ABSTRACT

Aims: To screen for phytochemicals present in *Aloe barbadensis* (Aloe vera) growing within the Main Campus of Nasarawa State University Keffi (NSUK), determine biofilm forming ability, and to investigate the antibiofilm activity of the plant extracts on biofilm formed by clinical isolates of *Candida* isolated from urinary catheters.

Study Design: The leaf of *Aloe vera* were collected and maceration method was used to extract the plant materials used for the screening of bioactive components. Swab samples were collected from the surfaces of patients’ urinary catheters presenting at Federal Medical Centre and Nagari Hospital, Keffi respectively, irrespective of their ages and sexes. Biofilm forming ability of the isolates was investigated and the antibiofilm activity of the plant extracts determined. Ordinary one-way ANOVA was used to analyze the data where *P* = .05.

Place and Duration of Study: The research was carried out in the Microbiology Laboratory of NSUK, using the *Aloe vera* plant collected within the University community and the biofilm analysis was conducted at National Veterinary Research Institute, Vom Plateau State, between October 2018 to March 2019.
INTRODUCTION

Biofilm is a complex community of microorganisms attached to either biotic or abiotic surfaces embedded in an extracellular polymeric substance (EPS) with characteristics sticky nature [1,2]. The biofilm EPS matrix is composed mainly of polysaccharides, proteins, nucleic acids, lipids, and some organic substances that helps in adhering to medical, industrial, food processing environments, water treatment plants and engineering surfaces [3,4].

Microorganisms found within the biofilm matrix are reported to be more resistant to antibiotics and other chemical substances, as well as resisting phagocytosis and host immune responses as compared to their planktonic counterparts in suspension, thereby causing chronic infections [5]. While planktonic cells (unicellular) are free-floating microbial cells that can easily be inhibited, the multicellular sessile forms also referred to as biofilms are complex community of microbial cells embedded in an extracellular polymeric substance (EPS). These biofilms have been shown to protect the bacterial community from being damaged by external factors as such, promoting persistence of chronic infections [6]. Additionally, other reasons contribute to this resistance nature, which include exclusion of antimicrobials by the EPS from getting to the cells, antibiotics degrading enzymes present in the matrix, protection from host defense, reduced rate of bacterial multiplication, quorum sensing, adaptation to physiological and environmental stress response. This resistance confer by biofilms, makes treatment with effective doses of antibiotics on planktonic cells embedded in the matrix, more difficult to achieve [7].

Candida albicans is the most common opportunistic fungal pathogen reported to cause diseases in humans ranging from mucosal to systemic. In addition, its ability to form biofilm on indwelling medical devices, such as urinary catheters, as well as its inherent tolerance to most antimicrobial therapy has led to a significant morbidity and mortality rate in hospitalized patients [8,9]. Inhibiting the cell attachment and biofilm formation of this nosocomial pathogen on medical devices is therefore paramount for public health.

Due to microbial resistance to the commonly used antimicrobials, and for the need to develop novel therapy, interest has shifted to plants with reported medicinal properties. Plants extracts have increasingly become useful in the pharmaceutical industries due to the role of their bioactive components in prevention and treatment of diseases [10]. Plants materials such as ethanolic extracts of Citrus sinensis, Allium cepa and Laurus nobilis were reported to have significant anti-quorum sensing activity against biofilms of Pseudomonas aeruginosa as well as, play a role in regulating the production of...
virulence factor such as pyocyanin [7]. These plant materials can be used as novel therapy for the treatment of several infections. Furthermore, due to the long history of use in folk medicine, plant materials are widely accepted owing to the belief that they are safe when used to cure diseases and illnesses [11].

*Aloe vera* (L.) Burm.f. (*Aloe barbadensis* Miller) is a perennial succulent xerophyte, which develops water storage tissue in the leaves to survive in dry areas with rainfall. The innermost part of the leaf is a clear, soft, moist, and slippery tissue consisting of large thin-walled parenchyma cells in which water is stored in the form of a viscous mucilage [12]. Many of the medicinal effects of *A. vera* leaf extracts have been attributed to the polysaccharides found in the inner leaf parenchymatous tissue. These biological activities include promotion of wound healing, hypoglycemic or antidiabetic effects, anti-inflammatory, anticancer, immunomodulatory and gastroprotective properties [13].

With little or lack of data on the antimicrobial and antifilm activities of *A. vera* on clinical isolates gotten from medical catheters such as *Candida albicans* around our locality, and considering the numerous medicinal uses of this plant, it has become necessary to conduct this study which may help the predominantly poor community in fighting nosocomial infections and in prevention of antimicrobial resistant strains in circulation.

2. MATERIALS AND METHODS

2.1 Study Location

The research was carried out in the Microbiology Laboratory of Nasarawa State University, Keffi. The swabs were inoculated in Tryptic Soy Broth (Sigma Aldrich) and incubated for 24 hours at 37°C.

2.3 Isolation of Microorganisms

The overnight broth cultures were standardized and streaked on nutrient agar plates and were incubated at 37°C for 24 hours. The random colonies were selected independent of their colony characteristics and streaked on differential and selective media such as Sabouroud Dextrose agar (Sigma-Aldrich), MacConkey agar (Sigma-Aldrich), Eosin Methylene Blue (HiMedia Lab), Mannitol Salt agar (HiMedia Lab), and Blood agar (TitanBiotech Ltd). Pure cultures were obtained by frequent sub-culturing which were later maintained on Nutrient agar slants at 4°C. Isolates were characterized culturally and morphologically by their appearance on agar plates, Gram's staining and biochemical tests.

2.4 Biochemical Characterization of the Isolates

Biochemical characterization of the isolates was conducted including Indole test, Methyl red test, Voges-Proskauer test, Citrate test (IMViC test), Catalase test and Sugar fermentation tests and the results were compared with already established findings [14].

2.5 Preparation of Crude Extracts and Screening for Phytochemicals

150 g of the *Aloe vera* grounded powder was macerated in 500 ml distil water and 500 ml ethanol respectively for 48 hours. After maceration, the solution of the plant extracts was filtered using Whatman No. 1 filter paper at room temperature and the resulting solutions were dried using a rotary evaporator. The remaining solvents were evaporated using water bath at 40°C to dryness. The extracts were then labelled as follows; distilled water extract of *Aloe vera* leaf (D.E) and Ethanolic extract of *Aloe vera* leaf (E.E). The phytochemical screening was carried out as described below.

2.5.1 Test for tannins

Extracts were treated with 1mL of 5% ferric chloride which was added. The presence of tannins was indicated by the formation of bluish black or greenish black precipitate.
2.5.2 Test for saponin
2 mL of distilled water was added to extracts suspended in ethanol and was shaken vigorously. The presence of saponins is indicated by the formation of large amount of foam layer.

2.5.3 Detection of triterpenes and steroids
1 mL of acetic anhydride and 5 drops of concentrated sulfuric acid (H₂SO₄) were added to the extract. A color change from violet to blue confirms the presence of steroids and formation of blue-green ring indicated the presence of terpenoids.

2.5.4 Test for aloin
3 mL of the extracts and 5 drops of concentrated sulfuric acid (H₂SO₄) were mixed properly and boiled. It was allowed to cool and filtered before adding 1 mL of chloroform, shaken and diluted ammonia was added. The formation of reddish-pink coloration of ammoniacal layer confirms the presence of aloin.

2.5.5 Test for glycosides
2 mL of each extract was mixed with 2 mL of Fehling’s solution 1 and 2 and was heat on water bath for 5 minutes. The formation of white color precipitate confirms the presence of glycosides.

2.5.6 Test for flavonoids
2 mL of each extract was added to 2 mL dilute sodium hydroxide and 2 mL concentrated hydrochloric acid. The solution turned yellow and after a short while, it turns colorless confirming the presence of flavonoids.

2.5.7 Test for reducing sugars
2 mL of each crude extract was added to 2 mL of mixtures of Fehling A and B reagents and boiled for 5 minutes. Appearance of brick red at the bottom of the test tube confirms the presence of reducing sugars.

2.5.8 Test for quinones
Few drops of sodium hydroxide were added to 2 mL of each extract and shaken vigorously for 1 minute. Appearance of blue-green or red colour signifies the presence of quinones.

2.5.9 Test for phenols
2 mL of each crude extract was mixed with 2 mL of 2% solution of FeCl₃. Appearance of visible coloration confirms the presence of phenol.

2.5.10 Test for alkaloids
1 mL of each crude extract was added to 2 mL of 1% hydrochloric acid and then heat. Meyer and Wagner reagents were then added to the mixture. Visible turbidity of the mixture confirms the presence of alkaloids.

2.6 Biofilm Formation
The biofilm formation was conducted using a 96-well microtiter polystyrene plate and spectrophotometer assay as described by [15] with little modifications. Briefly: Discrete colonies were scraped from Sabouroud dextrose agar (SDA) plate and were inoculated in Tryptic Soy broth (TSB) and incubated at 37°C for 24 hours to obtain enough microbial growth. The liquid culture was standardized using the same medium and 200µL final OD₆₀₀ of 0.05 was used to inoculate the wells of the 96 well plates (Linbro®) in triplicate while the remaining surrounding wells were filled with sterile supplemented TSB (negative control) and incubated at 37°C for 72 hours. After the 3 days incubation, the total growth was determined by measuring the OD₆₀₀ using an auto plate reader (LabSystems Type 355) and the content was poured off and the wells washed gently using Phosphate Buffer Saline (PBS) pH 7.4 to remove planktonic cells. The wells were stained with 0.1% (w/v) crystal violet (CV) solution and allowed to stand for 15 minutes. The CV was poured off and the plate inverted and allowed to dry. The microtiter wells were de-stained with 200µL of 95% ethanol-acetic acid and allowed to stand for 5 minutes. After the 5 minutes, the content was transferred to a new clear flat-bottom 96 well plate and the biofilm formed was measured at OD₅₇₅ using the auto plate reader (Labsystems Type 355) of the Brucellosis Division of National Veterinary Research Institute (NVRI) Vom-Jos, Nigeria. The biofilm formed was standardized against growth by dividing the crystal violet absorbance by the OD₆₀₀ initial results.

2.7 Determination of Minimum Inhibitory Concentration (MIC)
Minimum Inhibitory Concentration (MIC) determination was conducted using sterile Tryptic Soy Broth (TSB) by broth micro-dilution techniques as described by National Committee for Clinical Laboratory Standards (NCCLS,
The C. albicans were streaked on Sabouraud Dextrose Agar (SDA) and incubated at 37°C for 48 hours. Following incubation in agar, single colonies were picked from the plate and inoculated into a clean tube containing 10ml sterile TSB and incubated with constant shaking at 37°C for a period of 12 h to ensure that the organism were in the log phase. The fungal suspension was adjusted to an absorbance OD_{600} of 0.25 using a spectrophotometer. Stock solutions of the aqueous (water) and ethanolic leaf extracts in the first well of a 96-well microplate and two-fold serial dilution was carried out to obtain a final concentration range of 16–0.125 mg/ml. 100 µl of OD_{600} 0.25 C. albicans suspension was added to each well. Sterile TSB was used to fill the remaining surrounding wells and incubated at 37°C for 48 hours. Positive and negative controls were included in experiment. 100 µl Ketoconazole (1 mg/ml) was included as positive control (instead of leaf extracts) in the first well of a column in serial dilution. 100 µl of DMSO was used as negative control (instead of leaf extracts) with 100 µl TSB instead of the fungal solution. The reactions were done in triplicates. Following 48 h incubation, a loopful from each well was streaked on Mueller Hinton agar plates and incubated at 37°C for 24 h. The well of lowest concentration of extracts in which fungal growth was prevented was observed, and the corresponding concentration was referred to as the MIC value [16].

2.8 Determination of Anti-biofilm Activity of Aloe vera

2.8.1 Inhibition of cell attachment

Both the aqueous and ethanolic extracts were tested for their anti-adhesion properties at their respective MIC concentrations (1mg/ml and 0.25mg/ml). Briefly, 2 mg/ml stock solutions of the extracts were prepared in their respective solvents and, 100 µl of each extract was added to the 96-well polystyrene plates, also, equal volume of sterile TSB and ketoconazole (MIC concentration) were added to separate wells as negative and positive controls respectively in triplicates. 100 µl of OD_{600} 0.05 culture of C. albicans prepared in sterile TSB was added to the wells to yield a final volume of 200 µl. Equal volume of distil H_{2}O was used to fill the remaining surrounding wells to prevent dehydration of the set up. The 96-well plates were covered and incubated at 37°C for 3 hours in a static condition to allow for cell attachment and biofilm development. After the incubation, the modified crystal violet assay as described in section 2.6 was performed to assess biofilm biomass, and the results expressed using the equation below as percentage inhibition

\[
\text{Percentage inhibition} = \frac{\text{OD}_{\text{Negative control}} - \text{OD}_{\text{Experimental}}}{\text{OD}_{\text{Negative control}}} \times 100
\]

2.8.2 Inhibition of biofilm formation

Biofilm forming C. albicans confirmed earlier were subjected to biofilm formation in the presence of Aloe vera leaf extract that inhibits at least 50% cell attachment in section 2.8.1 according to the method described by [17]: Briefly: the ethanolic leaf extract and Ketoconazole at MIC values were investigated for their ability to inhibit C. albicans biofilm formation (anti-biofilm activity). 100 µl of OD_{600} 0.05 C. albicans suspension was pipetted into the 96-well plates and incubated aerobically at 37°C for 4 h to allow for biofilm formation. After the 4 hours incubation period, 100 µl of both ethanolic extract and ketoconazole were added to the respective wells of the 96-microtitre plate. 100 µl of Ketoconazole at MIC value was used as the positive control with the addition of 100 µl C. albicans culture, while in the negative control wells, it contains 100 µl of C. albicans plus 100 µl of sterile TSB instead of the leaf extract. Lastly, 200 µl of TSB was added in blank wells without the fungal culture. The plate was covered and incubated aerobically at 37°C for 72 h without shaking after which, crystal violet assay was performed as described in section 2.6 and then plotted using GraphPad Prism (version 8.4.2). Lastly, the percentage inhibition of the biofilm was compared with that of ketoconazole.

3. RESULTS

3.1 Phytochemical Screening

Both the aqueous and ethanolic extracts were tested for the presence of bioactive compounds as described in section 2.5 above. More phytochemicals were detected from ethanolic extract of Aloe barbadensismiller (Aloe
vera) as compared to the aqueous extract (Table 1).

### 3.2 Determination of Biofilm Formation

Clinical isolates of *Candida albicans* were subjected to biofilm formation in a 96-well plate, with sterile tryptic soy broth (TSB) only used as the negative control. After quantifying the crystal violet stained plate using a micro plate reader at OD575, the result shows that *C. albicans* is a strong biofilm former (Fig. 1).

### 3.3 Determination of Minimum Inhibitory Concentration (MIC)

Antimicrobial activities of the plant extracts were determined using broth microdilution, where the ethanolic extract recorded IC50 value of 0.25 mg while the aqueous extract had IC50 of 1 mg. On the other hand, the MIC value for ketoconazole is 0.125 mg. The least concentration of the plant extracts in the 96-well plate which inhibits *C. albicans* growth was recorded as the MIC. The MIC values are as presented in (Table 2).

#### Table 1. Phytochemical compounds detected in aloe vera leaf extracts

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Aloe vera extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>-</td>
</tr>
<tr>
<td>Glycoside</td>
<td>-</td>
</tr>
<tr>
<td>Tannin</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
</tr>
<tr>
<td>Quinones</td>
<td>-</td>
</tr>
<tr>
<td>Aloin</td>
<td>+</td>
</tr>
</tbody>
</table>

*Key:* (+) = Present and (-) = Absent

#### Table 2. MIC values of aqueous, ethanolic leaf extracts of aloe vera and ketoconazole against clinical isolates of *candida albicans*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>1.0</td>
</tr>
</tbody>
</table>

Fig. 1. Biofilm formation by clinical isolates of *candida albicans*
3.4 Determination of Antibiofilm Activities of Aloe vera Extracts

3.4.1 Inhibition of cell attachment

Although all the extracts significantly inhibit cell attachment, none was able to achieve complete biofilm eradication, including the positive control ketoconazole. Ethanolic extract inhibits 54.25%, Water extract 25.68% and Ketoconazole 48.54% C. albicans attachment, respectively.

Data represent the mean of three experiments, where each condition was repeated three times. Error bars=SEM. Data were calculated using Microsoft excel and GraphPad Prism (8.4.2) was used to plot the graph. Ordinary one-way ANOVA was used to analyze the data where \( P < .001 \).

3.4.2 Inhibition of biofilm formation

(Antibiofilm)

Only the extracts with at least 50% reduction in cell attachment were used in the preformed biofilm inhibition assay. Although the results in Fig. 2 indicated that both extracts have activity, only the ethanolic extract and ketoconazole achieved 50% reduction of initial cell attachment. There were no significant differences in the reduction of preformed C. albicans biofilm between the ethanolic extracts of A. vera and the positive control Ketoconazole.

Each plot represents the results from three independent experiments conducted in triplicate. Differences between mean values in this experiment were evaluated by student t-test, with \( P = .021 \) being taken as the level of significance.

4. DISCUSSION

Phytochemical screening of aqueous and ethanolic extracts of the Aloe vera leaf used in this study revealed the presence of numerous bioactive compounds with tannin, phenols, reducing sugars and aloin present in both extracts. In ethanolic extract however, alkaloid, flavonoid, glycoside, terpenoids and quinones were present while, saponin was present only in the aqueous extract (Table 1). [18], reported the presence of tannin, saponin, flavonoids and terpenoids in ethanolic extracts of A. vera. These bioactive compounds may be the reason why Aloe vera is used for burns and wound healing also, as topical agents in cosmetics as well as taken as health drink [13]. Other medicinal properties of this plant include its ingestion for diarrhea, electrolyte imbalance, kidney dysfunction, and conventional drug interactions; episodes of contact dermatitis, erythema, and phototoxicity have also been reported from topical applications [12]. Candida albicans has been implicated in causing diseases such as Candidiasis, oral thrush, vaginal yeast infections and other form of urinary tract infections.

![Fig. 2. The effects of aqueous and ethanolic extracts of A. vera on attachment of clinical isolates of candida albicans](image-url)
Biofilm formation by pathogenic microorganisms such as the opportunistic fungal pathogens of *C. albicans* have been reported to contribute to disease progressions and antimicrobial resistance [8]. *C. albicans* isolated from a urinary catheter used in this study was shown to be a strong biofilm former with a percentage formation of almost 70% (Fig. 1). This finding is in accordance with other researchers who reported the ability of *C. albicans* isolated from hospital settings to form strong biofilms which contributes to systemic infections such as urinary tract infections, Candidiasis, invasion of gastrointestinal tracts and resistance to commonly used antimicrobials resulting to rise in morbidity and mortality rates [19,20].

The success recorded with the leaf extracts of *Aloe vera* in inhibiting planktonic cells attachment and biofilm formation recorded in this study is a promising tool for reducing microbial colonization of mucosal and urogenital surfaces which more often lead to opportunistic infections.

Although both extracts have antimicrobial effects (Table 2) and significantly inhibit cell attachment, none of them was able to do that completely, including ketoconazole which is a known antifungal drug. Cell attachment which is the initial stage of biofilm formation is easier to be inhibited however, when the cells are embedded in biofilm matrix, it becomes more difficult to be dissolved. This may be reason for these observed characteristics in this present study. Among the crude extracts tested, ethanolic extract inhibits 54.25%, aqueous extract 25.68% and ketoconazole has 48.54% inhibition of *C. albicans* attachment respectively (Fig. 2). On the other hand, only the ethanolic extract and ketoconazole were tested for biofilm inhibition with both inhibiting more than 50% of the biofilms formed by the clinical isolates of *C. albicans*. Ethanolic extracts achieved 37.38% while ketoconazole had 33.98% biofilm inhibition respectively (Fig. 3). The reduced antibiofilm activities observed in this study further confirms that cells in biofilms are more resistant to antimicrobials than the planktonic cells [7]. Interestingly, [21] also reported the inhibitory effects of ethanolic extract of *Aloe vera* on *C. albicans* with minimum inhibitory zone comparable to that of Fluconazole. On a contrary, [22] reported a weak inhibitory activity of ethanolic extract of *A. vera* on *C. albicans* as compared to other plants extracts used in their study. While several papers reported the inhibitory effects of *Aloe vera* extracts on planktonic cells of *C. albicans*, few reported it antibiofilm properties against biofilms formed by *Candida albicans*. 

![Graph showing percentage inhibition of C. albicans preformed biofilms by ethanolic extract of A. vera and ketoconazole](image-url)
Previous research also reported that it is much easier to inhibit cell attachment to surfaces than to inhibit an already formed biofilms [17,19,8,23]. This is so because, cell attachment is the first stage in biofilm formation after surface conditioning which creates the needed environment for the attachment of cells. In addition, other factors like EPS surrounding the biofilms, the negative charges on EPS also prevent penetration of antimicrobials, efflux pumps which expels the antimicrobials, degradation of antimicrobials can confer resistance in biofilms to antimicrobials [24].

Although A. vera extracts were previously reported not to have inhibitory activity against pathogens such as Listeria monocytogenes [17], in this present study, both aqueous and ethanolic extracts of Aloe vera have shown a significant inhibition of both planktonic cells and biofilms formed by Candida albicans.

5. CONCLUSION

The findings in this study revealed the phytochemicals present in aqueous and ethanolic leaf extracts of Aloe vera harvested within the main campus of Nasarawa State University, Keffi. In addition, the ethanolic extract of this A. vera plant showed a significant antimicrobial activity against the planktonic cells of C. albicans and, reduced the C. albicans biofilm from strong to weak biofilm. In summary, due to increasing antimicrobial resistance by both bacteria and fungi, Aloe vera extracts could be combined with other antimicrobials to improve management of infections such as Candidiasis, wound healing, and other urinary tracts infections.

Lastly, determination of synergistic effects as well as the cytotoxic effects of these plant extract on host cells should be carried out.

CONSENT

Written informed consent of the patients was obtained, and approval given before their swab samples were taken.

ETHICAL APPROVAL

Ethical approval was obtained from the Ethical Committee of Federal Medical Centre and Nagari Hospital, Keffi, respectively.


© 2021 Galleh et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/65743