: *Phyllanthus niruri*; hydroalcoholic; plant extract; *Candida albicans*; HaCaT cells; antifungal agent.

INTRODUCTION

Candida is a dimorphic and commensal yeast present in the microbial flora of the human respiratory, gastrointestinal and genitourinary tract [1,2]. Even though it is considered as a harmless commensal fungus, the shifting from commensalism to pathogenic status can be observed in immune-deficient or in immune-compromised patients, those with uncontrolled diabetes mellitus, patients in extremes of age and nutritional status [3]. The genus *Candida* has approximately 200 species, however few are considered as opportunistic pathogens, which can lead to serious human infections [4]. *Candida* spp*.* is the most frequent fungal isolate from human infections (about 70% - 90%) and these infections can be superficial or severe life-threatening invasive infections. On the other hand, *Candida albicans* is the commonest etiological agent in cases of fungal infections [5].

Number of microbial factors are influencing the virulence and high pathogenicity of *Candida* spp*.* The evasion of the host immune defense mechanisms, the adhesion to biotic (host cells) or abiotic surfaces (medical devices), dimorphism, facilitating the tissue invasion through the germ tube formation and biofilms development are few examples for Candidal virulence factors [6].

Candida albicans is one of the commonest commensal fungal species that inhabits mucosal membranes and others areas of the human body, especially the gastrointestinal and genitourinary tracts of healthy individuals [7] and can become an opportunistic pathogen, which usually occurs due to immunological and endocrine disorders, nutritional deficiency states and prolonged hospitalizations, as well as the increase in AIDS cases. *C. albicans* infections can range from superficial to invasive infections involving multiple organs, such as bloodstream infections [8]. Oral and vulvovaginal candidiasis is considered a superficial fungal infection; however, it has a high recurrence, causing long-term patient discomfort [9]. *C. albicans* infections are epidemiologically important due to its high recurrence, causing long-term patient discomfort and high frequency [10]. Approximately 80% of hospital records are of *Candida* infections, with half of this turns into candidemia [8].

Candida albicans possesses several virulence characteristics, such as phenotypic switching, adhesion, invasion of host tissues and secretion of proteolytic enzymes, as well as the biofilm formation which contribute to the high pathogenicity of the species [11]. *Candida albicans* (ATCC MYA-2876) also known as SC5314 is a normal inhabitant of mucosal membranes in human, and also an etiological pathogen for infections of both skin and mucosa. Higher ability to form biofilms and tissue invasion contribute to the high virulence of *Candida albicans* (ATCC MYA-2876), the strain is also known to be susceptible to all antifungals [12].

There are several antifungal agents used topically or systemically in the treatment of candidiasis. Chlorhexidine has been used as a topical therapeutic agent due to its broad spectrum of antimicrobial activity against a variety of organisms. It acts as a fungicidal and fungistatic agent. In addition, chlorhexidine has the ability to inhibit adhesion of *Candida* cells to abiotic and biotic surfaces [13].

The available therapeutic modalities are becoming less effective with the ability of causative agents to adapt to different human body conditions as well as external chemical or physical stresses. This can cause increased length of hospital stay for patients, high levels of morbidity and mortality and increased hospital costs [14]. *C. albicans* infections as well as antifungal resistance due to arbitrary use of antifungal are becoming an emerging problem all over the world when the public health and economy is concerned [15].

With the increased antimicrobial resistance, the invention of novel therapeutic alternatives for treatment purposes is becoming a necessity when considering the treatment of infectious diseases [16]. Currently, medicinal plants derived antimicrobial alternatives are being widely used due to their availability, cost efficacy, low toxicity, as well as their high antimicrobial potential. However, it is very important to conduct extensive studies on their antimicrobial properties, chemical composition as well as the mechanism of action [17,18].

Phyllanthus niruri is a native Brazilian medicinal plant which can be found in several other countries, mainly in tropical and subtropical regions of the world [19]. The plant is known as "stone-breaker", “quebra-pedra” (in Portuguese) and have been extensively used in traditional medicine [20]. *P. niruri* possesses number of important medicinal properties such as antiglycaemic [21], antiviral [22], anti-inflammatory and antinociceptive [23], antigenotoxic [24] and antimicrobial activities [25,26,27]. However, the studies on effect of *P. niruri* on fungal pathogens and the toxicological assessments are not widely carried out yet. This study aimed to evaluate the antifungal action of the hydroalcoholic extract of *Phyllanthus niruri* (HE-Pn) against 16 *Candida* strains, as well as the antifungal activity against the virulence factors of *C. albicans* (ATCC MYA-2876) and the HE-Pn cytotoxicity on human cells.

MATERIAL AND METHODS

Candida strains and culture conditions

C. albicans (ATCC MYA-2876; ATCC 90028 and ATCC 18804), *C. dubliniensis* (ATCC MYA-646), *C. glabrata* (ATCC 5207), *C. guilliermondii* (ATCC 6260), *C. krusei* (ATCC 6258 and ATCC 749), *C. lusitaniae* (ATCC 4031 and ATCC 42720), *C. parapsilosis* (ATCC 22019 and ATCC 10232), *C. rugosa* (ATCC 10571), *C.* tropicalis (ATCC 40281 and ATCC 750), *C. utilis* (ATCC 9950) were used as test organisms. All *Candida* strains were obtained from the Microbiology and Immunology Area, Piracicaba Dental School, UNICAMP, Brazil.

Stock cultures were stored in 80% glycerol at -80 °C ultrafreezer. To reactivate the microorganisms, *Candida* was subcultured on freshly prepared Saboraud Dextrose Agar (SDA, OXOID, UK) and incubated aerobically at 37 ºC for 24 h. Standard inocula of test strains were prepared by adjusting the absorbance of the suspensions equivalent to 0.08-0.10 at 600 nm (0.5 McFarland turbidity).

Preparation of hydroalcoholic extract of *Phyllanthus niruri* (HE-Pn)

The crushed *Phyllantus niruri* plant was obtained from Florien Fitoativos Ltd. (Piracicaba/SP, Brazil). (Lot: 18K26-FL37-004769.Collected on: 05/2018. Origin: Brazil).

The hydroalcoholic extraction was performed using the protocol published by Silva and coauthors (2014) and Leão and coauthors (2017) with few modifications [28,29]. Three hundred grams (300 g) of crushed *Phyllanthus niruri* (stem, leaf and seed) was macerated in 3 L of 70 % hydroalcoholic solution (v/v), in the period of 10 consecutive days (at dark) and the suspension was filtered. Five hundred milliliters (500 mL) of the above extract was subjected to solvent evaporation at 40 rpm coupled in a heating bath system at 40 °C (SL-126 Rotary vacuum evaporator, Solab, Brazil). The final product was kept at –20 °C for 24 h. Then, aliquots of the extract were lyophilized at -46 °C and 0.07 mBar (Jouan, USA). Dried HE-Pn was stored in a freezer at -20 ºC for subsequent use.

The freeze-dried HE-Pn was dissolved in 1% analytical grade Dimethyl Sulfoxide (DMSO, Sigma-Aldrich, USA) followed by centrifugation and filter sterilization prior to experiments.

HE-Pn chemical analysis

Gas chromatography-mass spectrometry (GC-MS) was employed in determination of chemical composition of HE-Pn. HE-Pn was dissolved in ethyl acetate (20 mg/mL) and injected into the gas chromatography column HP-6890 (Agilent, USA) coupled with selective mass detector HP-5975 (Agilent, USA); [HP-5MS Capillary Column (30 m x 0.25 mm x 0.25 µm); temperatures: injector (280ºC), column at 50°C (2 min), 5°C/min, 240°C/min, 10°C/min, 300°C (34 min); detector (300ºC), carrier gas flow (1.0 mL/min). One microliter (1.0 µL) of HE-Pn was injected in split mode and the ionization source was 70 eV. The analytes identification was performed using the MSD ChemStation D. 02.00.275 (Agilent, USA) mass spectral database and NIST mass spectral search program (Version 2.0 g).

Antifungal screening

Antimicrobial screening was performed using the agar well diffusion method [30]. Briefly, standard cell suspensions of *Candida* spp*.* were prepared in sterile normal saline (0.9% NaCl) as previously explained and SDA plates were inoculated with prepared cell suspensions separately using a sterile cotton swab. Three holes were prepared on agar surface using a base of 1 mL sterile pipette tip*.* The bottom of the holes was sealed with molten agar. Prepared holes were then filled completely with 64 mg/mL HE-Pn, 2 mg/mL Chlorhexidine digluconate (Sigma-Aldrich, USA) (positive control) and 1% DMSO (negative control). After incubation at 37 °C for 24 h the agar surface was observed for presence or absence of growth inhibition zone.

Presence of an inhibitory zone was considered as an indicator of the organism sensitivity to relevant treatment.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicide Concentration (MFC)

CLSI M27-A3 broth microdilution technique was employed in determination of MIC [31]. HE-Pn (64 mg/mL) was prepared in RPMI 1640 (Sigma-Aldrich, USA) buffered with MOPS (3-(N-morpholino) propane sulfonic acid) (Sigma-Aldrich, USA). Series of doubling dilution of HE-Pn was prepared by diluting 64 mg/mL HE-Pn in RPMI 1640 in 96 well sterile flat bottomed microtiter plate (Kasvi, Brazil) (100 µL/well). The microtiter plate with HE-Pn dilutions was then seeded with standard *Candida* cell suspensions (100 µL/well) and plate was then incubated aerobically at 37 °C for 24 h. After incubation, the turbidity of the contents of the plate was visually observed. The lowest concentration of the HE-Pn capable of inhibiting the visible growth of yeast was considered as MIC [31].

To determine the MFC, 5 µL from the content of each well was subcultured on SDA plate and incubated aerobically at 37 °C for 24 h. MFC was defined as the lowest concentration of HE-Pn required to kill the *Candida* population completely [32]. Chlorhexidine digluconate (2 mg/mL) was used as the positive control.

Effect of EH-Pn on initial adhesion of *Candida albicans*

Effect on *C. albicans* germ-tube formation

Effect of HE-Pn on *C. albicans* (ATCC MYA-2876) morphological transition from blastoconidia to hypha was performed using the standard protocol published by Hammer and coauthors (2000) [34]. The concentrations of 0.5 and 2 mg/mL of HE-Pn were prepared in Fetal Bovine Serum (FBS, Gibco, United States) and mixed with equal volume of standard *Candida* cell suspension in YPD broth (Himedia, India). Then, these mixtures were aerobically incubated at 37 ºC. Subsequently, 10 µL from each sample was carefully removed at 2 h, 4 h and 6 h and the number of germinated cells was counted using a Neubauer improved counting chamber (Boeco, Germany) [23].

Transmission Electron Microscopy (TEM)

The internal morphology of *C. albicans* (ATCC MYA-2876) with exposure to HE-Pn was evaluated using TEM, by following the methodology published by Spinola and coauthors (2019) with modifications [35].

Standard *Candida* cell suspension ($1x10^6$ cells/mL) was prepared in RPMI 1640. Nine milliliters (9 mL) of 2.5 mg/mL and 20 mg/mL of HE-Pn in RPMI 1640 was mixed with 1 mL of standard cell suspension. HE-Pn final concentration used was 0.25 mg/mL and 2 mg/mL. After incubation in an aerobic incubator at 37 °C for 24 h, the treated Candidal cell suspension was centrifuged at 13000 rpm for 4 min. Resultant cell pellet was resuspended in Karnovsky fixative and kept for 48 h. Then the fixed cells were washed with sterile 0.9% NaCl and treated with 1% OsO_4 for 2 h followed by washing thrice with Sorensen solution. After fixation, the process of serial dehydration and resin infiltration followed, as previously explained by Spinola and coauthors (2019) and Wijesinghe and coauthors (2021) [35,36].

In vitro cytotoxicity of EH-Pn

In vitro cytotoxicity of HE-Pn was determined using the normal human keratinocyte cell line (HaCaT). HaCaT cell density was adjusted to 6.5×10^4 cells/mL and 96-well culture microplates were seeded (100 µL/well) with above mentioned cell suspension. Then the plate was incubated in a cell culture incubator with 5% CO_2 at 37 °C for 24 h. Afterwards, the remaining solution was carefully removed and 100 µL of the HE-Pn diluted in RPMI 1640 was added to each well, starting at the concentration of 64 mg/mL. Then the plate was further incubated at 37 °C for 24 h. Quantification of HaCaT of cell viability was performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma-Aldrich, USA) protocol explained by Zanette and coauthors (2011) [37].

Statistical analysis

All experiments were performed in three independent experiments, and the results expressed as the mean of the values obtained. One way and two-way Anova were used to compare mean values. $p<0.05$ was considered as statistically significant. Statistical analysis was performed using the BioEstat 5.3 software (Instituto Mamirauá) version 5.3.

RESULTS

Chemical Composition

Chemical constituents and their relative abundance of HE-Pn were represented in Table 1.

The most abundant compound of HE-Pn was Linolenic acid ethyl ester (23.38%) followed by Hexadecenoic acid ethyl ester (18.23%), Beta-Sitosterol (16.90%) and Phytol (10.22%).

Table 1. Chemical composition of the hydroalcoholic extract of *Phyllanthus niruri*.

Retention time (min)	Compound identification	% Abundance
16.49	Linolenic acid ethyl ester (9,12,15-octadecatrienoic acid ethyl ester)	23.38
13.40	Hexadecanoic acid ethyl ester	18.23
29.81	Beta-sitosterol	16.90
15.52	Phytol	10.22
29.10	Stigmasterol	7.41
27.66	Alpha-tocopherol	6.42
16.38	Linoleic acid ethyl ester (9,12-octadecadienic acid ethyl ester)	6.27
21.47	Dihydrocrisine	4.30
16.92	Octadecanoic acid ethyl ester	3.03

HE-Pn antifungal activity against *Candida* spp.

Growth inhibition zone diameters obtained from agar well diffusion technique were represented in Table 2. The presence of inhibition zone in any diameter on agar surface demonstrates the microorganism sensitivity to HE-Pn (Table 2). All test strains were sensitive to HE-Pn at working concentration (64 mg/mL).

Table 2. Average diameters of the zones of inhibition exhibited by *Candida* strains.

	Zone of inhibition (mm)	
Microorganism	**HE-Pn**	**Chlorhexidine digluconate**
C. albicans (ATCC MYA-2876)	28	22
C. albicans (ATCC 90028)	28	24
C. albicans (ATCC 18804)	14	18
C. dubliniensis (ATCC MYA-646)	24	18
C. glabrata (ATCC 5207)	10	16
C. guilliermondii (ATCC 6260)	18	28
C. krusei (ATCC 6258)	22	22
C. krusei (ATCC 749)	26	24
C. lusitaniae (ATCC 4031)	18	18
C. lusitaniae (ATCC 42720)	14	18
C. parapsilosis (ATCC 22019)	28	24
C. parapsilosis (ATCC 10232)	10	20
C. rugosa (ATCC 10571)	28	24
C. tropicalis (ATCC 40281)	14	24
C. tropicalis (ATCC 750)	12	16
C. utilis (ATCC 9950)	18	18

MIC and MFC

The MIC and MFC results are shown in Table 3. MIC values for HE-Pn were ranging from 0.03 mg/mL to 8 mg/mL. MFC values were ranging from 0.5 mg/mL to 32 mg/mL, except for *C. albicans* (ATCC MYA 2876) which exhibited the highest MFC (64 mg/mL). There was a significant difference in MIC values of HE-Pn and Chlorhexidine digluconate of all test strains ($p<0.05$) except *C. tropicalis* (ATCC 40281) *C. utilis* (ATCC 9950).

Table 3. MIC and MFC of *Candida* spp. * significant difference between MIC and MFC of each treatment (p<0.05)

Microorganism	HE-Pn (mg/mL)		Chlorhexidine digluconate (mg/mL)	
	MIC	MFC	MIC	MFC
C. albicans (ATCC MYA-2876)	0.5*	64*	1*	2*
C. albicans (ATCC 90028)	0.25*	32*	1*	2*
C. albicans (ATCC 18804)	0.25*	32*	1	1
C. dubliniensis (ATCC MYA-646)	1 *	32*	0.5*	1*
C. glabrata (ATCC 5207)	8*	16*	1*	2*
C. guilliermondii (ATCC 6260)	0.12*	32*	0.5	0.5
C. krusei (ATCC 6258)	0.06*	8*	1	1
C. krusei (ATCC 749)	0.03*	16*	0.5	0.5
C. lusitaniae (ATCC 4031)	0.12*	2*	0.5*	2*
C. lusitaniae (ATCC 42720)	0.12*	8*	1	1
C. parapsilosis (ATCC 22019)	0.12*	32*	1*	2*
C. parapsilosis (ATCC 10232)	0.12*	32*	1	1
C. rugosa (ATCC 10571)	0.12*	16*	0.5	0.5
C. tropicalis (ATCC 40281)	0.25*	0.5*	0.25*	0.5*
C. tropicalis (ATCC 750)	1*	16*	0.5*	2*
C. utilis (ATCC 9950)	0.25*	32*	0.25*	2*

Effect on *C. albicans* (ATCC MYA-2876) adhesion

Percentage reduction of adhesion of *Candida* cells compared to negative control was shown in Figure 1.

According to the data obtained, HE-Pn effectively reduced the *C. albicans* adhesion in comparison to the negative control. *C. albicans* obtained a 50% of adhesion reduction with a HE-Pn concentration of 3-4 mg/mL. Chlorhexidine digluconate exhibited a 50% reduction of *C. albicans* adhesion at 0.21 mg/mL (p=0.00).

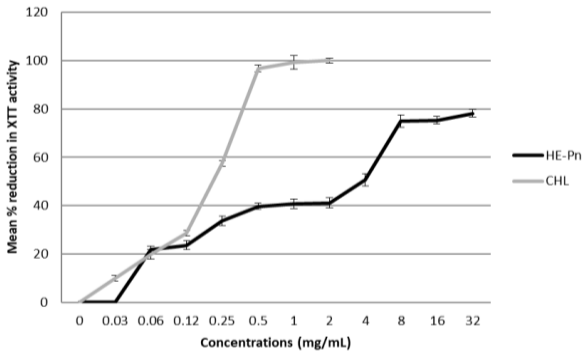

Figure 1. Percentage reduction in XTT metabolic activity of *Candida albicans* (ATCC MYA-2876) in different concentrations of hydroalcoholic extract of *Phyllanthus niruri* (HE-Pn) compared to negative control. All error bars represent ± 2 SD.

Effect on formation of *C. albicans* germ-tube

According to the data obtained, chlorhexidine digluconate and HE-Pn exhibited a significant reduction (all p values <0.05) in the formation of *C. albicans* germ-tube (Figure 2).

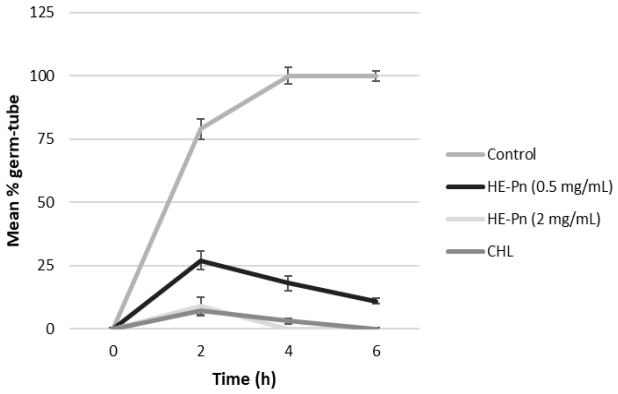

Figure 2. Percentage of germinated cells of *Candida albicans* (ATCC MYA-2876) with 0.5 mg/mL and 2 mg/mL of HE-Pn and chlorhexidine digluconate within 6 h test period. All error bars represent the ± 2 standard deviations (SD).

Transmission Electron Microscopy (TEM)

Structural changes in *C. albicans* cells after HE-Pn exposure were visualized by Transmission Electron Microscopy. TEM images obtained after 24 h exposure of 0.25 mg/mL of HE-Pn (Figure 3 B) indicate structural changes such as large amount of cytoplasmic granules and vacuoles. Post-exposure to 2 mg/mL of HE-Pn (Figure 3 C) Candidal cells showed cellular changes such as the cytoplasmic granules and vacuoles, chromatin condensation and several cytoplasmic membrane detachment areas. Figure 3 D demonstrates *C. albicans* cell (ATCC MYA-2876) with 2 mg/ml of HE-Pn treatment.

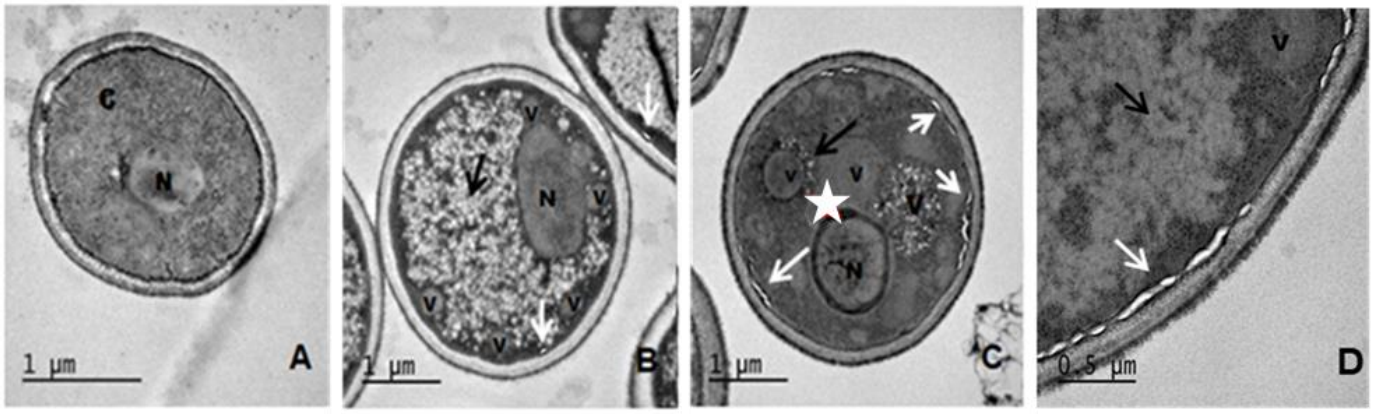

Figure 3. Transmission Electron Microscopic (TEM) images of *C. albicans* (ATCC MYA-2876) (A) Negative control, (B) Exposed to 0.25 mg/mL of HE-Pn, (C) Exposed to 2 mg/mL of HE-Pn (D) Exposed to 2 mg/mL of HE-Pn approximation (0.5 µm) for visualization of cytoplasmic membrane detachment. C: cytoplasm, N: nucleus, black solid arrow: cytoplasmic granules, white solid arrow: detachment of cell wall and plasma membrane, white star: nucleus with chromatin condensation.

HE-Pn *in-vitro* cytotoxicity

The *in-vitro* cytotoxic effect of HE-Pn and chlorhexidine digluconate on immortalized human keratinocyte cells (HaCaT) was evaluated by quantifying the HaCaT cell viability after treating the cell line with different concentrations of HE-Pn using the MTT metabolic assay. No toxicity/reduction of MTT metabolic activity of HaCaT cell line was noted at any concentration tested ranging from 0 – 64 mg/mL (Figure 4).

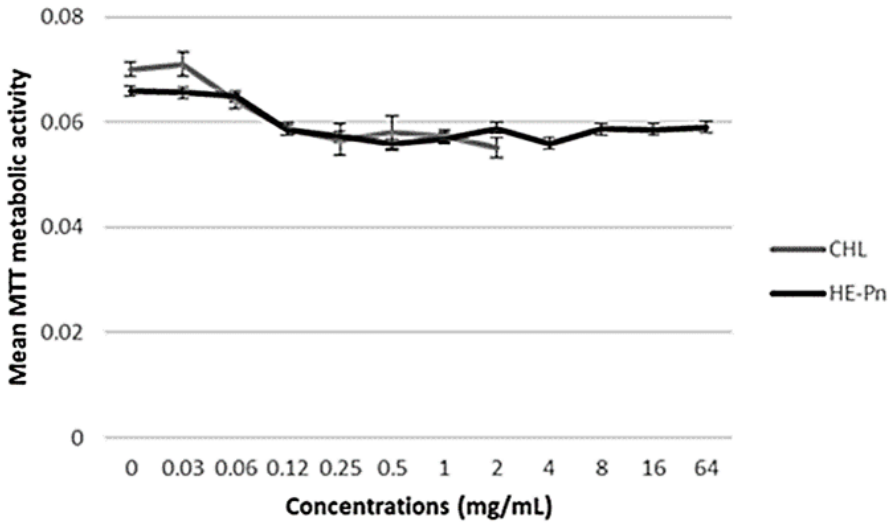

Figure 4. Average MTT activity/viability of HaCaT cell line after treatment with different concentrations of HE-Pn and chlorhexidine digluconate. All error bars represent the ± 2 standard deviations (SD).

DISCUSSION

From antiquity to the present day, especially in populations with medicines scarcity, the illnesses treatment is carried out through the use of medicinal plants [29]. Medicines can be originated directly or indirectly of natural products, most of which originates from plants (25 %). The products of natural origin are important resources for the formulation and the development of new of pharmaceutical products, due to the microorganism's acquired resistance to commonly used antifungal agents [38]

Phyllanthus niruri ("Stone-breaker") has been widely used as a medicinal plant in several types of diseases and also used by communities as a folk medicine in many regions of the world for centuries [2,39]. *P. niruri* plant is well known for treating urogenital system infections and diseases [27,40], so it would be a high candidate for the treatment of microorganisms associated with these sites, as well as microorganisms that are present in the human microbiome and that in an unbalanced condition can cause diseases.

The present study was carried out to discover the effectiveness of the *Phyllanthus niruri* hydroalcoholic extract as a phytomedicinal alternative against the strains of *Candida* spp., especially the strain with greater virulence, *C. albicans* (ATCC MYA-2876).

The *P. niruri* phytochemical analysis, in previous studies, registered the presence of several lignans, flavonoids, triterpenoids, phenols and tannins. However, there are still few studies correlating the plant activity with a single substance or the synergistic effect between the different constituent [40]. *P. niruri* has antimicrobial components that can be found in different types of plant extracts, there are reports that some extracts of *P. niruri* do not show activity against fungi, however this inactivity of the extracts can be attributed to extracts not prepared according to standard methods or different interpretations [41]. In this present research it was possible to verify the antifungal action against *Candida* spp. that the hydroalcoholic extract has.

The *Candida* strains inhibition can be attributed to the presence of antimicrobial compounds in HE-Pn. Such as linolenic and linoleic acid ethyl ester, phytol and beta-sitosterol, that have been shown to have antinociceptive, anesthetic, anti-inflammatory and antimicrobial potential [42,43]. As well as the presence of palmitic acid (hexadecanoic acidethyl ester) which showed antimicrobial activity against oral and non-oral microorganisms, including *C. albicans* yeast [44]. The data obtained corroborate the literature, which demonstrates the presence of these compounds in the *P. niruri* plant and these compounds may be responsible for the antimicrobial activity, including against several *Candida* strains [45,46].

The present research demonstrated that all *Candida* strains tested with 64 mg/mL of HE-Pn showed sensitivity to the HE-Pn. All strains tested exhibited growth inhibition zone with 64 mg/mL of HE-Pn in agar well diffusion experiment (Table 2). In the CLSI broth microdilution assay, all *Candida* strains showed growth inhibition. The lowest concentration of MIC found was 0.03 mg / mL for *C. krusei* (ATCC 749), and the lowest concentration of MFC was 0.5 mg / mL for *C. tropicalis* (ATCC 40281). MIC and MFC for the *C. albicans* (ATCC MYA-2876) was 0.5 mg / mL and 64 mg / mL, respectively. The highest MFC concentration was 64 mg/mL, just for *C. albicans* (ATCC MYA-2876). Chlorhexidine digluconate demonstrated the lowest MIC of 0.25 mg/mL, whereas the lowest MFC value was 0.5 mg/mL. *C. albicans* (ATCC MYA-2876) presented MIC and MFC of 1 mg/mL and 2 mg/mL, respectively. Interestingly, MIC values of *C. dubliniensis* (ATCC MYA-

646), *C. glabrata* (ATCC 5207) and *C. tropicalis* (ATCC 750) for HE-Pn treatment were comparatively lower to Chlorhexidine digluconate. Further studies were recommended to identify the potential causes such as cell wall structure and metabolic activities etc. for aforesaid difference.

The data of the present research showed that the hydroalcoholic extract of *P. niruri* (HE-Pn) had an antifungal activity against all *Candida* strains tested. All strains tested exhibited growth inhibition zone with 64 mg/mL of HE-Pn and 2 mg/mL Chlorhexidine digluconate in agar well diffusion experiment (Table 2).

According to a study conducted by Shilpa and coauthors (2018) *Phyllanthus niruri* does not show antifungal activity even though it demonstrates a significant antibacterial activity [41]. Similar observation was obtained by Njoroge and coauthors (2012) with methanol (MeOH) and aqueous extracts, however the methanol extract of *Phyllanthus niruri* at higher concentration (50 mg/μL^{-1}) exhibits more than 40% *Candida albicans* inhibition, as well as inhibition of bacterial species [47]. Further, *P. niruri* alcoholic extract showed an antimicrobial activity against *Streptococcus* and *Lactobacillus acidophillus*, as well as several other bacterial and fungal species [48,49].

Different results may be probably due to the difference in the extraction methods and solvents used (as infusion, aqueous, methanol or alcoholic extraction and others), differences in *Phyllanthus* species, as well as the environmental factors such as soil, location, etc. which can influence the plant metabolism [18,50]. Even though many studies reported the antibacterial activity of *P. niruri*, there are still lack of evidence-based studies conducted to evaluate the antifungal action against *Candida* species.

Despite the differences in the extraction method and solvent used, closely similar results were obtained for broth microdilution assay in the current study and a study conducted by Ferrante and coauthors in 2020. They observed an inhibitory activity of *Phyllanthus niruri* aqueous extract against bacteria and fungi strains including *Candida* species with a MIC of 0.09921 mg/mL and > 0.25 mg/mL on *C. tropicalis* and *C. albicans* respectively [51].

One of the most important virulence factors of *C. albicans* species is the ability to adhere to the biotic or abiotic surface in order to form a basal layer of yeast cell and matrix embedded biofilm structures. *C. albicans* is capable of forming extremely resistant biofilms by secreting an extracellular matrix during the development of the biofilm. Such structure promotes the protection of the microorganism against the host's immune defenses and the penetration of antifungal drugs into the core of the biofilm [52,53].

HE-Pn effectively reduced the *C. albicans* (ATCC MYA-2876) adhesion. *C. albicans* obtained a 50% of adhesion reduction with a concentration of 3-4 mg/mL, which could be due to the death of microorganisms during the experiment period [43]. Importantly, according to Raut and coauthors (2013) the adhesion of *C. albicans* to solid polystyrene surfaces is not reduced by the routine, first line antifungal agent, fluconazole [33]. This indicates the possibility of developing HE-Pn as a preventive strategy for harmful colonization and pathogenic biofilm formation of *C. albicans*.

Germ-tube formation is another unique virulence factor of *C. albicans* and *C. dubliniensis*, which enables the tissue invasion and nutrients absorbance. HE-Pn at MIC (0.5 mg/mL) and chlorhexidine digluconate at 1 mg/mL were able to reduce the formation of *C. albicans* germ tube, 2.0 mg/mL of HE-Pn completely inhibit the germ tube formation during the test period. Since there are few published scientific researches discussing the effect of the HE-Pn on the *Candida* adhesion and germ tube formation, the current study possibility and open space for further studies.

TEM images of *C. albicans* (ATCC MYA-2876) after the exposure to 0.25 mg/mL of HE-Pn demonstrate accumulation of cytoplasmic granules and intracellular vacuoles, and small detachments of the plasma membrane which are characteristics of cellular stress. Other than aforesaid characteristics, 2 mg/mL of HE-Pn treated cells showed chromatin condensation in the periphery of nucleus, nuclear granulation and perinuclear changes. Similar types of microbial structural alterations were observed previously by many scientists with the exposure to plant derived antimicrobial natural products and fluconazole [54,55].

Some antifungals can show toxic effects on human cells, since fungi show physiological and biochemical similarities to human host cells because both are eukaryot. Thus, it is necessary to carry out tests to determine the toxicity of plant extracts to the host using *in-vitro, in-vivo* or *ex-vivo* experimental models [14]. The current study evaluated the cytotoxic effect of HE-Pn on HaCaT cells and did not observe any toxic characteristics of HE-Pn. According to some previous studies which evaluates the toxicity of *P. niruri* on human cells, it is selectively toxic to cancer cell lines, however protective for normal cells [56]. Even though these data confirm the safe use of HE-Pn as an anti-Candidal agent on human subjects, further clinical and *in-vivo* experiments are recommended to get a complete idea about the toxicity of HE-Pn.

These data together with the literature, indicate that the medicinal and antimicrobial properties existing in the *Phyllanthus niruri* are not only derived from popular belief. Since the current study shows positive results, HE-Pn could be developed as a unique antifungal therapeutic alternative or with available antifungal. However further research on mode of antimicrobial action of HE-Pn, synergistic effects when combined with

available antimicrobials and *in-vivo* toxicology studies should be widely conducted by considering the contribution of plant derived therapeutics towards the reduction of healthcare costs and providing a reliable, cost effective and non-toxic therapeutic modalities.

CONCLUSION

- HE-Pn shows anti-*Candida* activity against selected test strains.
- HE-Pn decreases the adhesion and germ tube formation of *C. albicans* (ATCC MYA-2876) *in-vitro*.
- HE-Pn does not show any toxicity on HaCaT cell line.

Funding: This research was funded by Coordination for the Improvement of Higher Education Personnel (CAPES), Grant number: 88882.434532/2019-01.
Acknowledgments: The results described in this paper were part of the thesis presented by Flávia Camila Maia, to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Oral and Dental Biology, in Microbiology and Immunology area. This paper is dedicated to the memory of Prof. Rafael Nobrega Stipp. Authors acknowledge Florien Fitoativos LTDA for providing the crushed plant. Pluridisciplinary Center for Chemical, Biological and Agricultural Research - CPQBA for providing GC-MS facilities for the study.
Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Achkar JM, Fries B*C. Candida* infections of the genitourinary tract. Clin Microbiol Rev. 2010 Apr;23(2):253-73. doi: 10.1128/CMR.00076-09.
2. Maia FC, Wijesinghe GK, Oliveira TR, Barbosa JP, Feiria SNB, Boni GC, et al. *Phyllanthus niruri* L. (stone-breaker) as an alternative of anti-human diseases, antimicrobial agent, and its applicability to combat resistant microorganisms. A brief Review. Braz J Nat Sci. 2020; 3(2): 342-353. doi: https://doi.org/10.31415/bjns.v3i2.99
3. Dadar M, Tiwari R, Karthik K, Chakraborty S, Shahali Y, Dhama K. *Candida albicans* - Biology, molecular characterization, pathogenicity, and advances in diagnosis and control - An update. Microb Pathog. 2018; 117: 128-38. doi: 10.1016/j.micpath.2018.02.028.
4. Spampinato C, Leonardi D. *Candida* infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. Biomed Res Int. 2013; 204237. doi: 10.1155/2013/204237
5. Weerasekera MM, Wijesinghe GK, Jayarathna TA, et al. Culture media profoundly affect *Candida albicans* and *Candida* tropicalis growth, adhesion and biofilm development. Mem Inst Oswaldo Cruz 2016; 111(11): 697-702. doi:10.1590/0074-02760160294.
6. Ciurea CN, Kosovski IB, Mare AD, Toma F, Pintea-Simon IA, Man A. *Candida* and candidiasis-opportunism versus pathogenicity: A review of the virulence traits. Microorganisms 2020; 8(6): 857. doi: 10.3390/microorganisms8060857.
7. Nobile CJ, Johnson AD. *Candida albicans* biofilms and human disease. Annu Rev Microbiol. 2015; 69:71-92.
8. Colombo AL, Guimarães T, Camargo LFA, Richtmann R, Queiroz-Telles F, Salles MJC, et al. Brazilian guidelines for the management of candidiasis - a joint meeting report of three medical societies: Sociedade Brasileira de Infectologia, Sociedade Paulista de Infectologia and Sociedade Brasileira de Medicina Tropical. Braz J Infect Dis. 2013; 17(3): 283-312. doi: 10.1016/j.bjid.2013.02.001
9. Patil S, Rao RS, Majumdar B, Anil S. Clinical appearance of oral *Candida* infection and therapeutic strategies. Front Microbiol 2015; 6:1391.
10. Wu YM, Lee CH, Cheng YC, Lu JJ, Wang SH. Association between CAI microsatellite, multilocus sequence typing, and clinical significance within *Candida albicans* isolates. Med Mycol 2021 May 4; 59(5): 498-504.
11. Mayer FL, Wilson D, Hube B. *Candida albicans* pathogenicity mechanisms. *Virulence*. 2013;4(2):119-28. doi:10.4161/viru.22913.
12. Khalaf RA, Fattouh N, Medvecky M, Hrabak J. Whole genome sequencing of a clinical drug resistant *Candida albicans* isolate reveals known and novel mutations in genes involved in resistance acquisition mechanisms. J Med Microbiol. 2021; 70(4): 001351.
13. Machado FC, Portela MB, Cunha AC, Souza IPR, Soares RMA, Castro GFBA. Antifungal activity of chlorhexidine on *Candida* spp. biofilm. Rev Odontol UNESP. 2010; 39(5): 271-5.
14. Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJS. *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol. 2013; 62, 10–24. doi: 10.1099/jmm.0.045054-0.
15. Barbosa A, Araújo D, Ribeiro E, Henriques M, Silva S. *Candidaalbicans* Adaptation on Simulated Human Body Fluids under Different pH. Microorganisms 2020; 8(4):511. doi: 10.3390/microorganisms8040511.

16. Prestinaci F, Pezzotti P, Pantosti A. Antimicrobial resistance: a global multifaceted phenomenon. Pathog Glob Health. 2015; 109(7): 309-18. doi: 10.1179/2047773215Y.0000000030.
17. Silva NCC, Fernandes Júnior A. Biological properties of medicinal plants: a review of their antimicrobial activity. J VenomAnim Toxins incl Trop Dis. 2010; 16(3), 402-13. doi: 10.1590/S1678-91992010000300006
18. Wijesinghe GK, Feiria SNB, Oliveira TR, Maia FC, Joia F, Barbosa JP, et al. *Cinnamomum verum* (true cinnamon) leaf essential oil as an effective therapeutic alternative against oral and non-oral biofilm infections: A brief review. Braz J Nat Sci. 2020; 3(3): 556-67. doi: https://doi.org/10.31415/bjns.v3i2.119
19. Webster GL. A synopsis of the Brazilian taxa of *Phyllanthus* section *Phyllanthus* (Euphorbiaceae). Lundellia 2002; 5:1-26. doi: /10.25224/1097-993X-5.1.1
20. Narendra K, Swathi J, Sowjanya KM, Satya AK. *Phyllanthus niruri*: a review on its ethno botanical, phytochemical and pharmacological profile. J Pharm Res. 2012; 5(9): 4681-91.
21. Okoli CO, Ibiam AF, Ezike AC, Akah PA, Okoye T*C.* Evaluation of antidiabetic potentials of *Phyllanthus niruri* in alloxan diabetic rats. Afr J Biotechnol. 2010; 9 (2): 248-59.
22. Naik AD, Juvekar AR. Effects of alkaloidal extract of *Phyllanthus niruri* on HIV replication. Indian J Med Sci. 2003; 57(9):387-93.
23. Adedapo AA, Ofuegbe SO. Anti-inflammatory and antinociceptive activities of the aqueous leaf extract of *Phyllanthus amarus* Schum (Euphorbiaceae) in some laboratory animals. J Basic Clin Physiol Pharmacol. 2015; 26(1):89-94. doi: 10.1515/jbcpp-2013-0126.
24. Queiroz FM, Matias KWO, Cunha MMF, Schwarz A. Evaluation of (anti)genotoxic activities of *Phyllanthus niruri* L. in rat bone marrow using the micronucleus test. Braz J Pharm Sci. 2013; 49(1), 135-148. doi: 10.1590/S1984-82502013000100015.
25. Hoffman B, Delasalas H, Blanco K, Wiederhold N, Lewis R, Williams L. Screening of Antibacterial and Antifungal Activities of Ten Medicinal Plants from Ghana. Pharm Biol 2004; 42: 13-7.
26. Lee NYS, Khoo WKS, Adnan MA, Mahalingam TP, Fernandez AR, Jeevaratnam K. The pharmacological potential of *Phyllanthus niruri*. J Pharm Pharmacol 2016; 68: 953-69.
27. Maia FC. Efeito do extrato hidroalcoólico de *Phyllanthus niruri* L. (quebra-pedra) sobre células planctônicas e em biofilme de *Candida albicans* [Dissertation]. Brazil: State University of Campinas, Piracicaba Dental School; 2020. 101p.
28. Silva JJ, Cerdeira CD, Chavasco JM, Cintra ABP, Silva CBP, Mendonça NA, et al. In vitro screening antibacterial activity of *Bidens pilosa* Linné and *Annona crassiflora* Mart. against oxacillin resistant Staphylococcus aureus (ORSA) from the aerial environment at the dental clinic*.* Rev Inst Med Trop*.* 2014; 56(4): 333-40.
29. Leão MFM, Güez CM, Duarte JA, Schmitt EG, Quintana LD, Zambrano LAB et al. Avaliação dos efeitos anti-genotóxicos *Phyllanthus niruri* (Euphorbiaceae) em leucócitos humanos expostos a agente agressor. Rev. Sta Maria. 2017; 43(1): 133-9.
30. Wijesinghe G, Jayarathna P, Gunasekara T, Fernando N, Kottegoda N, Weerasekera M. Antibacterial and anti-*Candida* activity of chlorhexidine gluconate, Triphala and Munamal pothu (bark of *Mimusops elengi*). Sri Lankan J Infec Dis. 2018; 8(1), p.25–31. doi: 10.4038/sljid.v8i1.8166
31. CLSI. Performance Standards for Antifungal Susceptibility Testing of yeasts. 1st ed. CLSI Supplement M60. Wayne, PA: Clinical and Laboratory Standards Institute; 2017.
32. Gullo FP, Sardi JCO, Santos VAFFM, Sangalli-Leite F, Pitangui NS, Rossi SA et al. Antifungal activity of maytenin and pristimerin. Evid Based Complement Alternat Med. 2012; 2012: 340787. doi: 10.1155/2012/340787.
33. Raut JS, Shinde RB, Chauhan NM, Karuppayil SM. Terpenoids of plant origin inhibit morphogenesis, adhesion, and biofilm formation by *Candida albicans*. Biofouling 2013; 29: 87-96.
34. Hammer KA, Carson CF. Melaleuca alternifolia (tea tree) oil inhibits germ tube formation by *Candida albicans*. Med Mycol J. 2000; 38: 355–362.
35. Spinola MS, Nóbrega DF, Del Bel Cury AA, Ricomini Filho AP, Cury JA, Tenuta LMA. Fluoride penetration and clearance are higher in exopolysaccharide-containing bacterial pellets. Caries Res. 2019; 53: 16-23.
36. Wijesinghe GK, Oliveira TR, Maia FC, Feiria SB, Barbosa JP, Joia F, Boni GC, Höfling JF. Efficacy of true cinnamon (*Cinnamomum verum*) leaf essential oil as a therapeutic alternative for *Candida* biofilm infections. Iran J Basic Med Sci. 2021; 24:787-95.
37. Zanette C, Pelin M, Crosera M, Adami G, Bovenzi M, Larese FF et al. Silver nanoparticles exert a long-lasting antiproliferative effect on human keratinocyte HaCaT cell line. Toxicol in Vitro 2011; 25(5): 1053–60. doi: 10.1016/j.tiv.2011.04.005.
38. Calixto JB. 2019. The role of natural products in modern drug discovery. An Acad Bras Cienc 91: e20190105.
39. Nisar MF, He J, Ahmed A, Yang Y, Li M, Wan *C.* Chemical components and biological activities of the genus *Phyllanthus*: A review of the recent literature. Molecules. 2018; 23(10): 2567. doi: 10.3390/molecules23102567.

40. Pucci ND, Marchini GS, Mazzucchi E, Reis ST, Srougi M, Evazian D, Nahas WC. Effect of *Phyllanthus niruri* on metabolic parameters of patients with kidney stone: a perspective for disease prevention. Int Braz J Urol. 2018; 44(4): 758-64.
41. Shilpa VP, Muddukrishnaiah K, Thavamani BS, Dhanapal V, Arathi KN, Vinod KR. In vitro immunomodulatory, antifungal, and antibacterial screenning of *Phyllanthus niruri* against to human pathogenic microorganisms. Environ Dis. 2018; 3, 63-9.
42. Acikara OB, Citoğlu GS, Dall'Acqua S, Ozbek H, Cvacka J, Zemlicka M, et al. Bioassay-guided isolation of the antinociceptive compounds motiol and β-sitosterol from *Scorzonera latifolia* root extract. Pharmazie 2014; 69: 711-4.
43. Sarin B, Verma N, Martín JP, Mohanty A. An overview of important ethnomedicinal herbs of *Phyllanthus* species: Present status and future prospects. Sci World J 2014; 2014 (1): 1-12.
44. Huang CB, George B, Ebersole JL. Antimicrobial activity of n-6, n-7 and n-9 fatty acids and their esters for oral microorganisms. Arch Oral Biol 2010; 55: 555-60.
45. Ahmad MU, Husain S, Osman SM. Ricinoleic acid in *Phyllanthus niruri* seed oil. J Am Oil Chem Soc 1981; 58(6): 673–4.
46. Marques LC. *Phyllanthus niruri* (Quebra-Pedra) no tratamento de urolitíase: Proposta de documentação para registro simplificado como fitoterápico. Revista Fitos 2010; 5(3): 20-33.
47. Njoroge AD, Anyango B, Dossaji S*P.* Screening of *Phyllanthus* species for antimicrobial properties. Chem Sci J. 2012; 2012: CSJ-56.
48. Sunitha J, Krishna S, Ananthalakshmi R, Jeeva JS, Smiline Girija AS, Jeddy N. Antimicrobial effect of leaves of *Phyllanthus niruri* and *Solanum nigrum* on caries causing bacteria: An *in vitro* study. J Clin Diag Res. 2017; 11(6): KC01-KC04.
49. Shanmugam B, Shanmugam KR, Ravi S, Subbaiah GV, Mallikarjuna K, Reddy KS. Antibacterial activity and phytochemical screening of *Phyllanthus niruri* in ethanolic, methanolic and aqueous extracts. Int J Pharm Sci Res. 2014; 27(2): 85-89.
50. Ncube BJF, Finnie JF, Van Staden J. Quality from the field: The impact of environmental factors as quality determinants in medicinal plants. S Afr J Bot. 2012; (82): 11. doi: 10.1016/j.sajb.2012.05.009.
51. Ferrante C, Chiavaroli A, Angelini P, Venanzoni R, Angeles Flores G, Brunetti L, et al. Phenolic content and antimicrobial and anti-Inflammatory effects of *Solidago virga-aurea*, *Phyllanthus niruri, Epilobium angustifolium, Peumus boldus,* and *Ononis spinosa* extracts. Antibiotics (Basel) 2020; 6: 9(11): 783.
52. Wijesinghe GK, Maia FC, Oliveira TR, Feiria SNB, Joia F, Barbosa JP et al. Effect of *Cinnamomum verum* leaf essential oil on virulence factors of *Candida* species and determination of the in-vivo toxicity with *Galleria mellonella* model. Mem Inst Oswaldo Cruz 2020; 115: 1-13. doi: 10.1590/0074-02760200349
53. Gulati M, Nobile CJ. *Candida albicans* biofilms: development, regulation, and molecular mechanisms. Microbes Infect. 2016; 18(5): 310-321.
54. Hao B, Cheng S, Clancy CJ, Nguyen MH. Caspofungin kills *Candida albicans* by causing both cellular apoptosis and necrosis. Antimicrob Agents Chemother 2012; 57(1): 326-32.
55. Khan SN, Khan S, Iqbal J, Khan R, Khan AU. Enhanced killing and antibiofilm activity of encapsulated Cinnamaldehyde against *Candida albicans*. Front Microbiol 2017; 8: 1-15.
56. Araújo Júnior RF, Souza TP, Pires JGL, Soares LAL, Araújo AA, Petrovick PR et al. A dry extract of *Phyllanthus niruri* protects normal cells and induces apoptosis in human liver carcinoma cells. Exp Biol Med.2012; 237(11): 1281-8.