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Full Length Research Paper

Genotypic characterization of *Escherichia coli* strains isolated from dairy cattle environment

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The aim of this study was to characterize the diversity of *Escherichia coli* strains involved in the dispersion of virulence genes. 152 *E. coli* strains originated from dairy cattle environment were evaluated through phenotypic and proteomic assays. These samples were investigated for the presence of virulence genes (*eae*A, *stx*I, *stx*II, *ST*, *LT*, *eagg*, *ial*) and biofilm related genes (*fim*H, *csg*A, *flu*). Eighteen profiles were obtained and 30 isolates were selected for macrorestriction assay by *Pulsed-Field Gel Electrophoresis* (PFGE) technique. PFGE patterns of *Xba*I-digested performed to determine the clonal relatedness of *E coli* isolates. A total of 27 pulsotypes of *E. coli* were identified with a low percentage of genetic similarity (≤68%) demonstrating a high genetic diversity in the isolates tested. Furthermore, the presence of biofilm-associated related gene (*fim*H) can contribute to the dispersion and persistence of this pathogen into the milk environment. Also, all of the pulsotypes (n = 8) with genetic similarity from 95% were present in milk samples, feces and liner were found in different seasons of the year, sharing at least one gene associated with biofilm formation. So the characterization of the genetic diversity of *E. coli* is important for understanding its dispersion in order to assist in the implementation of measures to prevent the spread of these agents in milk production environment.

Key words: circulating clones, diversity, *Escherichia coli*, persistence, Pulsed-Field Gel Electrophoresis (PFGE), virulence gene.

INTRODUCTION

Escherichia coli have been isolated from a wide range of animal hosts and in mammals. However, this specie is able to adapt to various environmental conditions, being highly diversified with commensal and pathogenic strains that can colonize and persist in humans, animals and abiotic environments (Wirth et al., 2006; Tenaillon et al., 2010).

The pathogenic *E. coli* subtypes have caused diseases

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worldwide and the main route of human infection is through bovine meat and water contaminated with bovine fecal material (Leclerc et al., 2001). This specie may persist in the intestines of the animals and can be excreted in the environment through the feces, ensuring the spread of this pathogen in the animal production environment (Lambertini et al., 2015). Hussein and Sakuma (2005) discussed the importance of dairy cattle as reservoirs of STEC and its significant importance to health risk to humans. The authors demonstrated that fecal testing of dairy cattle worldwide showed wide ranges of prevalence rates for *E. coli* O157: H7 (0.2 to 48.8%). In animal residues, pathogenic *E. coli* can survive from 50 to more than 300 days, depending on environmental factors such as temperature, aeration and nutrient availability (Rogers and Haines, 2005).

In addition, the bovine gastrointestinal tract has been considered as a natural reservoir for commensal and pathogenic *E. coli* of high phylogenetic and genotypic diversity with the putative ability to cause mastitis (Houser et al., 2008). Thus, it has been proposed that several *E. coli* genotypes with specific phenotypes are more suitable to incite bovine mastitis than others (Shpigel et al., 2008; Blum et al., 2008; Blum et al., 2013).

A severe outbreak of O104: H4 in Germany in 2011 emphasized this versatility and adaptability of pathovar STEC (shiga - toxin producing *E. coli*), as it was caused by a newly developed hybrid strain that combines virulence properties of enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) (Denamur, 2011). Recently, Eichhorn et al. (2015) have shown that some atypical *E. coli* strains (aEPEC) and EHEC share the same phylogeny and are called post-pre-EHEC aEPEC isolates.

The constant horizontal transfer of genes allows triggering new strains STEC and EHEC with unpredictable risk potential for humans. Thus, STEC clones that persist in animals or farms over long periods of time, which are equipped with certain virulence and virulence-associated genes (VAGs), can serve as reservoirs of genes for the evolution of these new strains (Hoffman et al., 2006). However, the persistence of distinct EHEC subpopulations such as O157: H7 was shown to be related to the colonization properties and, for example, to the presence of the large EHEC virulence plasmid or long polar fimbriae (Lim et al., 2007).

Molecular typing techniques have been used to investigate several *E. coli* subtypes as O157 and PFGE is currently considered the gold standard for fingerprint DNA of these strains (Swaminathan et al., 2001). The ecology and epidemiology of this organism in cattle appear to be very complex, often involving multiple clones on a single farm (Renter et al., 2002; Liebana et al., 2003). Previous studies have reported on persistence on the farm in different livestock production systems. However, information on persistence in individual animals is very scarce (Renter et al., 2003; LeJeune et al., 2004).

Thus, the objective of this study was to characterize the diversity of *E. coli* of major importance in the dispersion of virulence genes within the milk production system using the technique of pulsed field gel electrophoresis.

MATERIALS AND METHODS

Ethics statement

This study was conducted according to ethical standards and approved by the Ethics Committee and Biosafety of the institution under protocol number: CEUA-3664040915. The samples used in the current study were obtained from samples submitted to routine veterinary diagnosis.

Sample collection

The present study was performed in the town of Barra do Piraí, Rio de Janeiro, Brazil between 2014 and 2015 (Figure 1). A pool of milk samples from 94 cows tested positive by the California Mastitis Test (CMT), collected over three consecutive weeks. Thus, 282 milk samples were obtained.

A total of 94 rectal feces samples from these same lactating cows were also collected. Representing milk line samples (n=48) were also collected: 10 samples from workers' hands, 10 nasal samples from workers, 20 milking machine samples and 8 nasal samples of pets (dogs and cats present in the milking parlor). Finally, one sample each from farm water supply was collected from well, weir, faucet, drinking fountain and brook, making a total of 19 water samples.

Bacterial Identification

The bovine milk and milk line samples were first inoculated on blood agar enriched with 5% sheep blood (MicroMed®, Rio de Janeiro, Brazil 2098), while the fecal and water samples were inoculated on Eosin Methylene Blue (EMB) agar (Acumedia®, Lansing, Michigan 48912) and incubated at 35°C (±2°C) for 24 h. Then, the isolates were submitted to routine microbiological diagnostics, including inoculation in selective medium for analysis of cultural propertie (Rodrigues et al., 2017).

The Gram negative bacteria identification was followed according to Koneman et al. (2012), glucose and lactose fermentation with gas production, H_2S (hydrogen sulfide), indole, motility, acetoin and mixed acid production and citrate were assayed. All tests were carried out in triplicate. The strain *E. coli* ATCC25922 was used as positive control.

Maldi-Tof MS used to confirm the species

All Enterobacterial strains previously identified by biochemical tests were evaluated for, Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) technique to confirm their species. Assay was performed in Laboratory of Investigation in Medic Microbiology of the Institute of Microbiology Paulo Góes, Federal University of Rio de Janeiro (UFRJ). The samples were inoculated in Brain Heart Infusion (BHI) agar (Merck KGaA®, Darmstadt, Germany 64271) at 37°C for 24 h and each culture was transferred to a microplate (96 MSP, Bruker - Billerica, USA).

The bacterial sediment was covered by a lysis solution (70% formic acid, Sigma-Aldrich). Furthermore, 1 µL aliquot of matrix

Figure 1. Map of study area, Barra do Piraí City.

solution (alpha-ciano-4-hidroxi-cinamic acid diluted in 50% acetonitrile and 2.5% trifluoracetic acid, Sigma-Aldrich) was added to each sediment. The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT Microflex, Bruker) equipped with a 337-nm nitrogen laser in a linear path, controlled by the FlexControl 3.3 (Bruker) program. The spectra were collected in a mass range between 2,000 to 20,000 m/s, and then analyzed by the MALDI Biotyper 3.1 (Bruker) program, using the standard configuration for bacteria identification by which the spectrum of the sample is compared to the references in the database. The results vary on a 0 to 3 scale, where the highest value means a more precise match and reliable identification. In this study, we accept values for matching greater than or equal to 2, as proposed by the manufacturer (Rodrigues et al., 2017).

DNA extraction and detection of virulence genes

DNA extraction was performed by thermal lysis method described by Buyukcangaz et al. (2013) and the virulence genes were analyzed by Polymerase Chain Reaction (PCR) technique.
The PCR technique was performed with a thermal cycler (Bio Rad, T100TM Thermal Cicler, Singapore). The reaction mix contained 10× Buffer (10 mM Tris-HCl (pH 9.0) (Invitrogen), 2.0 mM of 20 mM MgCl2 (Invitrogen), 0.2 mM deoxynucleotide triphosphate (Invitrogen), 1U Taq DNA polymerase (Fermentas), 1 mM of each primer, to a total volume of 20 ul of reaction containing 20 ng of the extracted DNA.

All strains were investigated for molecular markers related to intimin (*eae*A) production, Shiga toxins (*stx*I and *stx*II), heat-labile enterotoxins (*LT*), heat-stable enterotoxins (*ST*), invasivity (*ial*) and enteroaggregative *E. coli* (EAEC) gene (*eagg*). Besides, they were analyzed for genes associated with adherence such as fimbriae F1 (*fim*H), curli fimbriae (*csg*A) and antigen 43 (*flu*). Base sequences and predicted sizes of the amplified products for the specific oligonucleotide primers used are shown in Table 1. The amplified products were evaluated for electrophoresis at 70 volts for 1 h in 1.5% agarose gel and developed with SYBR Green dye (Invitrogen), allowing visualization in UV light and documentation of the amplicons by image capture by the L- PIX EX (Loccus Biotechnology). The size of the fragments was estimated by comparison using the 100 bp molecular weight marker (Fermentas®).

PFGE assay

Preparation of genomic DNA for pulsed field gel electrophoresis

Bacteria were incubated in Soy Tripticase agar at 37°C for 16 to18 h. The cells were removed to microcentrifuge tubes containing 2 ml of Buffer (CSB) (100 mM Tris-HCl 100 mM EDTA pH 8) and cell density were regulated at 1unit. The cell suspension (245 µl) was transferred to microcentrifuge tubes with proteinase K (20-mg/ml stock solution) added to the final concentration of 1 mg/ml each.

An equal volume of molten 2% of Low Melting Point Agarose (BRL®) prepared in TE buffer (10mM Tris, 1mM EDTA, pH8) was added to the cell suspension. The mixture was transferred into two plugs mold and allowed to cool at 4°C for 20 min. The agarose plugs were transferred to 1275 µL of lysis buffer I (50 mM Tris, 50 Mm EDTA, pH 8.0 and 1% sodium lauryl sarcosine) with 75 µL of proteinase K (20-mg/ml stock solution), incubated at 50°C for 90 min. The lysis buffer solution was removed and the plugs were washed twice with water at 50°C for 15 min. After, the plugs were washed with 10 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH8) for 4 times at 50°C for 15 min and stored in TE buffer at 4°C.

Digestion of genomic DNA in agarose plugs

Enzyme digestion was done by addition of the plug slice in microtubule containing restriction buffer (2 µl albumin, 20 µl Buffer 10×, 74 µl nuclease free water and 4 µl restriction enzyme *Xba*I).

Table 1. Primer sequence and predicted sizes of amplication products.

Target gene	Oligonucleotide Sequences of primers	Amplicon size (bp)	Reference*
*eae*A	5'-AGGCTTCGTCACAGTTG-3' 3'-CCATCGTCACCAGAGGA-5'	570	China et al., 1996
*stx*I	5'-AGAGCGATGTTACGGTTTG-3' 3-TTGCCCCCAGAGTGGATG-5'	388	CHINA et al., 1996
*stx*II	5'-TGGGTTTTTCTTCGGTATC-3' 3'-GACATTCTGGTTGACTCTCTT-5'	807	CHINA et al., 1996
ST	5'-ATTTTTCTTTCTGTATTGTCTT-3' 3'-CACCCGGTACAAGCAGGATT-5'	190	Lopez-Saucedo et al., 2003
LT	5'-GGCGACAGATTATACCGTGC-3' 3'-CGGTCTCTATATTCCCTGTT-5'	450	Lopez-Saucedo et al., 2003
ial	5'-GGTATGATGATGATGAGTCCA-3' 3'-GGAGGCCAACAATTATTTCC-5'	650	Lopez-Saucedo et al., 2003
eagg	5'-AGACTCTGGCGAAAGACTGTATC-3' 3'-ATGGCTGTCTGTAATAGATGAGAAC-5'	194	Pass et al., 2000
*fim*H	5'-TGCAGAACGGATAAGCCGTGG-3' 3'-GCAGTCACCTGCCCTCCGGTA-5'	508	Johnson & Stell, 2000
*csg*A	5'-GATCTGACCCAACGTGGCTTCG-3' 3'-GATGAGCGGTCGCGTTGTTACC-5'	178	Silva et al., 2014
flu	5'-CCGGCGGGCAATGGGTACA-3' 3'-CAGCTCTCACAATCTGGCGAC-5'	707	Restieri et al., 2007

*cycle.

The *Xba*I (Promega®) at concentration of 25 units U per plug was used for 3h at 37°C.

Electrophoresis, staining and destaining of agarose gel

PFGE was executed with the CHEF DR-III (Bio-Rad Laboratories, Hercules, C.A) system. Plug peaces were loaded and electrophoresed in 1% SeaKem gold agarose (SIGMA Chemical Company, USA) with 2 l of 0.5 X TBE (Tris Borate EDTA) running buffer. The electrophoretic criterion used: initial switch time, 2.2 s; final switch time, 54.2 s; run time, 19 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14°C; ramping linear. The gels were stained after electrophoresis for 30 min in 150 ml of TBE buffer containing 6 µl of ethidium bromide (10 mg/ml).

A molecular size standard (*Salmonella* enterica serotype Braenderup H9812, CDC PFGE marker) was used at each run along with the *E. coli* isolates to be tested (Romero et al., 2004). The electrophoresis conditions adopted were those recommended by Pulsenet protocol for *E. coli* O157:H7.

The *Xba*I macrorestriction patterns were analyzed using BioNumerics 6.0 software (Applied Maths, A., USA). Clustering was created using unweighted pair group with arithmetic average UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and Dice coefficient interprets according to Tenover et al. (1995). In the comparison of the similarity between clusters the cut tolerance was applied as 1.5%.

RESULTS AND DISCUSSION

The clustering analysis was conducted based on the eighteen profiles of the virulence genes selecting 30 isolates to the PFGE technique (Figure 2). The PFGE patterns of *Xba*I-digested were evaluated to determine the clonal relatedness of *E. coli* isolates in the dairy production during 2014 to 2015.

The detection of 28 different pulsotypes of *E. coli* with a low percentage of genetic similarity (≤ 68%) presents a high heterogeneity among the circulating clones within the dairy establishment studied. This high variability of pulsotypes may be related to the fact that, the target of the present study is strains that present only general characteristics of the species. The *E. coli* is a very diverse species considering virulence, resistance and other genes. Different panorama would be detected if a subgroup of such species had been selected as STEC and EHEC, among others. However, this selection was not possible due to the low prevalence of genes related to toxin production. On the other hand, regarding the presence of genes related to biofilm production, some results were interesting.

Two pulsotypes presented two strains with a clonal ratio of 100% similarity between them, being N46 and C59 belonging to pulsotype 1 and N66 and F86 to pulsotype 17. N46 and C59 strains were found respectively in milk and in the Liners at different periods (spring and summer) and obtained the same virulence profile (only positive to *fim*H). N66 and F86 strains isolated in autumn, presented the same virulence profile characterized by the presence of *fim*H and *csg*A both associated with biofilm formation.

4 strains (F64, F17, F76, C69) with pulsotypes being 97% of genetic similarity presented the identical biofilm-

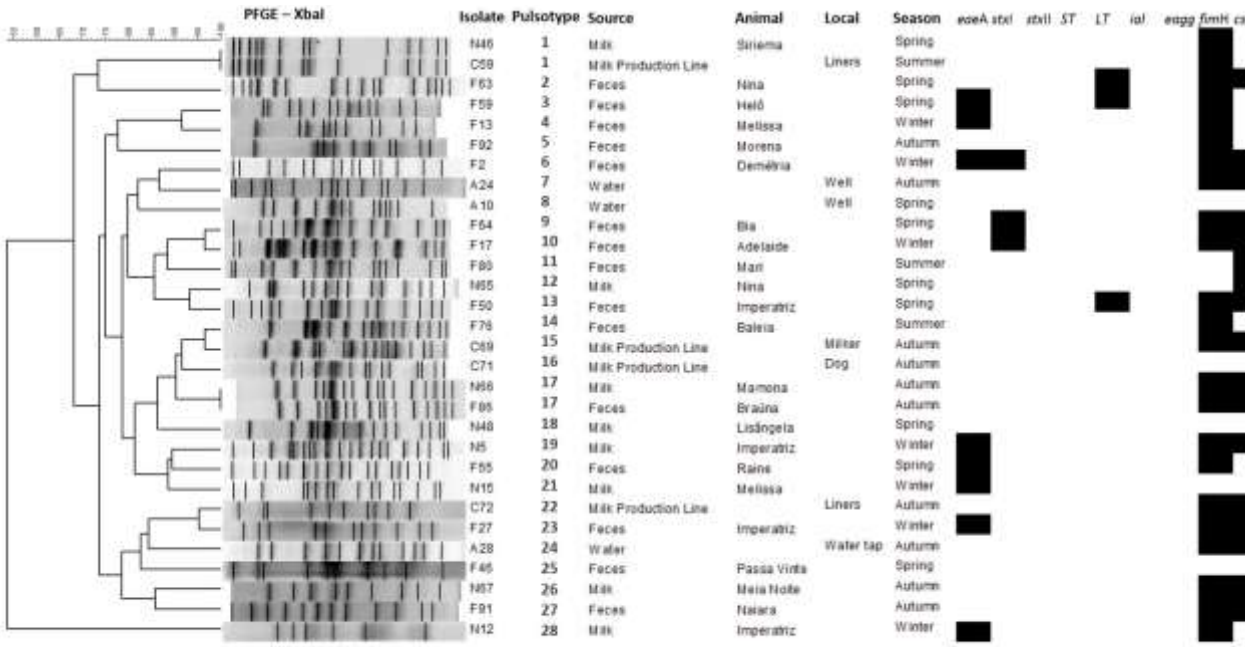

Figure 2. Dendrogram of 30 *E. coli* isolates digested with *Xba*I using the UPGMA method.

related virulence profiles. Furthermore, two of them (F64, F17) also amplify a gene related to shiga toxin (*sxt*I +) production, both isolates from feces samples. 2 strains (C72 and F27) belong to pulsotypes with 95% genetic similarity, they also presented the identical biofilm-related virulence profiles and additionally the F27 was positive for the *eae*A adhesion gene. The Figure 2 presents virulence profiles obtained with the PCR technique.

Dairy cattle and farms are known as reservoirs of several lineage of *E. coli* (Hussein and Sakuma, 2005; Gyles, 2007). This microorganism can enter a dairy farm environment through new animals inserted on herd; beyond the environmental medio such as air, water, and soil; wildlife; or organic materials, such as cattle feed and bedding (Lambertini et al., 2015). The prevalence of *E. coli* O157:H7 in cattle can be related with the seasonal influence obtaining highest rates of infection in the hottest months (Hancock et al., 1994). In this study, the clonal strains were observed in three different seasons: summer, spring and autumn that correspond to the hottest periods in Brazil.

Oliver and Page (2016) observed significant interactions between treatment and season when evaluating the effects of seasonal climatic variables on *E. coli* persistence in cattle feces through different treatments of open field exposure or with polytunnel protection. In their work the season had expressive impact *E. coli* persistence (P< 0.001) and in 2012, both seasons, summer and autumn, were observed higher in *E. coli* counts when compared to spring and winter for all treatments combined. Already in 2013, the highest *E. coli* counts (P< 0.001) was presented only in summer and the spring 2013 corresponded with the period whereby, counts of *E. coli* were considerably higher at the end of the experiment relative to day 0 (P<0.001).

Besides being present in different seasons, this microorganism was also found in different sources such as liners, milk and feces and these conditions can be related to factors that help in the persistence of *E. coli* in milk production environment. The persistence of this microorganism in mammary tissue is associated with virulence factors involving toxins which include enterotoxins that cause food poisoning and leukocidins that promote tissues/leukocyte destruction and also biofilms which enables the adherence and colonization of the mammary gland epithelium (Peacocok et al., 2002; Santos et al., 2003; Argudín et al., 2010). Although the dynamics and routes of introduction, colonization and persistence in animals and the farm environment are not well characterized, these virulence factors are important due to its intensification of the animal disease (Lambertini et al., 2015).

In our study, the presence of biofilm-associated gene (*fim*H) in 21 strains (77.7%) was found and it was observed that, this virulence factor contribute to the dispersion of this pathogen into the milk environment. Researchers have reported that dispersion can be promoted by the interaction between biofilm formation and horizontal gene transfer (Madsen et al., 2012). High frequency of *fim*H gene, the adhesion-encoding gene is associated with epithelial cell invasion in uropathogenic *E. coli*. This gene, detected in 76.6% of isolates was similar with the findings of Dogan et al. (2006), by investigating the possibility that *E. coli* strains associated

with persistent intramammary infections are more capable of adhering, invading and surviving within cultured mammary epithelial cells. Although another study has shown 100% prevalence in mastitis *E. coli* isolates (Fernandes et al., 2011).

According to Madsen et al. (2012) both biofilm formation and horizontal gene transfer have been main areas of research in microbiology showing their relevance for bacterial adaptation and evolution. They observed that plasmid and biofilm community structure and functions are interconnected through numerous complex interactions, as community and genetic level, pointing towards a main role of the action of these activities in bacterial evolution and showing its relevance about this evidence of the connection between horizontal gene transfer and biofilm formation. The premise for interconnectedness between these two strands is that conjugation happens at higher frequencies in that, biofilm are dense communities that speed up the propagation of mobile genetic elements. This happens through a spatial and structural advantage though keeps the conjugative pili intact. Moreover the high horizontal transfer frequencies of mobile plasmids can possibly be the microorganism persistence as molecular parasites (Sorensen et al., 2005; Hausner and Wuertz, 1999; Sorensen et al., 2005). Therefore horizontal transfer can, however, still be an advantage to any mobile genetic element even though is not the main strategy.

Similarly, it has been shown in studies with *E. coli* and *Lactococcus lactis* that horizontal gene transfer within the biofilm community can introduce new dynamics, due to increased expression of clumping factors simultaneously as plasmid transmission which can also cooperate to biofilm formation by their new hosts (Ghigo, 2001; Luo et al., 2005). Conjugative plasmids are inserted to various incompatibility groups, expressing different types of conjugative pili which conferred in stimulatory effect on *E. coli* K-12 biofilm formation in study realized by Ghigo (2001). These results were supported by Reisner et al. (2006) who observed that, natural *E. coli* strains which housed conjugative plasmids was more frequent in biofilm formation and this virulence factor was more expressed during derepression of plasmids. Naturally repressed incF plasmids also are capable to form biofilm, even in a lower frequency. They believe that the expression of the conjugative pili occurs in biofilm priming but it appears that, the pili is not the main structure which directly facilitates the cell surface adherence. Such process is being initiated mainly by activating the host biofilm system (Madsen et al., 2012). This was also reported by May and Okabe (2010), who discussed the expression of colonic acid and curli in *E. coli* induced by a natural incF plasmid. The conjugative pili allow cell–cell contact while the induction of the acids production promote cell-surface adherence to global cohesion and structure of the biofilm. Also Madsen et al. (2012) demonstrated that, interactions are important in understanding the interconnectedness between biofilm arrangement and plasmid biology.

All pulsotypes (n = 8) with genetic similarity of 95% were present in samples of milk, feces and liner, found in different seasons of the year which share at least one gene associated with biofilm formation. The prevalence of biofilm-associated genes indicated the persistence of the microorganism in the production environment due to the presence of biofilm factor which was possible to observe the occurrence of bacterial interaction during milk collection, owing to the fact that they were found in samples which were evaluated as closely related to the animal.

Thus, through PFGE patterns it was possible to determine that this particular farm presented flaws in relation to the hygienic criteria, since it was proven persistence to Enterobacteria in this study in the production environment, during different seasons of the year as well as different sites of collection in a same station. Therefore, the characterization of the genetic diversity of *E. coli* is important in understanding the pathogen dispersion pattern within the environment of milk production, aiding in notes the measures of control and surveillance of these microorganisms in order to control and prevent diseases caused by them.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Phytochemical screening, toxicity and antimicrobial action of *Solanum paniculatum* Linn extract against dental biofilm bacteria

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The aims of study were to determine the phytochemical composition of hydroalcoholic extract of *Solanum paniculatum* Linn (jurubeba) root, to evaluate its *in vitro* antimicrobial action, as well as to determine the acute toxicity and potential cytotoxic effects of this extract. The extract was characterized by phytochemical screening and thin-layer chromatography. The following oral bacteria were used to determine the minimum inhibitory concentration (MIC): *Streptococcus mitis, Streptococcus mutans, Streptococcus sanguinis, Streptococcus oralis, Streptococcus salivarius* and *Lactobacillus casei.* Each assay was carried out in duplicate and the positive control (0.12% chlorhexidine digluconate) was subjected to the same procedure. Results were analyzed by Student t-test or Mann-Whitney test, with the level of significance set at 5%. Preclinical acute toxicity assays were performed using the median lethal dose of the extracts in animals. In addition, the cytotoxic effects of the extracts on human erythrocytes were evaluated. *Solanum paniculatum* showed MIC values of 7.81 mg/mL. The extract had no acute effects at concentrations of 0.97 to 500 mg/mL. The *S. paniculatum* extract was only cytotoxic at a concentration of 250 mg/mL. Phytochemical screening revealed predominance of phenolic compounds such as flavonoids and tannins. In conclusion, *S. paniculatum* Linn showed *in vitro* antimicrobial activity against bacterial monocultures. No toxicological effects were observed. The predominance of phenols may explain the pharmacological activity of this extract. However, randomized controlled clinical trials should be conducted to evaluate the effect of *S. paniculatum* added to mouthwash solution.

Key words: Microbiology, phytotherapy, chromatography, toxicity, *Solanum paniculatum* Linn.

INTRODUCTION

Diseases affecting the oral cavity are of infectious origin. Depending on factors such as diet and regular

mechanical removal of plaque, the type of microbiota prevailing in the oral cavity may vary, having a high or low degree of pathogenicity. There are difficulties in achieving patients' adequate mechanical control of dental plaque accumulation, therefore, antimicrobial substances could compensate with regards to good teeth cleaning (Torres et al., 2000).

Many cultures commonly use medicinal plants for the treatment of various disorders and diseases affecting humans. From this perspective, the benefits of these herbal medicines are a source of promising research in countries like Brazil, known for its immense floral diversity, as well as for a high prevalence and variability of infectious diseases (Vieira et al., 2010).

Research involving herbs and plants in dentistry is increasing in order to elaborate new therapies against oral infections. In this context, there are many studies that relate the oral microbiota to the use of medicinal plants, with the objective of eliminating pathogenic agents (Majid and Omid, 2011).

The oral cavity often has a diverse microbiota and the eruption of dental elements leaves this microbiota even more complex. The pioneer organisms in the mouth are the *Streptococcus mitis.* After tooth eruption, other commensal species of *Streptococcus*, including *Streptococcus sanguinis* and *Streptococcus gordonii* are present as pioneers of the dental biofilm. From these species, it is possible that other opportunistic pathogens such as the are *mutans* colonize, which is the primary species associated with dental caries. Thus, the state of oral health or disease suffers crucial influence of colonization by the commensal species of *Streptococcus* because the oral microbiota is occupied by them (Moraes et al., 2014).

The "jurubeba" (*Solanum paniculatum* Linn) is a species found in America, in tropical regions, mainly in the Brazilian Cerrado. It is used not only in folk medicine for the treatment of liver and gastric diseases, but also in culinary practices (Vieira Junior et al., 2015; Vieira et al., 2010). In addition, as a highlight of its utility, "jurubeba" tea is great for hangovers and extracts are used for respiratory and stomach diseases. It has properties that prevent gastric secretion, and also anti-inflammatory and antioxidant characteristics (Vieira et al., 2010, 2013).

The *Solanaceae* family is constituted by species and toxic characteristics and pharmacological properties. Some previous studies with the ethanolic extract of the leaf or fruit of *S. paniculatum* have shown that they have no genotoxic activity in rats or bacterial strains, despite their cytotoxicity and antigenotoxicity in high doses. The active constituents of jurubeba have been reported as steroid saponins, glycosides, alkaloids and tannins in the roots, stems and leaves (Vieira et al., 2013).

The aim of the study was to evaluate the antimicrobial action, toxicity and cytotoxicity of the *S. paniculatum* and to analyze the plant material with respect to its phytochemical aspects.

MATERIALS AND METHODS

Preparation of the *Solanum paniculatum* Linn. extract

The roots of *S. paniculatum* were collected in the town of Teixeira, Paraíba, Brazil. The crude extract was prepared at the Laboratory of Chemical and Biological Sciences, Federal University of Campina Grande (UFCG), Center for Health and Rural Technology (CSTR). A voucher specimen of the plant was deposited at the Dárdano de Andrade Lima Herbarium, Regional University ofCariri (URCA), Crato, Ceará (Registration No. 4016).

After collection, the *S. paniculatum* root samples were desiccated in an oven under circulating air at an average temperature of 45°C and then ground to powder in a mechanical grinder. The dry and ground material was macerated with 2 L of 95% ethanol for 72 h. The resulting crude *S. paniculatum* extract was concentrated in a rotary evaporator under reduced pressure at a temperature that did not exceed 40°C. For phytochemical analysis, the crude extract was resuspended in distilled water to a final concentration of 10%.

Phytochemical analysis and thin layer chromatography (TLC)

The crude extract of *S. paniculatum* was resuspended in distilled water (10% solution) and then was submitted to various qualitative tests of color change, excitation by ultraviolet light and precipitation, to identify several classes of secondary metabolites. These tests investigated the presence of saponins, tannins, gums, mucilages, flavonoids, quinones, lactones, coumarins, steroids, triterpenoids, carotenoids, alkaloids, catechins and resins through specific chemical reactions.

Next, the extract of *S. paniculatum* was analyzed by thin layer chromatography (TLC) through the resuspension in methanol (1% solution) for application in chromatographic plates (Merck®, Darmstadt, Germany). Silica gel chromatoleaves were used as a fixed-phase and various mobile phases were assayed for the presence of specific phenols. The extract and standards were eluted in saturated chromatographic vats, using a methanol solution of ferric chloride to 5% for the revelation of tannins (catechin, epicatechin, tannic acid, ellagic acid and gallic acid), while the plates which researched flavonoids (quercetin, isoquercetina, rutin, vitexin and isovitexin) were used a natural reagent (Wagner and Bladt, 1996; Lionço et al., 2001).

Determination of minimum inhibitory concentration (MIC)

Antimicrobial activity on plates was determined according to the methodology proposed by Bauer et al. (1966), by diffusion in solid medium in Petri dishes for the determination of minimum inhibitory concentration (MIC) of extract of *S. paniculatum* Linn.

The bacterial strains (*S. mitis* -ATCC 903-, *S. mutans* -ATCC 25175-, *S. sanguinis* -ATCC 15300-, *S. oralis* -ATCC 10557-, *S. salivarius* -ATCC 7073- and *Lactobacillus casei* -ATCC 9595) were cultured in nutrient broth (BHI - brain heart infusion - Difco,

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Michigan, USA), incubated at 37°C for 18-20 h under microaerophilic conditions by the candle flame method. Mueller-Hinton agar plates (Difco, Michigan, USA) were prepared and after 24 h (sterility control), they were flooded with saline inoculated with each bacterial growth at a concentration of 10^{-1}, and below, and standard holes were prepared measuring approximately 6 mm in diameter. In each plate, five holes were made receiving numbering ranging from 1 to 5, which corresponded to the solution of the crude extract (CE), which were then diluted in distilled water (CE to 1:512). After the introduction of 50µL of the test substances, the plates were incubated inside a bacteriological greenhouse at 37°C for 24 h. Each assay was carried out in duplicate of each selected strain. The same procedure was used for the positive control, 0.12% chlorhexidine digluconate (Periogard®, Colgate-Palmolive Company, New York, USA.).

The lowest concentration of the extract that could inhibit growth was regarded as MIC, represented by the presence of an inhibition halo, measured in mm with the aid of the caliper (Digimess®, São Paulo, Brazil).

Determination of the lethal dose (LD_{50})

For the LD_{50}, adult animals (Swiss albino mice) were used, weighing between 18 and 40 g, provided by Vivarium professor Thomas George LTF-UFPB. All were placed in polyethylene cages, with five animals per cage, maintained under temperature conditions of 27 ± 2°C, without any medication, having free access to food (pellets type purine diet) and water. The animals were kept in light/dark cycles of 12 h before performing any experimental protocol. The animals were divided into 21 groups of five units and treated with the extract of *S. paniculatum* Linn (at different concentrations - CE to 1:512) intraperitoneally (I.P.) in a single dose of 0.1 mL/animal. The control group was given distilled water.

Then, the animals were observed for a period of 24 h with the objective of mapping possible behavioral changes, suggestive activity on the central nervous system (CNS) or on the autonomic nervous system (ANS). The animal observation method was based on the experimental protocol developed by the psychopharmacology sector LTF/UFPB (Almeida et al., 1999). After treatment, the general comparative effects on the animals in the control group were observed in intervals of 30, 60, 120, 180 and 240 min.

In the end, the number of dead animals was counted to determine the dose which killed 50% of the experimental animals (lethal dose = LD_{50}).

Evaluation of the cytotoxic potential of extract of *S. paniculatum* Linn in human erythrocytes

The human erythrocytes (A, B, O and AB) were derived from blood that could not be used for transfusion (blood being discarded), obtained in the Transfusion Unit of the University Hospital Lauro Wanderley/UFPB. The handling and disposal of blood was performed according to security norms followed by the unit.

A human blood sample was mixed with NaCl 0.9% in the ratio 1:30 and centrifuged (model 206, FANEM) at 2500 rpm for 5 min to obtain the erythrocytes. This procedure was repeated twice and the pellet from the last centrifugation was resuspended in 0.5%, at NaCl 0.9%. Samples of the test products (extract) at different concentrations were added to 2 mL of the red cell suspension to a final volume of 2.5 mL. The negative control was assembled with red cell suspension + NaCl 0.9% (0% hemolysis), and the positive control with red cell suspension + Triton X-100, 1% (100% hemolysis). Samples were incubated for 1 h at 25°C under slow and constant stirring (100 RPM). After this time, they were centrifuged at 2500 rpm for 5 min and hemolysis was quantitated by spectrophotometry (DU 640 BECKMAN) 540 nm (Rangel et al. 1997). All experiments were performed in triplicate.

Ethical considerations

The research was approved by the Research Ethics Committee of UFCG (Protocol / CEP / UFCG No. 34/2010) to determine LD50. The research was approved by the Ethics Committee in Research with Human Beings UFPB (Protocol / CEP / HULW No. 743/10), Cover Sheet 390665, CAAE 6396.0.000.126-10 for the evaluation of the cytotoxic potential of extracts in human erythrocytes.

RESULTS

Phytochemical analysis

Phytochemical screening of the *S. paniculatum* Linn. extract revealed the presence of alkaloids, gums and lactones, as well as a high content of phenols (flavonoids and tannins). Qualitative analysis of phenols by TLC showed a spot suggestive of isovitexin (flavonoid) and the presence of tannic acid (tannin) in the extract.

Determination of minimum inhibitory concentration

The *S. paniculatum* extract exhibited antibacterial activity against all bacteria tested. The extract was effective against *S. sanguinis* and *L. casei* up to a dilution of 1:64 (7.81 mg/mL). The antibacterial activity of the *S. paniculatum* extract was significantly higher than that of chlorhexidine at concentrations/dilutions of 1:4 (125 mg/mL) and 1:8 (62.5 mg/mL) (Table 1).

Determination of the lethal dose (LD_{50})

This study observed that the mice showed behavioral changes such as piloerection and intense movement of the vibrissae up until the first 60 min for the *S. paniculatum* extract (up to 1:16 dilution- 31.25 mg/ml). There were no serious side effects and the animals showed behavioral changes, suggestive of stimulating the central nervous system.

Evaluation of the cytotoxic potential of extract of *S. paniculatum* Linn in human erythrocytes

The *S. paniculatum* extract at a concentration of 7.81 mg/mL (MIC) haemolysed 41.2% of human erythrocytes of type A, 45.1% of type B, 16.4% of type O and 24.8% of type AB. However, it showed cytotoxicity at a dilution of 1:2 (250 mg/ml).

DISCUSSION

The control of dental biofilm is the primary goal to be

Table 1. Student t-test or Mann-Whitney U test (MIC *Solanum paniculatum* extract vs. chlorhexidine digluconate).

Concentrations/dilutions of extract/chlorhexidine	MIC – Mean (rank[#])		Statistic	Significance
	Extract	Chlorhexidine	(t ou U)	p-value
EB (500 mg/ml) vs. C	18.33 (7.33)	18.00 (5.67)	t=0.291. gl=10[++]	0.777
E 1:2 (250 mg/ml) vs. C1:2	17.17 (7.58)	16.33 (5.42)	U=11.50	0.310
E 1:4 (125 mg/ml) vs. C1:4	16.50 (8.75)	14.33 (4.25)	U=4.50	0.026*
E 1:8 (62.5 mg/ml) vs. C1:8	14.50 (8.75)	13.33 (4.25)	U=4.50	0.026*
E 1:16 (31.25 mg/ml) vs. C:1:16	13.00 (7.67)	10.50 (5.33)	U=11.00	0.310
E 1:32 (15.65 mg/ml) vs. C1:32	8.17 (7.33)	5.67 (5.67)	U=13.00	0.485
E 1:64 (7.81 mg/ml) vs. C1:64	4.00 (7.00)	2.00 (6.00)	U=15.00	0.699

[(#)] Rank = mean of Student t-test or Mann-Whitney U test CE: crude extract CD: chlorhexidine digluconate; [(++)] Teste de Levene; * (*p-value* < 0,05) (significant results).

achieved in order to prevent caries and periodontal disease, which can be accomplished by employing methods of oral hygiene, using instruments that provide the removal and mechanical disruption of these biofilms such as toothbrushes and dental floss. Furthermore, the combined association of using these mechanical devices with chemical procedures have demonstrated their efficiency and highlighted them as being ideal for the control of dental biofilm, especially in patients with high potential for developing oral diseases (Filogônio et al., 2011).

Although, the joint use of mechanical and chemical methods is recommended as “ideal” with positive effects in controlling biofilm and for preventing or minimizing the development of diseases, herbal medicines represent an alternative that is gaining more space as a therapeutic option in fighting various pathologies. Obviously, the mere fact of being natural is not indicative of being totally free from side effects, but studies suggest that they present a considerable safety margin, as their medicinal properties do not represent a risk to human health (Queiroz et al., 2014).

Brazil has a very diverse flora, which is a result of the climatic and geological aspects of its extensive territory. This facilitated the development of several species of plants, which are the object of current experimental studies in order to identify the presence of antimicrobial, antiseptic, anti-inflammatory and antioxidant properties with healing characteristics. Thus, it is important to investigate the use of these leaves and roots of plants as substances that are capable of inhibiting the onset and progression of some oral and respiratory diseases.

The aim of the study was to evaluate *in vitro* antimicrobial action, toxicity and cytotoxicity of the *S. paniculatum,* and to analyze the plant material in relation to its phytochemical aspects. The results of the phytochemical analysis make it clear that high activity against various species of microorganisms occurs due to the presence of large quantities of phenols (among which flavonoids and traces of pyrogallic tannins can be found), gums, lactones and alkaloids (in the presence of reactives of Dragendorff and Bertrand) in jurubeba extract, which is consistent with the findings of Mesia-Vela et al. (2002), Silva et al. (2005) and Cheng et al. (2008). Moreover, the chromatogram highlighted a stain which suggested the presence of isovitexin and tannic acid in the jurubeba, important finding to illustrate its pharmacological effect. The results of phytochemical screening and TLC of the extract were in accordance with those reported by Mesia-Vela et al. (2002), Silva et al. (2005) and Cheng et al. (2008). The antimicrobial potential of these compounds may explain the pharmacological activity of the extract studied. Also, the presence of sulfur compounds is interesting, since one of the main characteristics of these compounds, despite their structural differences, is the antimicrobial activity (Heinzmann, 2007).

The presence of products classified as polyphenols justified the analysis of *in vitro* antibacterial activity by determining the minimum inhibitory concentration (MIC) of the plant root extracts in question, against the activity of the microorganisms *S. mutans*, *S. mitis*, *S. sanguinis*, *S. oralis*, *S salivarius* and *L. casei*. This demonstrates that there was homogeneous inhibition of growth of these bacteria, which were verified by the concentration of the extract in this study directly related to the diameters of halos, indicating that the progressive reduction of the extent of such halos thereby reduce the extract concentration.

The *S. paniculatum* extract showed an antibacterial effect in the dilution of 1:64 (7.81 mg/mL) for *Streptococcus sanguinis* and *L. casei*. Chlorhexidine had an antibacterial effect on all microorganisms as expected, and *S. mutans* bacteria was more sensitive to the extract, with zone of inhibition up to 1:64 dilution. The *S. paniculatum* extract had a higher antimicrobial activity than that of chlorhexidine digluconate at concentrations of 125 mg/mL (1:4) and 62.5 mg/mL (1:8). This data showed the potential bacteriostatic effect *in vitro* of *S. paniculatum* in all strains (Table 1).

Although, the beneficial effects *in vitro* are noticeable, it is necessary to investigate the toxic effects, to allow its

use *in vivo*. For this, pre-clinical trials were developed in mice by using a lethal dose (LD_{50}), did not cause mortality in any of the tested concentrations (500 mg/mL [undiluted] and 0.97 mg/ml [diluted]) after 1, 3 and 15 days. However, behavioral modification is seen at time intervals of 30, 60, 90 and 240 min, verifying piloerection and vibrissae movements within 60 min and even 31.25 mg/mL (1:16). There were no serious side effects and the animals showed only minor behavioral changes, suggestive of CNS stimulation, thus from these findings extracts could be applied clinically in the concentrations tested. Regarding *S. paniculatum,* there are no other tests to determine its lethal dose, however, Vieira et al. (2010) evaluated the mutagenic and cytotoxic activities of ethanolic extracts of leaves and fruits of *S. paniculatum*, using the micronucleus test in bone marrow of mice, and found that there was no mutagenic action in mouse bone marrow. Nevertheless, in higher doses, both extracts showed cytotoxic activity. This study did not show an increase in cytotoxicity dose-response at 200 and 300 $mg.kg^{-1}$.

The cytotoxicity of the extract of *S. paniculatum* in human erythrocytes induced a low hemolytic activity (Prokof'eva et al., 2004), rate lower than 50% in all four blood types in MIC 7.81 mg/mL, and cytotoxic at 250 mg/mL (1:2). Studies involving antimicrobial activity and the adverse effects of *S. paniculatum* are still little mentioned in the literature. Although, they do not report anything that would compromise human health, they require further research to verify the effectiveness of this present study in the prevention of oral diseases.

The results showed that *S. paniculatum* has a significant potential of having a bacteriostatic reaction *in vitro* and only presented deleterious effects toxicologically in high concentrations making possible, the performance of a randomized controlled clinical trial, and evaluating the effect of *S. paniculatum* incorporated in a mouthwash, solution over the oral bacteria and dental biofilm.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Phytochemical screening and antifungal activity of leaves extracts of *Luffa cylindrica* (Roem)

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Mycosis constitutes a common health problem, especially in developing countries like Nigeria. The current cascade of antifungals are either too toxic or require long term use for total eradication. This study evaluates antifungal activity and phytochemical constituents of *Luffa cylindrica* leaves extract which were screened for the presence of bioactive phytochemicals and extracted by cold maceration in n-hexane, ethyl acetate, methanol and water. The ethyl acetate extract was further fractionated using bio-assay guided column chromatography. *In vitro* antifungal activities were investigated against three types of fungi which were, *Candida albicans* ATCC 2876, *Candida tropicalis* ATCC 19092, *Trichophyton rubrum* ATCC 28188; and four clinical isolates of *C. albicans, C. tropicalis*, *Microsporum canis* and *Epidermophyton flocossum* using agar diffusion and micro broth dilution methods. The crude extracts revealed the presence of sterols, saponins, flavonoids, phenols and alkaloids. The ethyl acetate extract produced the strongest antifungal activity with diameter zones of inhibition ranging from 13.00 to 16.00 mm at an exposure concentration of 2500 µg/mL. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values of the ethyl acetate extract against the test fungi were 500 to 1000 µg/mL and 2000 to 4000 µg/mL, respectively. L5 and L6 fractions produced fungal inhibitory activity comparable to the crude ethyl acetate extracts of *L. cylindrica* with MIC values of 1000 to 2000 µg/mL and 500 to 2000 µg/mL, respectively. The ethyl acetate extracts of *L. cylindrica* possess antifungal properties that could serve as leads for the development of novel antifungal drugs.

Key words: *Luffa cylindrica* leaves, antifungal, phytochemical screening, minimum inhibitory concentration.

INTRODUCTION

In the past few decades, there has been a worldwide increase in the incidence of fungal infections due to a rise In the resistance of some species of fungi to current antifungal agents used in medicinal practice (Abad et al., 2007; Senguttuvan et al., 2013; Dzoyem et al., 2014). The rise in the incidence of fungal infections has

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exacerbated the need for the next generation of antifungal agents, since many of the currently available drugs have undesirable side effects, and are ineffective against new or re-emerging fungi, or lead to the rapid development of resistance (Kullberg and Filler, 2002; Kreander et al., 2005).

There has been little break-through in the research into development of new antifungal drug unlike antibacterial agents (Scorzoni et al., 2017). Superficial mycosis are the most prevalent fungal infections, with infection rate in humans worldwide of about 25%, however invasive fungal infections (systemic) are more life-threatening, difficult to diagnose with limited amount of therapeutic options and accounting for approximately 1.5 million deaths annually (Souza and Amaral, 2017). Owing to the few antifungal arsenal, researchers have explored several approaches, with the most recent and effective development being the application of nanotechnology thus employing nanoparticles as carrier for antifungal drugs (Scorzoni et al., 2017).

Luffa cylindrica (L.) M. Roem belongs to the family Curcubitaceae. It is also known as *Momordica cylindrica* L. (1753), *Luffa aegyptica* Mill. (1768). *L. cylindrica* is widely distributed in the tropics and subtropics, as a cultivated and naturalized plant. Its cultivation is of ancient origin and it is hard to determine whether the native home is Africa or Asia. *L. cylindrica* has been reported to possess both medicinal and nutritional properties (Partap et al., 2012). Its seeds have been used in the treatment of asthma, sinusitis and fever (Sashikala et al., 2009). Its use in AIDS management can be linked to the presence of proteins such as luffaculin with ribosome-inhibiting properties on the replication of HIV infected lymphocyte and phagocyte cells (Otimenyin et al., 2008).

Abirami et al. (2011) reported that juice extracted from the stem is used in the treatment of respiratory disorders and the seed has emetic action. The aim of this study was to screen for phytochemical constituents and evaluate the antifungal activities of the *L. cylindrica* leaf extracts.

MATERIALS AND METHODS

Chemicals and media

Dimethyl sulphoxide (DMSO), fluconazole (Cat No. F8929), terbinafine HCl (T8826), n-hexane, ethyl acetate, and ethanol were obtained from Sigma Aldrich Laboratories, Germany. Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were obtained from Oxoid, Germany.

Plant collection

The fresh leaves of *L. cylindrica* were collected from the botanical garden of the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria between September and October, 2014. Voucher specimen (NIPRD/H/6643) of plant was deposited in the herbarium at the Department of Medicinal Plant Research and Traditional Medicine, NIPRD, Abuja Nigeria. The fresh leaves were separated, shade dried and grinded into powder using mortar and pestle.

Test organisms

Candida albicans ATCC 2876, *Candida tropicalis* ATCC 19092, clinical strains of *C. albicans*, *C. tropicalis*, *Trichophyton rubrum* ATCC 28188, clinical strains of *Microsporum canis* and *Epidermophyton floccosum* were obtained from Department of Microbiology and Biotechnology, NIPRD, Abuja, Nigeria. Suspensions of fungi were made in SDB. Subsequent dilutions were prepared from the above suspensions and used in the tests.

Extract preparation

Five hundred grams (500 g) each of the powdered leaves was macerated in various solvents (n-Hexane, ethyl acetate, methanol and water) with random shaking for 72 h and filtered. After each extraction, the extracts were concentrated using a rotary evaporator and water bath, dried and weighed.

Phytochemical screening

The freshly prepared extracts of the powdered *L. cylindrica* were evaluated for the presence of carbohydrate, tannins, flavonoids, phlobatannins, saponins, alkaloids, terpenes, sterols, phenols, resins and anthraquinone using simple qualitative and quantitative methods of Sofowora (1993) and Evans (2004).

Fractionation of ethyl acetate extract of *L. cylindrical*

Column chromatography was used to further simplify the solvent extract with highest antifungal activity from previous extraction (Masoko and Eloff, 2005). The wet method for packing of chromatographic columns was used; silica gel 60 was made into slurry with the least polar solvent and then poured slowly into a column (40.5 cm × 3.0 cm), on top of a small amount of cotton wool. The sample was dissolved in small quantity of appropriate solvent and then triturated thoroughly with equal weight of silica gel 60 in a mortar and pestle and the mixture was allowed to air dry. The extract- silica gel mixture was made into slurry with the most non polar solvent and poured neatly on top of the silica in the column. Filter paper cut to the internal diameter of the column and cotton-wool was neatly placed on top of the sample to prevent disturbance at the surface during solvent introduction. The appropriate elution systems were added slowly in the increasing order of their polarity. The fractions were eluted with n-hexane (100%), n-hexane – ethyl acetate (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 20:80, 10:90), ethyl acetate (100%), ethyl acetate – methanol (90:10, 80:20, 60:40, 40:60), methanol (100%), methanol – water (90:10, 80:20, 60:40). With the addition of solvent into the column, the vacuum was switched on. The solvent was allowed to run through the column; until all the solvent had been collected in the beakers through a separating funnel. The solvents collected in the beakers were concentrated using a rotary evaporator and TLC analysis carried out.

Preparation/ standardization of fungi

The yeast (*Candida* sp.) was standardized by inoculating sterile

normal saline solution with a 48 h pure culture by adjustment of turbidity to match 0.5 Mc Farland standards. Standardization of the dermatophytes included harvesting fungal spores from a 7 day old culture on SDA slant. Ten milliliters (10 mL) of sterile normal saline containing 3% (w/v) Tween 80 was used to disperse the spores with the aid of sterilized glass beads (Olowosulu et al., 2005). Standardization of the spore suspension to (1.0 x 10^6 spores/mL) was achieved with a UV spectrophotometer (Spectronic 20D; Milton Roy Company, Pacisa, Madrid, Spain) at 530 nm (OD530) of the suspensions and adjusted to a transmittance of 70 to 72%. The standardized fungal suspensions were quantified by spreading 100 µL on Sabouraud dextrose agar plate. The plates were incubated 24 h at 37°C for yeast and 72 h at 30°C for dermatophytes (Aberkane et al., 2002).

In vitro assessment of antifungal activity

Cup plate agar diffusion method (Etuk et al., 2008) was used to assess the antifungal activity of the extracts. Eighteen hours culture of *Candida* spp. and inoculum suspensions of the dermatophytes prepared from fresh, mature (7 to 14 day old) cultures in Sabouraud dextrose liquid medium were standardized to produce inoculum size of 10^6 cfu/mL. One millilitre (1 mL) of the diluted culture of each test organism was used to flood Sabouraud dextrose agar media and excess aseptically drained. The plates were allowed to dry at 37°C in a sterilized incubator. Adopting the agar diffusion cup plate method (Olowosulu et al., 2005), a sterile cork borer (6 mm) was used to bore holes in the agar plates. The bottoms of the wells (holes) were sealed with the appropriate molten Sabouraud dextrose agar. Using micropipette, 0.1 ml each of the different graded concentrations of the ethyl acetate extract was dispensed into the holes marked 'A' (20 mg/mL), 'B' (10 mg/mL), 'C' (5 mg/mL) and 'D' (2.50 mg/mL). Distilled water and the solvents used in diluting the extracts were used as control. These were allowed to diffuse into the agar at room temperature for 1 h before incubation at 37°C for 18 h (yeast) and 30°C for 72 h (dermatophytes). The zones of inhibition of the test organisms were measured to the nearest millimetre, using a well-calibrated meter ruler and pair of dividers. The experiment was carried out in triplicates (Olowosulu et al., 2005).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC value of the extracts and fractions against the fungal strains was determined using broth microdilution bioassay with tetrazolium violet reduction as an indicator of growth (Pereira et al., 2011). The 96-well plates were prepared by dispensing 50 µL of Sabouraud dextrose broth into each well and 50 µL from the stock suspension of plant extracts and fractions was added into the first wells. Then, 50 µL from their serial dilutions was transferred into consecutives wells, excluding the last ones. The last well contained 50 µL of broth inoculated with fungal inoculum to confirm the cell viability (viability control). At the same way positive controls were carried out with standard antifungal using terbinafine HCl and fluconazole. Sterility control was performed to verify whether the broth used in antifungal assay was contaminated before test procedures. For that, 50 µL of broth was dispensed into a well, without both extract and inoculum. As an indicator of growth, 40 µL of 0.2 mg/mL of p-iodonitrotetrazolium violet was added to each of the microplate wells.

Determination of the Minimum Fungicidal Concentration (MFC)

Minimum fungicidal concentration was determined using the micro dilution method to verify if the inhibition was reversible or permanent. Aliquot of 50 µL from the wells that did not show growth in MIC procedure (inactivated with 10% tween 80) was transferred to 96-well plates previously prepared with 50 µL of SDB. The plates were aseptically sealed followed by mixing on plate shaker (300 rpm) for 30 s, incubated at 30°C for 2 to 7 days. The test was performed in triplicate and the geometric mean values were calculated. Minimum fungicidal concentration was defined as the lowest extract concentration in which no visible growth occurred when sub cultured on the 96-well plates containing broth without antifungal products.

Table 1. Percentage yield of extracts from 1000 g of *L. cylindrica* leaves using various solvents.

Solvents	Extract mass (g)	Yield (%)
n-Hexane	20.00±0.33	2.00±0.33
Ethyl acetate	36.52±0.33	3.65±0.33
Methanol	40.00±0.67	4.00±0.67
Water	45.60±0.00	4.56±0.00

Statistical analysis

Data obtained were expressed as mean ± standard deviation and analyzed for significance using Students t-test and one way ANOVA (GraphPad Prism 5) at $p < 0.05$.

RESULTS

The yield of the different solvent extracts as shown in Table 1 revealed an increase in the extraction yield with increase in the polarity of the extraction solvents. As a result, water (most polar) yielded the greatest quantities and less polar solvent n-hexane extracted the least amount. The yield of the hexane, ethyl acetate, methanol and water were 2.0, 3.65, 4.0 and 4.56% respectively.

The phytochemical screening of the extracts *L. cylindrica* revealed the presence of carbohydrates, sterols, saponins, flavonoids, alkaloid and phenols; while resins, tannins, terpenes, balsams and anthraquinones were not detected. Phytochemical screening of Fractions F5 and F6 revealed the absence of flavonoids and phenols (Table 2). The result on the susceptibility of the various extracts at a concentration of 20 mg/mL on the growth of the test fungi is represented in Table 3.

Generally, all the extracts showed a level of inhibition against all the fungi tested with exception of n-hexane extracts which exhibited no inhibitory activity against *E. flocossum*. However, the ethyl acetate and methanol extract produced the strongest antifungal activities which were comparable with the standard antifungals used. The strongest inhibitory activity was exhibited by the ethyl acetate extracts with a diameter zone of inhibition range of 18.0 to 21.0 mm, this was followed by methanol extracts (15.0 to 18.7 mm) and least by the water and n-hexane extracts with zones of inhibition range of 9.0 to 12.0 and 9.0 to 11.0 mm, respectively.

Result of the effects of increasing concentration of the crude ethyl acetate and methanol extracts of *L. cylindrica*

Table 2. Phytochemical analysis of powdered leaves and crude extracts of *Luffa cylindrica* leaves.

Secondary metabolites	Hexane	Ethyl acetate	Methanol	Water	F5	F6
Carbohydrates	+	+	+	+	+	+
Terpenes	-	-	-	-	-	-
Sterols	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Tannins	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-
Phlobatannins	-	-	-	-	-	-
Resins	-	-	-	-	-	-
Alkaloids	+	+	+	+	+	+
Flavonoids	+	+	+	+	-	-
Phenols	+	+	+	+	-	-

+ : Present; - absent.

Table 3. Susceptibility of the test fungi to the solvent extracts of *Luffa cylindrica* at concentration of 20 mg/mL.

Organisms	Zone of Inhibition (mm)					
	HEX	ETA	MT	W	FCZ (30 µg/ml)	TER (30 µg/mL)
C. albicans ATCC 2876	9.00±0.57^{a}	20.00±0.00^{b}	18.0±0.00^{c}	9.0±0.00^{a}	29.00±0.57^{d}	NA
C. albicans	10.67±0.33^{a}	20.00±0.57^{b}	18.0±0.00^{c}	10.0±0.00^{a}	30.00±0.33^{d}	NA
C. tropicalis ATCC 19092	10.67±0.33^{a}	18.00±0.00^{b}	17.0±0.00^{b}	10.0±0.00^{a}	20.67±0.33^{c}	NA
C. tropicalis	10.33±0.57^{a}	18.00±0.00^{b}	17.0±0.00^{b}	10.0±0.00^{a}	20.33±0.33^{c}	NA
M. canis	11.00±0.00^{a}	16.33±0.33^{b}	15.0±0.00^{b}	10.0±0.00^{a}	NA	28.33±0.33^{c}
T. rubrum ATCC 28188	9.00±0.00^{a}	20.67±0.33^{b}	18.0±0.00^{c}	11.0±0.00^{a}	NA	29.33±0.33^{d}
E. flocossum	IA	21.00±0.00^{a}	18.67±0.33^{b}	12.0±0.00^{c}	NA	29.33±0.33^{d}

Values are mean inhibition zone (mm) ± S.D of three replicates, $p < 0.05$; Values with different superscript (a, b, c, d) on the same row are significantly different ($p < 0.05$). HEX- hexane; ETA- ethyl acetate; MT- methanol; W- water; FCZ- fluconazole; TER- terbinafine; NA- not applicable; IA –inactive.

are shown in Tables 4 and 5, respectively. The results revealed a gradual increase in the inhibitory action of the extracts with increase in concentration of the extracts. However the ethyl acetate extracts (13.0 to 20.0 mm) showed stronger antifungal activity than the methanol extract (10.0 to 18.0 mm) against all the fungi tested.

Fractions L5 and L6 (ethyl acetate extract) produced fungal inhibitory activity comparable to the crude ethyl acetate extracts of *L. cylindrica* with MIC values of 1000.0 to 2000.0 µg/mL and 500.0 to 2000.0 µg/mL, respectively, and MFC values range of 4000.0 to 8000.0 µg/mL and 2000.0 to 4000.0 µg/mL, respectively. The MIC of fraction L5 though comparable with the crude extract, however is not as active against *T. rubrum* (1000.0 µg/ml) while L6 was most active against *C. albicans* (500.0 µg/mL) with MIC similar to the crude extract.

DISCUSSION

The biological activities of the extract have also been linked to the solvent polarity (Zohra and Fawzia, 2011). Ahmad et al., (2009) reported that the yield of extraction is greatly influenced by the polarities of solvents; the more polar solvent produces higher yields than the less polar solvents. The extraction yield of the aqueous extract was lower than those previously reported by Mhya et al. (2014), who reported a percentage yield of 17.75. The lower yield recorded in this study could be linked to differences in the season of collection and location of the plant.

Plants possess bioactive phyto-compounds like saponins, tannins, flavonoids, and alkaloids etc. which have been shown to be responsible for their antimicrobial potentials (Thamaraiselvi and Jayanthi, 2012). The presence of carbohydrates, sterols, saponins, alkaloids, flavonoids and phenols in the study plant agrees with studies reported in the past (Aboh et al., 2012; Sharma, 2012; Mhya et al., 2014). However, the absence of glycosides in the study contrasts previous reports (Sharma, 2012; Mhya et al., 2014), tannins (Mhya et al., 2014). This could be related to the period of collection, methods of extraction or location of the plants. The

Table 4. Antifungal susceptibility testing of crude ethyl acetate extract of *Luffa cylindrical.*

Organisms	Zone of Inhibition (mm)			
	20 mg/mL	10 mg/mL	5 mg/mL	2.5 mg/mL
C. albicans ATCC 2876	20.00±0.00^{a}	20.00±0.33^{a}	17.50±0.33^{b}	15.00±0.00^{c}
C. albicans	20.00±0.57^{a}	19.00±0.00^{a}	18.00±0.0^{b}	15.00±0.00^{c}
C. tropicalis ATCC 19092	18.00±0.00^{a}	16.00±0.00^{b}	15.00±0.00^{b}	14.67±0.33^{b}
C. tropicalis	18.00±0.00^{a}	15.67±0.33^{b}	15.00±1.00^{b}	13.00±0.33^{c}
M. canis	16.33±0.33^{a}	15.00±0.33^{b}	13.00±0.33^{c}	13.00±0.33^{c}
T. rubrum ATCC 28188	20.67±0.33^{a}	18.00±0.00^{b}	16.67±0.33^{c}	15.00±0.00^{d}
E. flocossum	21.00±0.00^{a}	19.67±0.33^{a}	18.00±0.00^{b}	16.00±0.00^{c}

Values are mean inhibition zone (mm) ± S.D of three replicates, $p < 0.05$, Values with different superscript (a, b, c, d) on the same row are significantly different ($p < 0.05$).

Table 5. Antifungal susceptibility testing of crude methanol fraction from *Luffa cylindrical.*

Organisms	Zone of Inhibition (mm)			
	20 mg/mL	10 mg/mL	5 mg/mL	2.5 mg/mL
C. albicans ATCC 2876	18.67±0.00^{a}	18.00±0.33^{a}	16.00±0.33^{b}	12.00±0.00^{c}
C. albicans	18.00±0.00^{a}	18.00±0.00^{a}	15.00±0.0^{b}	13.00±0.00^{c}
C. tropicalis ATCC 19092	17.0±0.00^{a}	15.00±0.00^{b}	14.00±0.00^{b}	11.67±0.33^{c}
C. tropicalis	17.0±0.00^{a}	15.00±0.33^{b}	13.00±1.00^{c}	10.00±0.00^{d}
M. canis	15.0±0.00^{a}	14.67±0.33^{a}	12.00±0.33^{b}	10.67±0.33^{c}
T. rubrum ATCC 28188	18.0±0.00^{a}	16.33±0.33^{b}	14.67±0.33^{c}	13.00±0.00^{d}
E. flocossum	18.67±0.33^{a}	16.67±0.33^{b}	15.00±0.00^{c}	12.67±0.00^{d}

Values are mean inhibition zone (mm) ± S.D of three replicates, $p < 0.05$; Values with different superscript (a, b, c, d) on the same row are significantly different ($p < 0.05$).

botanical source and location of plants have been shown to affect or alter the presence and abundance of phytochemicals present in a plant, which can in turn affect its biological properties (Aliero and Wara, 2009; Prashant et al., 2011).

Solvents employed in extraction of phyto-compounds from medicinal plants play a vital role in the degree of biological activities the plant will exhibit. The phyto-constituents present in plants possess varying degree of solubility in different solvents, which is due to the different classes of constituents present in the plant and the polarities of the solvents (Olowosulu et al., 2005).

The strong growth inhibition and broad spectrum (yeast and dermatophytes) of activity displayed by the ethyl acetate and methanol extract as compared to the water and hexane extracts could be linked to the ability of these solvents to extract more anti-fungal components of the plants than the other solvents. The large growth inhibition (17.0 to 18.67 mm and 18.0 to 21.0 mm) of *Candida* sp by the methanol and ethyl acetate extracts of *L. cylindrica* which was found to be comparable with the standard antifungals is in agreement with previous studies (Aboh et al., 2012; Ahmad and Khan, 2013; Aladejimokun et al., 2014).

The crude extracts however showed the least anti-fungal activity against *M. canis* with zone of inhibition range between 10.0 and 16.33 mm at a concentration of 20 mg/mL. Ahmad and Khan (2013), reported linear growth inhibition of 70 and 75% against *M. canis* by the ethyl acetate fraction and crude methanolic extract, respectively, of *L. cylindrica* at a concentration of 24 mg/mL. Generally, the ethyl acetate fraction produced strongest antifungal activities against the fungi tested. This could be linked to the ability of ethyl acetate to solubilize and extract the antifungal components of the plant. Aboh et al., 2014, reported that non polar solvents like ethyl acetate and acetone are able to extract antifungal constituents from medicinal plants than polar solvents.

The increasing antifungal activities of the crude ethyl acetate extract with increase in concentration, is an indication of the potency of the extracts. However the significance of the increment varied among the test organism, as in most cases the difference in activity at concentrations of 5mg/mL and 10 mg/mL was not significant. The antifungal activities of the crude methanolic extracts also followed a similar pattern, however with lower antifungal inhibitory potentials. This agrees with the work by Ahmad and Khan (2013), which recorded a stronger antifungal (dermatophytes) activity by the ethyl acetate extract of *L. cylindrica* over the methanolic extract.

The reduced antifungal activities of fractions (F5 and F6) as compared to the crude ethyl acetate extract are not surprising. Also the absence of flavonoids and phenols could be associated with their reduced antifungal action. . Previous study has reported a reduced biological action of the fractions of some medicinal plants as compared to the crude plants (Hefferon, 2012). This suggests that the biological activity of some plant is due to a combination of phytochemicals which are separated into smaller entities with varying biological efficacy during the process of fractionation (Shafi et al., 2013). These lower antifungal activities of the fractions to the crude as explained suggest loss of active components during fractionation process.

Conclusion

The extracts and fractions L5 and L6 of the leaves of *L. cylindrica* produced good antifungal activity against a broad class of fungi. The ethyl acetate extract was shown to be most effective and could serve as a lead for development of novel antifungals in the nearest future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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