In Vitro effects of *Plantago major* extract, aucubin and baicalein on *Candida albicans* biofilm formation, metabolic activity and cell surface hydrophobicity

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Running title: Inhibition of *Candida albicans* biofilm formation and adhesion

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Abstract

Purpose: The aim of this study was to determine the in vitro effectiveness of *Plantago major* extract, along with two of its active components, aucubin and baicalein, on the inhibition of *Candida albicans* growth, biofilm formation, metabolic activity and cell surface hydrophobicity.

Materials and Methods: Two-fold dilutions of *Plantago major*, aucubin and baicalein were used to determine the minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and the minimum biofilm inhibitory concentration (MBIC) of each solution. Separately, two-fold dilutions of *Plantago major*, aucubin and baicalein were utilized to determine the metabolic activity of established *C. albicans* biofilm using a 2,3-bis (2- methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-carboxanilide reduction assay. Two-fold dilutions of *Plantago major*, aucubin and baicalein were used to determine the cell surface hydrophobicity of treated *C. albicans* biofilm by a two-phase assay using hexadecane. The hydrophobicity percentage of the cell surface was then calculated. A mixed-model ANOVA test was used for intergroup comparisons.

Results: The MIC's of *P. major* extract (diluted 1:2-1:8), aucubin (61-244 µg/ml) and baicalein (0.0063-100 µg/ml) on the total growth of *C. albicans* were noticeable at their highest concentrations, and the inhibition was dose-dependent. The MFC was evaluated after 48 h of incubation and aucubin (244 µg/ml) exhibited a strong fungicidal activity at its highest concentration against *C. albicans* growth. The MBIC indicated no growth or reduced growth of *C. albicans* biofilm at the highest concentrations of aucubin (61-244 µg/ml) and baicalein (25-100 µg/ml). Similarly, the effects of these reagents on *C. albicans* biofilm metabolic activity and hydrophobicity demonstrated high effectiveness at their highest concentrations.

Conclusion: *P. major* extract, aucubin and baicalein caused a dose-dependent reduction on the total growth, biofilm formation, metabolic activity and cell surface hydrophobicity of *C. albicans.* This demonstrates their effectiveness as anti-fungals and suggests their promising potential use as solutions for *C. albicans* biofilm related infections.

Keywords:*;* Candidiasis; anti-fungal; denture stomatitis; biofilm; crystal violet; hexadecane.

1. Introduction

Candida albicans is a dimorphic fungus that can be either a commensal or an opportunistic pathogen with the ability to cause a variety of infections in the oral cavity as well as other parts of the human body.[1-4] Opportunistic fungal pathogens are often responsible for common superficial infections, but sometimes are the cause of life threatening deep – seated mycoses.[5] *C. albicans* is not only capable of invading and forming biofilm on virtually every site on the human body, but can also attach to biomaterials.[2,4] There is an increased prevalence of *Candida* infections worldwide that are difficult to treat such as invasive candidiasis. [4]

Oral candidiasis is the collective term for a number of distinct clinical pathologies caused by *Candida* species, predominantly *C. albicans*. Oral lesions of *Candida* origin are categorized as: pseudomembranous candidiasis (oral thrush), erythematous candidiasis (atrophic) and hyperplastic candidiasis (*Candida* leukoplakia).[3,6] Many factors can predispose individuals to candidiasis. Some are mechanical such as ill-fitting dentures, some are short-term such as a course of antibiotic therapy, and some are associated with simply old age. Additionally, recent studies have found that certain type of proteins in saliva may predispose some patients to dentures stomatitis, causing disease from non-fungal relation.[7] Other types of predisposing factors are related to underlying diseases such as immunosuppressive therapy, HIV-infection and diabetes mellitus.[2,6,8]

The most common clinical manifestation of oral candidiasis is denture stomatitis. [2,6,9] It occurs in up to 67% of denture wearers and its multifactorial etiology makes it difficult to treat.[2,4,7] Increased colonization of *C. albicans*, and other types of candida species like *C. glabrata* seem to be involved in the development of denture stomatitis even when the normal bacterial flora seems not to be greatly altered.[6,7,9] This is supported by several studies, which describe the entrapment of yeast cells in irregularities of denture-base and denture-relining materials, that could serve as a reservoir of microorganisms.[4,10,11] Hence the recurrence of stomatitis is very likely to occur after anti-fungal therapy is discontinued. Anti-fungal agents commonly used to treat oral candidiasis patients are of a wide spectrum and if indiscriminately used can cause undesirable side-effects and permit the emergence of resistant organisms. This leads to the need to find effective natural solutions capable to inhibit the growth and biofilm formation of *Candida* related fungus not only at tissue level but also in biomaterials such as dentures.

The pathogenesis of *Candida* infections is complex, involving yeast and host-factors. The ability of *Candida* strains to overcome host clearance mechanisms and colonize surfaces depends on the effectiveness of the yeast mechanisms, its capability of adherence, and its growth rate.[11] According to Pereira-Censi *et al*.[4] many studies have demonstrated the adhesion of *C. albicans* to denture surfaces and they proposed that the more hydrophobic the surface, the more cell adherence. In other words, the higher the surface free energy, the higher will be the adhesion of the microorganisms. Furthermore, there seems to be a close relationship between the adherence of *C. albicans* to plastic surfaces and yeast cell surface hydrophobicity (CSH). This interaction is essential for initial resistance or adherence of yeast to acrylic surfaces, because the more hydrophobic the microorganism, the more adherent the microbes are to surfaces that are likewise hydrophobic.[13-14] Adhesion often leads to colonization and subsequently induces pathology.[4] Should adherence occur, there is greater opportunity for further *Candida* binding and in cases of denture stomatitis further formation of "denture plaque".[1] Irreversible adhesion to host tissues and prostheses often leads to biofilm formation.[5]

Biofilm is defined as a surface-associated, highly structured community of microorganisms that is enclosed by self-production of a protective extracellular matrix called extracellular polymeric substance (EPS).[15] These oriented aggregations of microorganisms attach to one another on living or non-living surfaces, and being embedded within EPS helps maintain biofilm structures.[16] In contrast to planktonic cells, biofilm cells display unique phenotypic traits, the most outstanding of which is their notorious resistance to both anti-fungal agents and to host immune factors.[8] Biofilm-associated *Candida* infections are difficult to treat and are a danger to patients.[5] The limitations and restricted target range of current anti-fungal medications, have led to a search for more effective anti-fungal treatments.[6] In order to reduce further development of anti-fungal medication resistance, there is a need to identify new methods of preventing and treating candidiasis. For this reason, new therapeutic strategies using medicinal plants and their components would be of significant importance as alternatives in the prevention and management of *C. albicans* biofilm formation.

P. major is a perennial herb that belongs to the family of *Plantaginaceae*.[17] It originated in Europe and Asia. In English it is known as greater Plantain, in French it is known as Plantain, in Portuguese as Tranchagem, and in Spanish as Llantén.[12,18] It is well known for its wound healing,

oral wound healing, analgesic, anti-inflammatory, anti-oxidant, anti-viral, and anti-fungal properties.[12,19] It contains five classes of eleven biologically active compounds: benzoic compound (vanillic acid), flavonoids (baicalein, baicalin, luteolin), iridoid glycoside (aucubin), phenolic compounds (caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid) and triterpenes (oleanolic acid, ursolic acid).[20] Many of *P. major's* medicinal properties may be attributed to two of its biologically active components, aucubin (AU) and baicalein (BE).

AU can be isolated from the leaves of *P. major*, *P. asiatica* and *Eucommia ulmoides*.[20-22] It has numerous pharmacological effects such as being anti-microbial, anti-inflammatory, hepatoprotective, choleretic, hemodynamic, anti-spasmodic, anti-nociceptive, and inhibition of RNA and protein biosynthesis in sarcoma 180 cells, and promoter of dermal wound healing.[21-23] Additionally, Shim *et al*.[21] concluded in his study that AU is useful for oral wound healing due to its anti-inflammatory effect and that it may be applied as a topical agent to oral wounds. Similarly, Kang *et al.*[23] recommended that AU be further researched and utilized due to its extensive pharmacological properties.

BE can be isolated from *P. major* and the traditional Chinese medicinal plant *Scutellaria baicalensis* Giorgi.[20,24] Several pharmacological studies of BE have demonstrated its anti-oxidative, anti-microbial and neuro-protective effects.[8] Other studies have demonstrated BE has anti-inflammatory and anti-cancer effects.[20,25] With respect to its anti-fungal aspect, BE has been found to inhibit *C. albicans* growth and its biofilm formation.[8,26] Additional studies have found that BE also exhibits an in vitro synergism with fluconazole on *C. albican*s.[27,28] Kang *et al*. [29] found in his study that BE could not induce apoptosis in *C. albicans* and suggested that BE affected fungal growth via different pathways.

To date, only a few studies have investigated the role of *P. major* extract, AU and BE as anti-fungal agents against *C. albicans* growth, biofilm formation, metabolic activity and hydrophobicity. Therefore, it would be of significant advantage to find new prospective natural resources to eradicate *C. albicans* biofilm related infections. The aim of this study was to assess the in vitro effects of *P. major* extract, along with two of its active components, (AU and BE), on the inhibition of *C. albicans* growth, biofilm formation, metabolic activity and cell surface hydrophobicity. The hypothesis was that *P. major* extract, AU and BE will cause dose-dependent

reductions on *C. albicans* growth, biofilm formation, metabolic activity and cell surface hydrophobicity, when compared to the negative control group.

2. Materials and Methods

2.1 Fungal Strain, Growth Medium and Anti-fungal Agents

C. albicans was obtained from the American Type Culture Collection (ATCC). *C. albicans* ATCC 10231 was chosen because is a widely used clinical isolate in many *C. albicans* studies. The strain was originally isolated from a patient with bronchomycosis.[30] Stock cultures were stored at -80°C, passaged onto a blood agar plate and incubated in 95% O_2/5% CO_2 at 37°C prior to use. *C. albicans* cells were inoculated into 5 ml of yeast-peptone-dextrose (YPD) broth medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose; Fisher Scientific, Newark, DE, USA). Thereafter, batches of inocula were incubated overnight in an orbital shaker (120-150 r.p.m) at 30°C.[1,5,8,12,15,22] *C. albicans* grew in the budding-yeast phase under these conditions.[2,15]

P. major extract was purchased in 30X potency containing 20% alcohol (Washington Homeopathic Products, Berkeley Spring, WV).[31] *P. major* extract was diluted with YPD media by two-fold dilutions up to 1:512. This potency of *P. major* extract contains 0.05 µg/ml of AU and 0.0425 µg/ml of BE.[20] (Table 1)

AU and BE were obtained from Sigma-Aldrich, St Louis, MO. Both components were dissolved in concentrations ranging from 0-244 $\mu g/ml^{-1}$ in dimethyl sulfoxide (DMSO) and stored at –20°C.[8,20] The final concentration of DMSO used alone in all assays was not inhibitory towards *C. albicans* in agar diffusion assays (data not shown).

2.2 Minimum Inhibitory Concentration and Minimum Fungicidal Concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of an agent that inhibits the visible growth of a microorganism, and the minimum fungicidal concentration (MFC) is the lowest concentration of the agent that kills the fungus.[32] The MIC of *P. major* extract, AU and BE against *C. albicans* were determined by two-fold dilutions with YPD media in sterile 96-well flat-bottom microtiter plates (MTP) (Fisher Scientific, Newark, DE, USA). *P. major* extract 30X in $1:2^n$ nine dilutions, AU concentrations ranging from 0.96-244 µg/ml and BE from 0.006-100 µg/ml in YPD (190 µl) were added to 96-well MTP with 10 µl of an overnight culture of *C. albicans* and incubated for 24 h or 48 h at 37°C. The total yeast growth in the presence of the agents was estimated using a microtiter plate reader (Spectra Max 190; Molecular Devices, Sunnyvale, CA,

USA) at a wavelength of 595 nm after incubation. The MIC was determined as the lowest concentration of the reagents that yielded a change in OD from the control of ≥0.050. The negative control group was YPD inoculated with *C. albicans.*

To determine the MFC, 10 µl from the 96-well MTP with reagent concentrations equal to or higher than the MIC were transferred onto blood agar plates (Fisher Scientific) and incubated in 95% O_2/5% CO_2 at 37°C. The MFC was defined as the lowest concentration of the reagents that had no visible yeast colonies on the agar plates after 48 h of incubation.

2.3 Minimum Biofilm Inhibitory Concentration

The minimum biofilm inhibitory concentration (MBIC) is the lowest concentration of an agent that inhibits the visible biofilm formation of a microorganism.[32] The MBIC was determined by the same two-fold dilution of *P. major* extract, AU and BE. The same 96-well MTP was used after the MIC was determined. The planktonic culture fluid was gently shaken out, fixed with 200 µl of 10% formaldehyde for 30 min, and stained with 200 µl of 0.3% crystal violet for 30 min. After washing the biofilm twice with sterile water, crystal violet was extracted from the biofilm cells by incubation of 200 µl of 2-propanol for 1 h. The absorbance was read at 490 nm. The control group was YPD inoculated with *C. albicans*.

2.4 Biofilm Metabolic Activity

The metabolic activity of *C. albicans* was measured using a method described by Pierce *et al*.[15] This technique requires 24 h established *C. albicans* biofilms on the bottom of the 96-well MTP, coupled with a colorimetric method that measures the metabolic activities of biofilm cells based on the reduction of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-carboxanilide (XTT; Sigma). Upon processing by metabolically active cells, XTT yields a water-soluble formazan-colored product that is measured in a spectrophotometer. The XTT-reduction assay indicates an excellent correlation between cellular density and metabolic activity, thus providing a semi-quantitative measurement of biofilm formation. This colorimetric assay is noninvasive and nondestructive, requiring minimal post-processing samples. Furthermore, this correlates with cell

viability, which is particularly useful for measuring the effects of anti-fungal agents on biofilm cells.[2,15,33]

The 96-well MTP were seeded for 24 h with 10 µl of an *C. albicans* overnight culture in 190 µl of YPD without the reagents to allow biofilm to grow, followed by another 24 h of growth in YPD supplemented with two-fold dilutions of *P. major* extract, AU and BE. *C. albicans* planktonic cells in the 96-well MTP were gently shaken out and 200 µl of XTT/menadione reagent added.[2,17] The plates were kept in the dark for 2 h at 37°C in an atmosphere of 95% O_2/5% CO_2. After incubation, 120 µl of the solution was transferred to a blank MTP and any color change was detected by measuring the absorbance at 490 nm in a spectrophotometer. The negative control group was YPD inoculated with *C. albicans*.

2.5 Cell Surface Hydrophobicity Assay

The cell surface hydrophobicity (CSH) of *C. albicans* was determined by measuring the yeast adherence to a water-hydrocarbon two-phase assay using hexadecane.[14] Two-fold dilutions of *P. major* extract, AU and BE in YPD (3 ml) were added to six-well tissue culture plates with 150 µl of an overnight inoculum of *C. albicans* and incubated for 24 h at 37°C. Briefly, biofilm cells were scraped off the bottom of the culture plates and suspended in 1.5 ml of YPD to yield an optical density of approximately 1.0 at 620 nm. From this cell suspension, 1.3 ml was pipetted into borosilicate glass tubes (18 mm × 75 mm). From each tube, 100 µl of the cell suspensions were pipetted into wells of a 96-well MTP. The plate was read at 620 nm and designated as the initial OD of the cell suspension. The 1.2 ml of cell suspension remaining in the glass tubes was overlaid with 0.3 ml of hexadecane. The phases were vigorously mixed and vortexed for 3 min and allowed to separate for 15 min. The lower aqueous phase was carefully withdrawn with a pipette and 100 µl was added to each microtiter well. The absorbance was read and noted as the final OD of the aqueous phase. Tubes without the reagents served as the control group. The percentage hydrophobicity of the yeast cell surface was calculated by the following formula: %CSH = (1-final OD of aqueous phase/initial OD of cell suspension) × 100.[5,10]

2.6 Statistical Analysis

The different experiments were compared for differences in total growth, biofilm formation, biofilm metabolic activity, and cell surface hydrophobicity using a mixed-ANOVA. The ANOVA included a fixed effect for the $1:2^n$ nine dilutions of the *P. major* extract, AU and BE, and a random effect for the experiment. Fisher's Protected Least Significant Differences was used to control the overall significance level at 5%. Distribution of the data was examined and a transformation of the data was used.

With a sample size of 144 two-fold dilutions for each reagent for each of 4 experiments, the study had 80% power to detect differences between groups of 0.05 for total growth, biofilm formation and cell surface hydrophobicity and differences between groups of 0.025 for biofilm metabolic activity. The calculations assumed two-sided tests each conducted at a 5% significance level and within-group standard deviations of 0.05 for biofilm formation,[32] biofilm metabolic activity,[10] and cell surface hydrophobicity.[8] All data were expressed as mean values with the corresponding standard deviations.

3. Results

P. major extract (diluted 1:2-1:8), AU (61-244 µg/ml) and BE (0.0063-100 µg/ml) demonstrated clear inhibition against *C. albicans* growth (Fig. 1). These differences were statistically significant ($p < 0.0001$). The data also indicated BE was more effective at lower concentrations (≥ 0.0063 µg/ml) than *P. major* and AU.

The MFC, after 48 h of incubation, indicated that AU has a noticeable fungicidal activity against *C. albicans* growth at its highest concentration of 244 µg/ml (Table 2). In contrast, *P. major* extract and BE demonstrated some fungicidal activity against *C. albicans* but this was not significant.

AU (61-244 µg/ml) and BE (25-100 µg/ml) revealed a significant dose-dependent reduction against *C. albicans* biofilm formation when compared to the negative control group ($p < 0.0001$; Fig. 2). Lower concentrations of aucubin (1-31 µg/ml) and baicalein (0.4-12.5 µg/ml) also produced some effect on *C. albicans* biofilm formation, but this was not statistically significant as well as all the concentrations of *P. major* extract.

To provide a more quantitative assessment of cellular activity of established *C. albicans* biofilm in the presence or absence of the reagents, the ability of the yeast to reduce XTT was measured (Fig. 3). The inhibitory effects of *C. albicans* metabolic activity were limited to *P. major* extract (diluted 1:2; $p < 0.0159$) and AU (244 µg/ml; $p < 0.0001$). Clearly the inhibition was dose-dependent only at the highest concentrations of these two reagents and was statistically significant. The results also demonstrated that the inhibitory effects were active on *C. albicans* that had already grown in an established biofilm, which usually are the most problematic to treat. On the other hand, BE demonstrated no inhibition on the metabolic activity of *C. albicans*.

To examine the effect of *P. major*, AU and BE on the yeast adherence mechanism, the cell surface hydrophobicity of treated *C. albicans* biofilm was measured by a two-phase separation method using hexadecane (Fig. 4). The water-hydrocarbon two-phase assay demonstrated a decrease in hydrophobicity of *C. albicans* biofilm at only the highest concentrations of AU (244 µg/ml; $p < 0.0048$) and BE (100 µg/ml; $p < 0.0375$) when compared to their control group. However, *P. major* extract had little effect on the CSH of the yeast.

4. Discussion

Biofilm associated *Candida* infections are a danger to patients and are challenging to treat because of the complex pathogenesis that involves not only the yeast but host factors as well.[1,17] The present study demonstrated that the *P. major* extract, aucubin and baicalein effectively inhibited *C. albicans* growth, while the active components of the plant (AU and BE) at higher concentrations, exerted strong inhibitory effects against *C. albicans* biofilm formation and additionally displayed strong fungicidal activity against the yeast. These results are in agreement with earlier data that depicted the usefulness of the reagents in having antimicrobial activities.[8,12,17,19,21-23,27-29,34] Ever since ancient times, people have looked to nature for cures. Plants have been used because of their effectiveness, low cost, and accessibility. Medicinal plants are a valuable natural resource due to their antimicrobial activities. Their side effects are often less severe than synthetic medications.[12,34] They play a principal role in the health care systems of rural and remote communities where the population depends on folklore and herbal medicines. *P. major* is an herb that has been used worldwide for hundreds of years for its medicinal properties. This natural resource would potentially benefit the oral health of at risk populations, such as denture wearers, debilitated elderly, immunocompromised individuals like neonates and other populations with fungal infections caused by *C. albicans*.

Appropriate further studies will be necessary to investigate alternative medical uses of the plant extract and components for their routes of administration such as, and not limited to, mouth rinses, troches, creams, ointments and/or in combination with existing anti-fungal agents. Topical application of the reagents would be rather safe and their cytotoxicity should not be a deterrent as this is supported by the study of Reina *et al*.[20] that concluded that *P. major* and BE were not cytotoxic at any of the concentrations examined, and AU was cytotoxic only at the concentration of 100 µg/ml, when studied on human neutrophils. Even though in the present study AU (244 µg/ml) at a higher concentration had an effect on *C. albicans* viability, a medication such as a mouthwash containing this solution would not have much potential of toxicity in the oral cavity tissues and other tissues not in direct exposure to neutrophils.

Furthermore, in accordance with Shim *et al*.[21] AU was suggested as a potentially safe drug to use in the oral cavity. Most importantly, it should be taken into consideration that the reduction in *C. albicans* viability and biofilm formation obtained from treatment with *P. major*, AU and BE

at lower concentrations may be sufficient for the subject's immune system to completely eradicate *C. albicans* related infections.

The ability of these three agents to inhibit *C. albicans* biofilm formation was dose-dependent to their concentrations. Other studies[8,27-29] have also supported this dose-dependent relation to the inhibition of *C. albicans* biofilm formation. Additionally, it was found that AU was the most effective in the decrease of *C. albicans* biofilm formation and the only agent to maintain the inhibition of viability of the yeast for over 48 hours.

The metabolic activity of *C. albicans* biofilm formation was assessed using XTT-reduction of tetrezolium to a tetrazolium formazan product by mitochondrial-active *C. albicans* in the presence of menadione.[29] The correlation between cellular density and metabolic activity provides an excellent semi-quantitative measurement of biofilm formation.[15] The colorimetric assay is noninvasive and nondestructive, requiring minimal post-processing sampling. This also correlated with cell viability, which is particularly useful for measuring the effects of anti-fungal agents on biofilm cells.[2,15] Among the three reagents, AU (244 μg/ml) and the *P. major* extract (diluted 1:2) exerted an effect towards *C. albicans* established biofilm metabolic activity. However, BE did not exhibit inhibition of the metabolic activity of *C. albicans* biofilm at any of its concentrations. This correlates with the data from Kang *et al.*[29] who demonstrated that BE could not induce apoptosis in *C. albicans* yet affected fungal growth via a different pathway. The results in this study indicated that BE affected *C. albicans* biofilm formation via cell surface hydrophobicity and in correlation with the findings of Cao *et al*.[8] BE affects the growth surface of *C. albicans* composition from true hyphae to yeast cell and pseudo-hyphae after treatment. *P. major* extract and AU behaved similarly to BE at their highest concentrations, and based on the growth inhibitory effects they contributed to poor biofilm formation. This, therefore, suggests their effectiveness in preventing *C. albicans* colonization and more importantly in preventing biofilm from becoming resistant.

The resistance of biofilm is likely multifactorial and among many mechanisms it may be due to the yeast cell surface hydrophobicity (CSH). CSH is important for the regulation of the pathogenicity of *C. albicans* biofilm, since elevated CSH causes increased adhesion of the organism to tissue surfaces and acrylic surfaces. Many studies have demonstrated that the more hydrophobic the microorganism the more adherent the cells are to acrylic surfaces. Furthermore, other studies have shown that *C. albicans* are less hydrophobic when compared to *C. tropicallis*

and relatively hydrophilic when compared to *C. glabrata*.[4,11] Raut *et al.*[5] stated that *C. albicans* is known to regulate cell surface hydrophobicity status according to growth phase, environment and nutritional conditions, making this mechanism difficult to study. In the present study, decreased CSH was observed at the highest concentrations of AU (244 µg/ml) and BE (100 µg/ml), where the increased effects of biofilm inhibition was noted. This negative correlation was similarly described by Cao *et al.*[8]

Further investigations to examine the role of *P. major* extract, AU and BE in acrylic and nylon denture base materials and their role in the inhibition of *C. albicans* biofilm formation are needed. Also, further studies may test the agents' solution in human affected tissues and their effectiveness against the growth of other non-*albicans Candida* species. Kang *et al.*[23] suggested that AU be further researched and utilized as a feasible anti-fungal medication, and in accordance with this study AU has the potential to be used as anti-fungal rinse against the growth of *C. albicans* due to its outstanding shown fungicidal properties.

Several other biologically active components of *P. major* extract should also be taken in consideration because it was shown that AU and BE at concentrations within the plant had little or no inhibitory effect against *C. albicans* biofilm formation when tested individually. However, those same concentrations, within the plant extract, displayed inhibition against *C. albicans* growth and metabolic activity, therefore, our findings show that *P. major* benefits from its anti-fungal properties from a combination of the plants eleven biologically active components working together, giving the plant its medicinal properties.

5. Conclusion

Within the limitations of this study the following conclusions can be drawn:

1. Collectively, *P. major* extract, and AU and BE demonstrated anti-fungal activity against *C. albicans* total growth.
2. *P. major* extract demonstrated weak inhibition of *C. albicans* biofilm formation and viability when compared to its components (AU and BE) in higher concentrations.

3. AU exhibited strong anti-fungal activity against *C. albicans* growth, biofilm formation, biofilm metabolic activity and CSH and was the only agent that provided continuous fungicidal activity against *C. albicans* viability.
4. BE revealed strong inhibition of *C. albicans* growth and its biofilm formation by inhibiting the cell surface hydrophobicity pathway.

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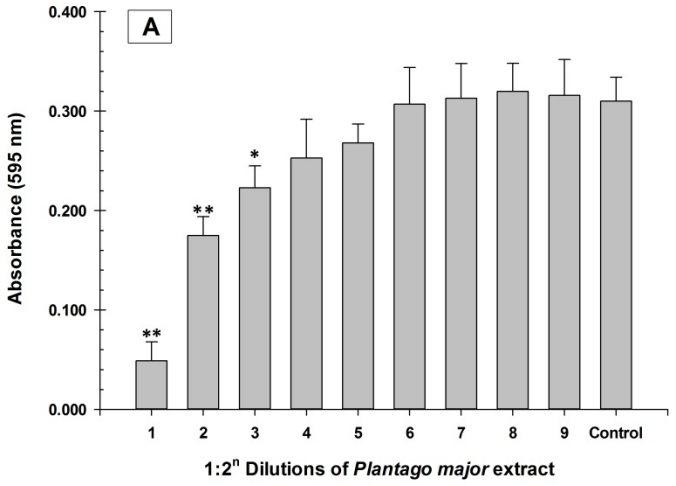
Table 1: Aucubin and baicalein concentrations in *Plantago major* extract 30X[19]

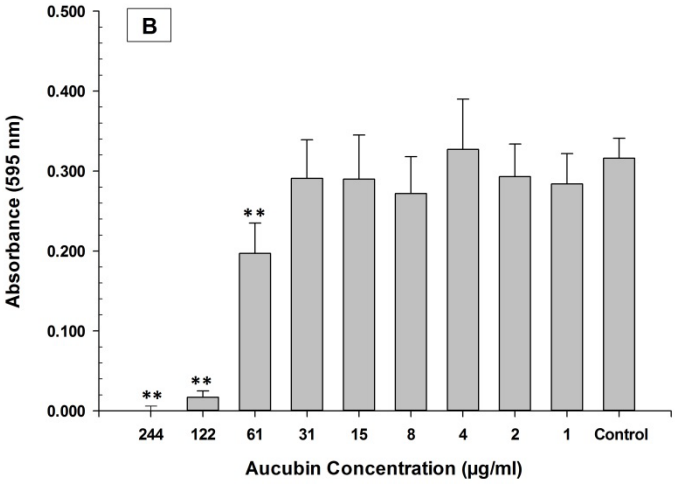
Dil. of P. major	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Aucubin (µg/ml)	0.25	0.125	0.062	0.031	0.015	0.0078	0.0039	0.0019	0.0009
Baicalein (µg/ml)	0.212	1.106	0.053	0.026	0.013	0.0066	0.0033	0.0016	0.00083

Table 2: The anti-fungal effects on *Candida albicans* viability of *Plantago major*, aucubin and baicalein (MFC)

Anti-Fungal Reagents	Minimum Fungicidal Concentration (MFC)
***Plantago major* extract**	> 60X
Aucubin	244 µg/ml
Baicalein	> 100 µg/ml

Figure 1


A
Absorbance (595 nm)
0.400
0.300
0.200
0.100
0.000
1
2
3
4
5
6
7
8
9
Control
1:2n Dilutions of Plantago major extract


B
Absorbance (595 nm)
0.500
0.400
0.300
0.200
0.100
0.000
244
122
61
31
15
8
4
2
1
Control
Aucubin Concentration (µg/ml)

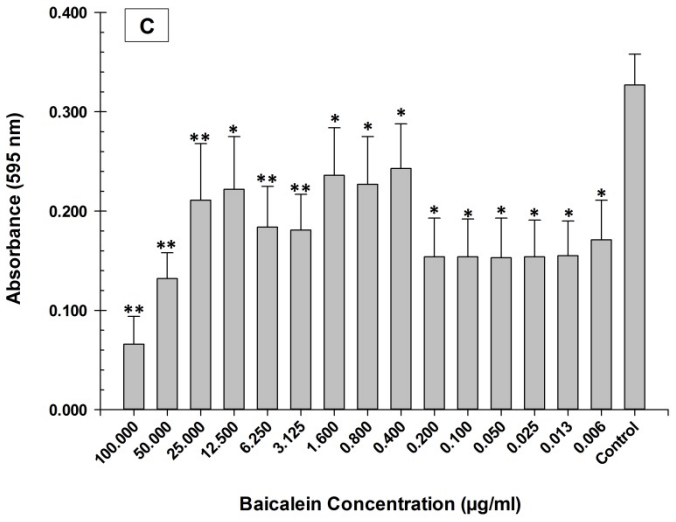

C
Absorbance (595 nm)
0.400
0.300
0.200
0.100
0.000
100.000
50.000
25.000
12.500
6.250
3.125
1.600
0.800
0.400
0.200
0.100
0.050
0.025
0.013
0.006
Control
Baicalein Concentration (µg/ml)

Figure 2

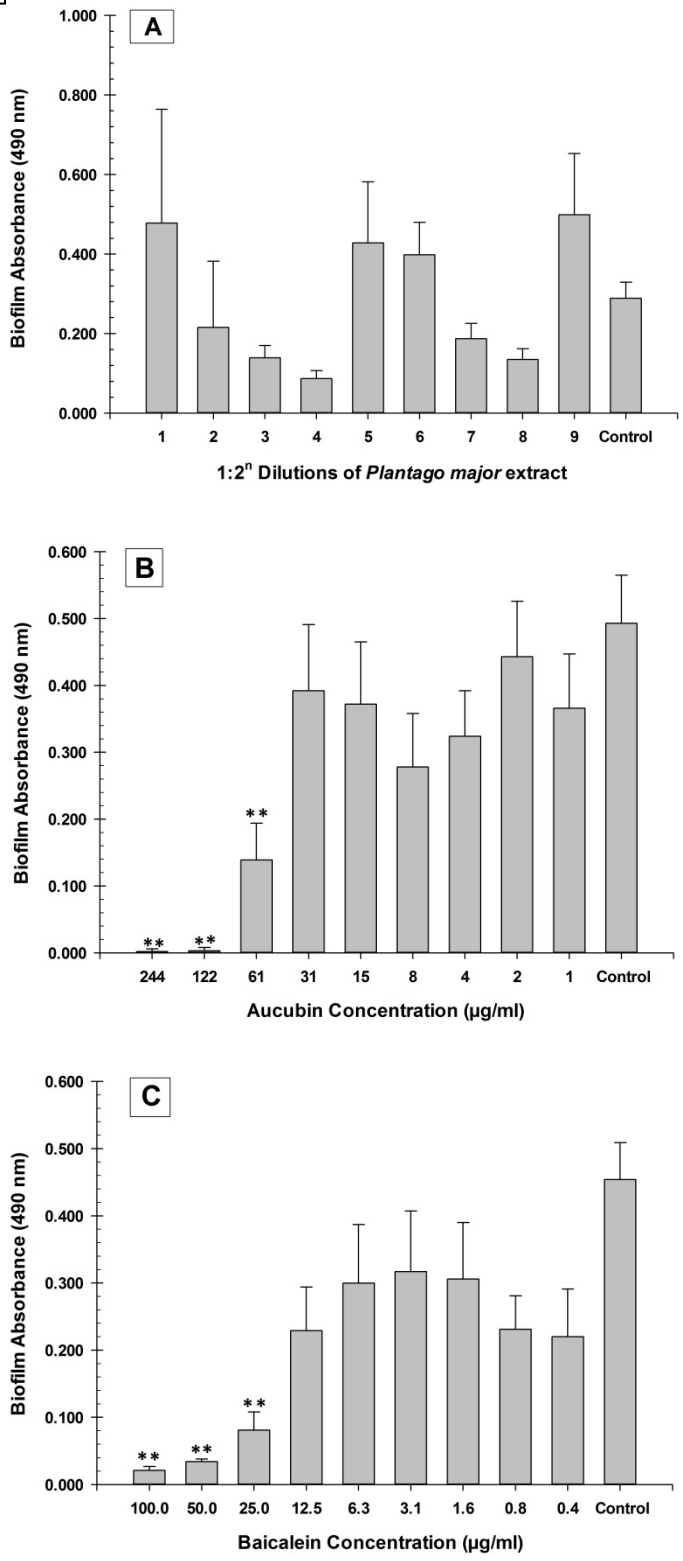

A
Biofilm Absorbance (490 nm)
1.000
0.800
0.600
0.400
0.200
0.000
1
2
3
4
5
6
7
8
9
Control
1:2n Dilutions of Plantago major extract
B
Biofilm Absorbance (490 nm)
0.600
0.500
0.400
0.300
0.200
0.100
0.000
**
244
122
61
31
15
8
4
2
1
Control
Aucubin Concentration (µg/ml)
C
Biofilm Absorbance (490 nm)
0.600
0.500
0.400
0.300
0.200
0.100
0.000
100.0
50.0
25.0
12.5
6.3
3.1
1.6
0.8
0.4
Control
Baicalein Concentration (µg/ml)

Figure 3

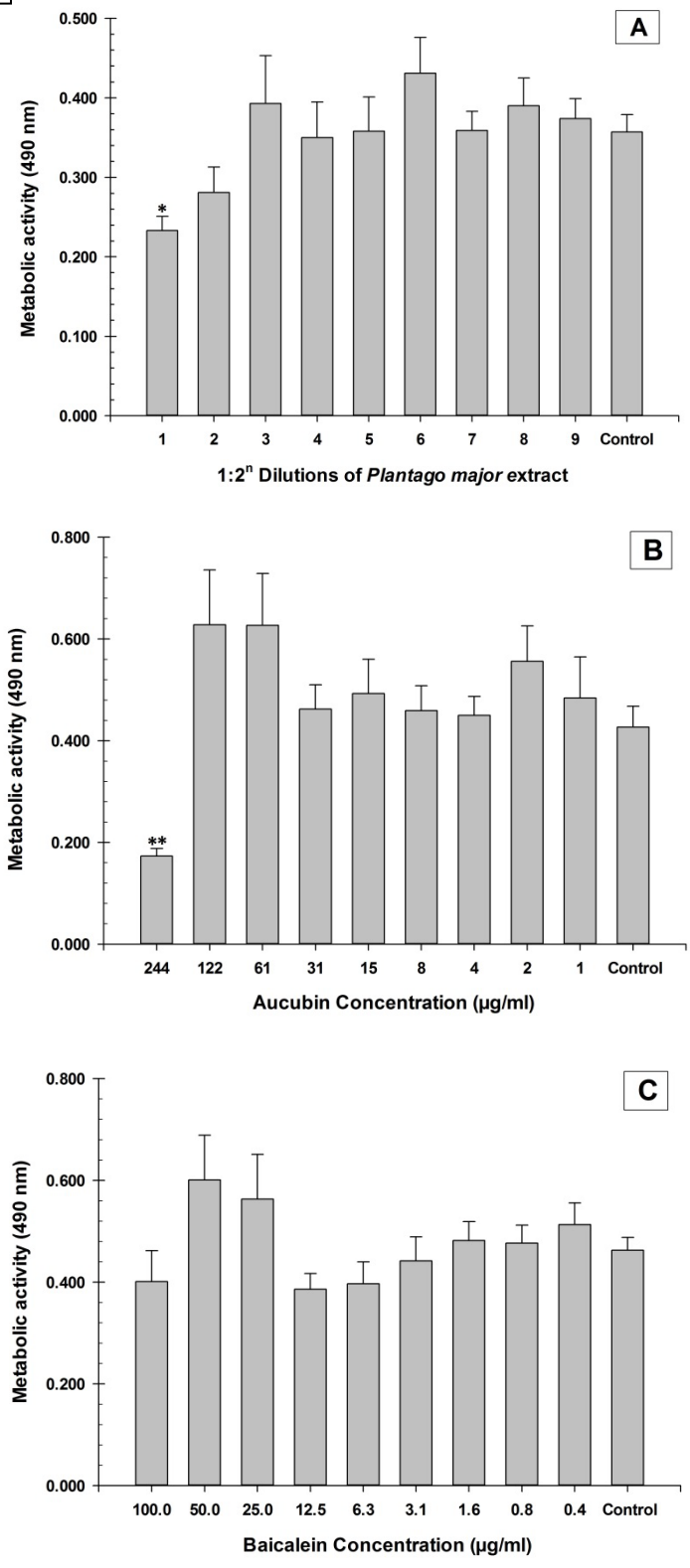
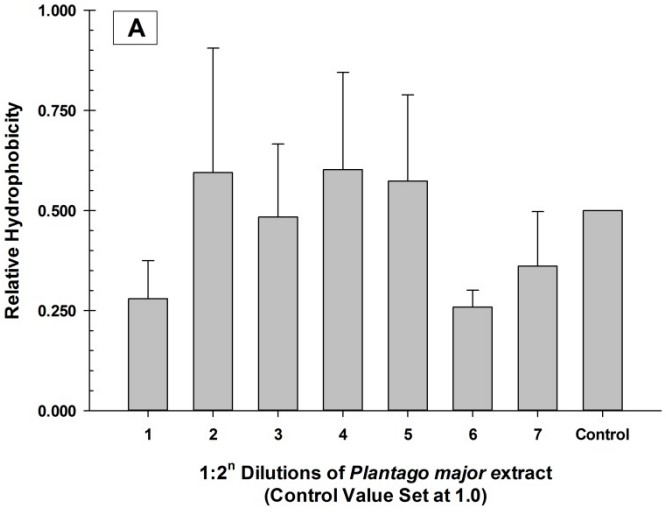
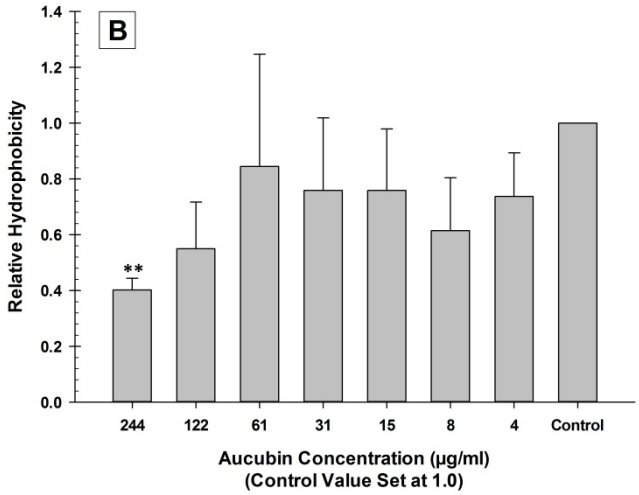
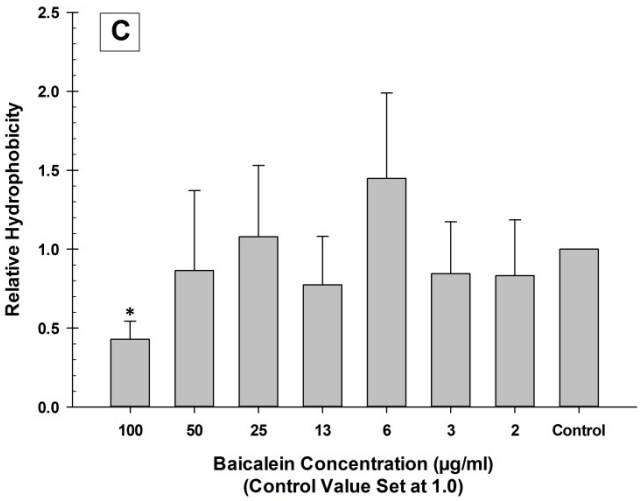

A
Metabolic activity (490 nm)
0.500
0.400
0.300
0.200
0.100
0.000
*
1
2
3
4
5
6
7
8
9
Control
1:2^n Dilutions of Plantago major extract
B
Metabolic activity (490 nm)
0.800
0.600
0.400
0.200
0.000
**
244
122
61
31
15
8
4
2
1
Control
Aucubin Concentration (µg/ml)
C
Metabolic activity (490 nm)
0.800
0.600
0.400
0.200
0.000
100.0
50.0
25.0
12.5
6.3
3.1
1.6
0.8
0.4
Control
Baicalein Concentration (µg/ml)

Figure 4


A
Relative Hydrophobicity
1.000
0.750
0.500
0.250
0.000
1
2
3
4
5
6
7
Control
1:2n Dilutions of Plantago major extract
(Control Value Set at 1.0)


B
Relative Hydrophobicity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
244
122
61
31
15
8
4
Control
Aucubin Concentration (µg/ml)
(Control Value Set at 1.0)


C
Relative Hydrophobicity
2.5
2.0
1.5
1.0
0.5
0.0
*
100
50
25
13
6
3
2
Control
Baicalein Concentration (µg/ml)
(Control Value Set at 1.0)

Legends

Figure 1 MIC's of (A) *Plantago major* extract, (B) aucubin and (C) baicalein on the total growth of *C. albicans* after 24-48 h at 37 °C. The data are expressed as mean absorbance (595 nm) ± S.E.M * $P < 0.05$, ** $P < 0.01$ when compared with the respective control groups.

Figure 2 MBIC's of (A) *Plantago major* extract, (B) aucubin and (C) baicalein on *C. albicans* biofilm formation after 24-48 h at 37 °C. The data are expressed as mean absorbance (490 nm) ± S.E.M * $P < 0.05$, ** $P < 0.01$ when compared with the respective control groups.

Figure 3 The biofilm metabolic activity of (A) *Plantago major* extract, (B) aucubin and (C) baicalein on established *C. albicans* biofilm formation after 48 h at 37 °C. The data are expressed as mean absorbance (490 nm) ± S.E.M * $P < 0.05$, ** $P < 0.01$ when compared with the respective control groups.

Figure 4 The cell surface hydrophobicity of (A) *Plantago major* extract, (B) aucubin and (C) baicalein on *C. albicans* biofilm after 48 h at 37 °C. The relative hydrophobicity of the control group was set at 1.0. The data are expressed as mean relative hydrophobicity ± S.E.M * $P < 0.05$, ** $P < 0.01$ when compared with the respective control groups.