

Antifungal activity of some *Sternbergia* taxa: effects on germ tube and biofilm formation

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Natural products are rapidly becoming the primary sources of novel antimicrobial agents, as resistance to existing antimicrobial agents is increasing. Apart from determining the antimicrobial activity of natural products, it is also important to understand their effects on the virulence factors of microorganisms. This study aimed to determine the antimicrobial activity of *Sternbergia* species prevalent in Turkey and investigate their role in the inhibition of germination tube and biofilm formation, both of which are known to be important virulence factors of *Candida albicans*. The antimicrobial activities of the plant extracts were evaluated using bore-plate and broth microdilution method. The extracts' capacity to inhibit the formation of the germ-tube was also evaluated. The findings of our study revealed that *Sternbergia lutea*, *Sternbergia vernalis* possessed antimicrobial activities, with MIC values ranging between 0.048 mg/mL and 0.39 mg/mL. The highest antimicrobial activity was observed against *Candida dubliniensis* (0.048 mg/mL). While evaluating the inhibition of fungal germination activities, *S. vernalis* extract (at a concentration of 0.09 mg/mL) was found to be the most effective against *C. albicans* ATCC 90028 strain. The results also indicated that *S. vernalis* extracts at sub-MIC levels inhibited germ tube formation and modulated the tail-length of germinated cells, both of which are important virulence factors of *C. albicans*. Furthermore, the inhibition of biofilm-formation was also investigated, and it was found that two *Sternbergia* spp. extracts at or below MIC levels inhibited biofilm formation.

Keywords: Antifungal activity. Biofilm inhibition. *Candida albicans*. Germ tube formation. Virulence factor. *Sternbergia* spp.

INTRODUCTION

Sternbergia spp., belonging to the Amaryllidaceae family, are represented by five species in Turkey: *S. lutea* (L.) Ker Gawl. Ex Spreng., *S. colchiciflora* Waldst. & Kit., *S. vernalis* (Mill.) Gorer & J.H.Harvey, *S. candida* B. Mathew & T. Baytop (endemic to Turkey), and *S. clusiana* (Ker Gawl.) Ker Gawl. ex Spreng. (theplantlist.org, 2018). *Sternbergia* spp. hold pharmaceutical importance because of their content of biologically active compounds such as Amaryllidaceae alkaloids, which are used in the drug industry. Of them, galantamine has been used in the treatment of Alzheimer's disease,

whereas lycorine has antiviral, antimalarial, analgesic, cytotoxic, and acetylcholinesterase inhibitory activities (Gabrielsen *et al.*, 1992; Tanker *et al.*, 1996; Campbell *et al.*, 1998; Lopez *et al.*, 2002; Unver, Kaya, Öztürk, 2005). The analgesic and antimicrobial activities of the extracts and alkaloids of *S. clusiana*, *S. lutea* and the anti-acetylcholinesterase and antioxidant activities of the five *Sternbergia* species prevalent in Turkey have also been reported (Unver, Kaya, Öztürk, 2005; Orhan *et al.*, 2011). In addition to evaluating the antimicrobial activity, it is also important to investigate the effects of plant extracts on the virulence factors of microorganisms, because they are important mechanisms adopted by the microorganisms to prevent their elimination by host defense systems. Thus, this study aimed to evaluate the antimicrobial activity of *Sternbergia* species and document their inhibitory effect on the formation of germ

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tube and biofilms, both of which are the most important virulence factors of *C. albicans*.

MATERIAL AND METHODS

Extraction of plant material

Three *Sternbergia* species: *S. lutea* and *S. vernalis* were included in the study. The designations and origins of the plants are summarized in Table I. *Sternbergia* species were collected from different parts of Anatolia as shown in Table I. Plant species were identified by Prof. Dr. Hayri Duman from Gazi University Faculty of Science Department of Biology. Voucher specimens are kept at the Herbarium of Ankara University, Faculty of Pharmacy with their herbarium numbers (Table I).

Fifty grams of the air-dried and powdered bulbs of each of the two taxa were macerated with water at room temperature. The extracts were then filtered using filter paper and lyophilized to obtain crude extracts. Before assaying the antimicrobial activities, the lyophilized extracts were dissolved in physiological saline to obtain a final concentration of 50 mg/mL.

Microorganisms

All microorganisms including *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 29213, Methicillin-resistant *Staphylococcus aureus* MFBF 10679, *Candida albicans* ATCC 90028, *Candida albicans* FC/S MFBF 10778, *Candida albicans* FC/R MFBF 11100, *Candida krusei* ATCC 14243, *Candida tropicalis* ATCC 750, *Candida dubliniensis* CBS 7987, and *Cryptococcus neoformans* MFBF 10828 were obtained from stock cultures of microorganisms at the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

Antimicrobial activity

Cylinder-plate diffusion and broth microdilution assays were performed to test the antimicrobial activity of the extracts (Cazedey, Salgado, 2011). Fungal inoculums were prepared from fresh cultures of *Candida*

spp. in Mueller-Hinton Broth (MHB). The density of the inoculums was set to 0.5 McFarland units using a nephelometer (ATB 1550, bioMerieux, France).

Initially, the cylinder-plate diffusion method was used for the determination of inhibition zones. Inoculums of fungi were spread onto MHA plates, after which wells (d = 6 mm) were made using sterile stainless-steel cylinders. Fifty microliters of the plant extracts (50 mg/mL) were added to the wells. The plates were kept at 4 °C for 1 h to accelerate the diffusion of the extracts into agar, and then were incubated aerobically at 37 °C for 18 h. After incubation, the diameter of inhibition zone around each well was measured and recorded. All the experiments were repeated five times and the inhibition zone diameters (mm) were documented as mean ±SD.

In the second step, minimal inhibitory concentrations (MIC) of the extracts against the microbial cells were determined by serial two-fold micro-dilution method in RPMI medium supplemented with glucose (Merck, Germany) contained in 96-well microtiter plates, following the Clinical and Laboratory Standards Institute (CLSI) procedure for *Candida* species. The *Candida* cells were seeded to the wells and treated with varying concentrations of extracts. After the incubation period, 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT)/menadione solution (0.5 mg/mL XTT and 1 µM menadione in acetone, at final concentrations) was added for determination of fungal cell viability and incubated further for 3 h. Absorbance in each well was determined using a microplate reader at 490 nm (Labsystems UV-Visible Reader MF). After incubation, 10 µL sample from each well was spread on an SDA plate and incubated at 37 °C for 48 h. MIC was defined as the lowest concentration of the extract that allowed no more than 20% growth of the microorganisms after re-incubation. All the experiments were repeated thrice.

Germ tube inhibition assay

The germ tube inhibition assay was performed using *C. albicans* ATCC 90028 strain according to Zuzarte *et al.* (2011) with slight modification. *N*-acetyl-D-glucosamine medium (0.5% *N*-acetyl-D-glucosamine (Sigma-Aldrich),

TABLE I - Location and Herbarium numbers of *Sternbergia* species investigated

Plant names	Location and Herbarium numbers
<i>Sternbergia lutea</i> (L.) Ker Gawl. Ex Spreng.	İzmir, Torbalı-Yeniköy Metropolis ruins, 11.11.2004, AEF no.23694
<i>Sternbergia vernalis</i> (Mill.) Gorer & J.H.Harvey	Antakya-Yayladağ, 23.04.2006, AEF no. 23793

0.5% peptone (Sigma-Aldrich), and 0.3% KH_2PO_4 , dissolved in distilled water at a final volume of 100 mL and filtered) was used for the assay. Thirty microliters of the inoculum suspension in physiological saline, adjusted to 2 McFarland units (6×10^8 CFU/mL) were added to 170 μL of *N*-acetyl-D-glucosamine media containing 90 $\mu\text{g/mL}$ (final concentration) of the *Sternbergia* extracts. The samples were incubated at 37 °C for 3 h. Using the 400x magnification of a phase-contrast microscope, at least 100 yeast cells from each sample were counted and tail length was determined using a Dinocapture software program 2.0 version 1.5.6.

Biofilm inhibition assay

For testing the inhibition of yeast biofilm formation, each well was pretreated with 250 μL FBS (fetal bovine serum; Sigma Aldrich, USA). The wells were then filled with 100 μL of *C. albicans* ATCC 90028 suspension at a concentration of 5×10^6 CFU/mL. The plates were incubated at 37 °C for 4 h to allow attachment, and then the wells were gently washed with phosphate-buffered saline (PBS). The wells containing uninoculated broth served as negative controls. The plates were covered and incubated aerobically at 37 °C for 24 h. All the extracts were diluted to concentrations between 0.5 and 0.03125 mg/mL in triplicate.

Following the incubation period, each well was aspirated and washed thrice using 250 μL of PBS. The plates were vigorously shaken in order to remove the non-adherent yeast cells. The remaining adherent cells were fixed with 200 μL of 99% methanol per well, which was removed after 15 min and the plates were left to dry overnight. The plates were then stained for 5 min with 200 μL of 1% crystal violet per well. Excess stain was rinsed by placing the plates under running tap water. After the plates were air-dried, the adherent cells were resuspended in 160 μL of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm (Multiscan plate reader, Thermo, USA) (Abdi-Ali, Mohammadi-Mehr, Agha Alaei, 2006).

Statistical analysis

The results of the inhibition of germination assay and yeast germination tail lengths were compared using one-way ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism v. 5.0 demo version, GraphPad Software, La Jolla California, USA). P-values less than 0.05 were considered significant.

HPLC analyses

HPLC analyses were carried out using Agilent LC 1200 model chromatograph (Agilent Technologies, California, USA). The diode array detector (DAD) was set at wave lengths 210 nm, 214 nm, 230 nm, 254 nm, 270 nm, 280 nm, 330 nm and 360 nm and. The chromatograms were plotted and processed using the above mentioned software. Separation was carried out using a ACE 5 C18 (250 mm \times 4.6 mm; 5 μm) column. The mobile phase was made up of 0.2% phosphoric acid in water (A), acetonitrile (B) and in gradient elution: initial 0 min, A–B (90:10, v/v); then 0–20 min, linear change from A–B (90:10, v/v) to A–B (75:25); and 20-25 min isocratic elution is from A–B (0:100). The flow rate was 1 ml/min and column temperature was maintained at 40°C. The sample injection volume was 10 μL .

All tested *Sternbergia* solutions were prepared as 1 mg/ml by dissolving in water.

RESULTS AND DISCUSSION

Initially, antimicrobial activities of *Sternbergia* taxa against some microorganisms including *E. coli* ATCC 10536, *S. aureus* ATCC 29213, MRSA MFBF 10679, *C. albicans* ATCC 90028, *A. niger* ATCC 16404, and *Mycobacterium fortuitum* MFBF 11440 were investigated. It was observed that *Sternbergia* taxa were only effective against *Candida*. However, the antimicrobial efficacy on organisms apart from *C. albicans* was either very low or non-existent. Hence, the antimicrobial activities of these *Sternbergia* taxa were investigated against other strains of *Candida*, including *C. albicans* FC/S MFBF 10778, *C. albicans* FC/R MFBF 11100, *C. krusei* ATCC 14243, *C. tropicalis* ATCC 750, and *C. dubliniensis* CBS MFBF 11098. The extracts from all two taxa exhibited the highest activity against *C. dubliniensis* CBS MFBF 11098, with MIC values of 0.048 and 0.19 mg/mL, respectively. The results of the antifungal activities of *Sternbergia* extracts are listed in Table II. Being members of the Amaryllidaceae family, *Sternbergia* species are rich in alkaloid content (Tanker *et al.*, 1996; Çitoğlu *et al.*, 1998). Lycorine is one of the major alkaloids found in the members of the Amaryllidaceae family. The quantification of lycorine in the bulbs of *Sternbergia* species was carried out using RP-HPLC external standard method in our previous study (Saltan Çitoğlu, Sever Yılmaz, Bahadır, 2008), wherein the highest content of lycorine was observed in *S. lutea* (1.069%) extract. The lycorine contents in the other species were recorded as 0.543%, 0.43%, and 0.429% for *S. vernalis*, *S. candida*, and *S. clusiana*, respectively.

TABLE II - The antimicrobial activities of *Sternbergia* spp. as diameter of inhibition zone (mm) and MIC value (mg/mL)

Strains	<i>S. lutea</i>		<i>S. vernalis</i>	
	Inhibition zone (mean±S.D.)	MIC	Inhibition zone (mean±S.D.)	MIC
<i>C. albicans</i> ATCC 90028	25 ± 0.8	0.39	24 ± 0.5	0.39
<i>C. albicans</i> FC/S MFBF 10778	24 ± 0.8	0.39	25 ± 0.8	0.19
<i>C. albicans</i> FC/R MFBF 11100	24 ± 0.7	0.39	26 ± 0.7	0.19
<i>C. krusei</i> ATCC 14243	23 ± 0.8	0.39	25 ± 0.8	0.19
<i>C. tropicalis</i> ATCC 750	-	-	-	-
<i>C. dubliniensis</i> CBS MFBF 11098	30 ± 0.7	0.048	21 ± 0.8	0.19

In current study the results of the HPLC analyses showed that water extract of the *S. vernalis*, and *S. lutea* contain mainly similar compounds in different amounts. According to the UV absorbances of the peaks it may be suggested that tested *Sternbergia* species may contain lycorine and galantamine when we compared to literature data (Emir *et al.*, 2013). Therefore activities of the extracts could be related to their alkaloid content which should be proved by further studies.

Lycorine, a widespread alkaloid in Amaryllidaceae species, has been proven to possess antiviral, cytotoxic, and antimalarial activities (Sener, Orhan, Satayavivad, 2003; Liu *et al.*, 2004). Amarbellisine, a lycorine-type alkaloid isolated from *Amaryllis belladonna* L., exhibits antibacterial activity against Gram-positive *S. aureus*. Furthermore, amarbellisine and lycorine, obtained from *A. belladonna*, displayed antifungal activity against *C. albicans* (De Laurentis *et al.*, 2004), while lycorine isolated from *Pancreatum maritimum* also inhibited the growth of yeast (Kaya, 2011). Unver, Kaya and Öztürk (2005) investigated the antimicrobial effects of n-hexane, ethyl acetate, ethanol, and aqueous extracts of *S. lutea* ssp. *lutea* and *S. lutea* ssp. *sicula* using the disc-diffusion assay. They observed that all the extracts were effective against the Gram-positive *S. aureus*, while only some extracts were effective against Gram-negative bacteria such as *E. coli* and *S. typhimurium*. The extracts were ineffective against *Pseudomonas* and *Enterobacter* spp. They also reported significant inhibition of the growth of *C. albicans*, similar to our study. However, the findings of antibacterial activity in their study were in contrast to those of our study on the

Sternbergia extracts. These differences could be due to the different geographical distribution of the collected plants, which could differently influence the levels of active compounds. We have previously reported the presence of bioactive ingredient lycorine in extracts from bulbs of plants (Saltan Citoglu, Sever Yilmaz, Bahadır, 2008). Haemantamine-, tazettin-, lycorine-, homolycorine-, crinine-, and galantamine-type alkaloids, together with lycorine, form the main constituents of *Sternbergia* species (Evidente *et al.*, 2004). Hippeastrine, homolycorine-type, vittatine and 11-hydroxyvittatine, and crinine-type alkaloids from different *Sternbergia* species have also been reported to show antifungal activity against *C. albicans* (De Laurentis *et al.*, 2004; Bastida *et al.*, 2011). The antifungal activities of compounds suggested to be responsible for the antimicrobial activity of *Sternbergia* species, including lycorine, vittatine and 11-hydroxyvittatine, and alkaloids, have not been tested yet. The antifungal activities of a mannose-binding lectin (Saito, Misaki, 1997); polysaccharides (Evidente *et al.*, 2004); and phenolic acids such as protocatechuic acid, 4-OH benzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, and ferulic acid (Nikolova, Gevrenova, 2005) have been previously evaluated in *Sternbergia* species.

It has been shown that the extracts of *Sternbergia clusiana* have relatively high levels of total phenol content (ranging from 250 to 1126 gallic acid equivalents per 100 g sample) (Mammadov, Kara, Vaizogullari, 2011), which is probably associated with their antioxidant and free radical scavenging activity. These extracts were tested for antimicrobial activity and it was observed that they

were relatively ineffective against the majority of the tested bacteria, although some of them (leaf methanol and ethanol extract, and bulb ethanol extract) had high anti-candida activity with inhibition zone diameter between 6 and 9 mm.

The anti-candida activity is highly dependent on the compound's influence on virulence factors, which are the most important factors in the development of candidiasis. Because the transition from yeast to hypha is an important virulence factor of *C. albicans*, we assessed the ability of the extract to inhibit the germ tube formation at concentrations below the MIC, as prevalent in clinical use. The hyphal development of *C. albicans* is an important factor influencing the ability of the yeast to damage cells and progress to deep-rooted infection (Tsuchimori *et al.*, 2000). The extent of germ tube inhibition of *C. albicans* ATCC 90028 following treatment with sub-inhibitory concentrations (90 µg/mL) of the two *Sternbergia* species is presented in Figure 1. The extracts of *S. lutea* was observed to insignificantly inhibit the germination process. In contrast, *S. vernalis* extract strongly inhibited germination by approximately 50% ($p=0.001$). These findings indicated that *S. vernalis* extract at sub-MIC level is a potent inhibitor of the germination of *C. albicans* cells. Furthermore, the extract of *S. vernalis* at the same concentration also strongly reduced the tail-lengths of germinated cells of *C. albicans* (Figure 2 and Figure 3), confirming the potent anti-virulence effect of *S. vernalis*. Although all two *Sternbergia* taxa with antifungal activities inhibited germ tube formation of *C. albicans* with varying efficiency, *S. vernalis* extract at concentration 0.09 mg/mL was found to be the most effective. The extract of *S. vernalis* strongly inhibited germination by more than 50% and also reduced the tail length, both of which are major virulence factors of *Candida* spp. These results are promising for the introduction of *Sternbergia* extracts into medicine for the treatment of *Candida* infections subsequent to further research and in-depth analysis of their mechanism of action.

The extracts of *Sternbergia* spp. were also assessed for their potential in the inhibition of biofilm formation. As shown in Figure 4, all extracts with potent antifungal activity against planktonic cells also inhibited biofilm formation of *C. albicans*. It was observed that the biofilm-inhibitory concentrations were at or below MIC values. All the two extracts tested for biofilm inhibition displayed immense potential in the reduction of biofilm mass, as evident by relatively high percentage of biofilm inhibition even at low concentrations (31.25 and 62.25 µg/mL).

To the best of our knowledge, this is the first study reporting the inhibition of germination and biofilm formation by extracts of *Sternbergia* taxa. Our results demonstrated that all extracts were potent inhibitors of *Candida* growth. Particularly, the extract of *S. vernalis* at sub-MIC level was a potent inhibitor of germination and biofilm formation of *C. albicans* cells, which are major virulence factors and consequences of *Candida* infection in living organisms and on surfaces.

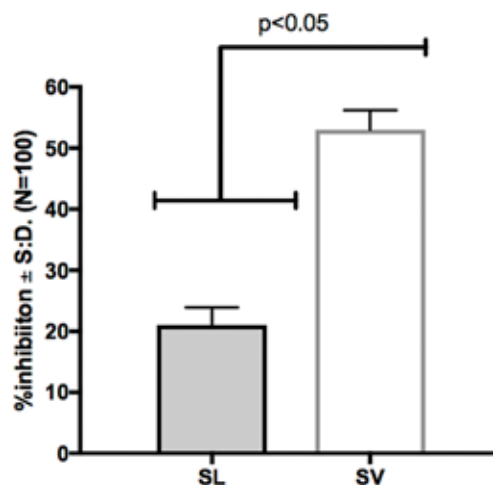


FIGURE 1 - The germ tube inhibition percentage of *C. albicans* ATCC 90028 after treatment with extracts of *Sternbergia* spp. Legend: SL= *Sternbergia lutea*, SV= *Sternbergia vernalis* extract; All results were obtained with concentration of 90 µg/mL.

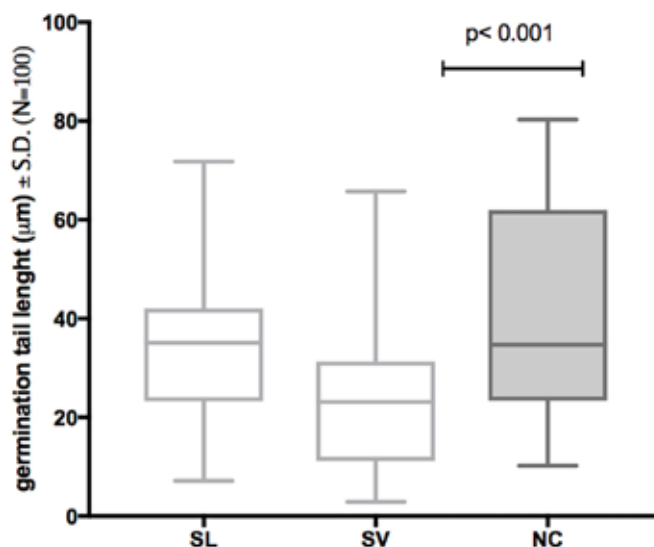


FIGURE 2 - The comparison of tail length of germinated *C. albicans* ATCC 90028 cells inhibited by *Sternbergia* spp. Extracts. Legend: SL= *Sternbergia lutea*; SV= *Sternbergia vernalis* extract; NC=negative control (non-treated cells). All results were obtained with concentration of 90 µg/mL.

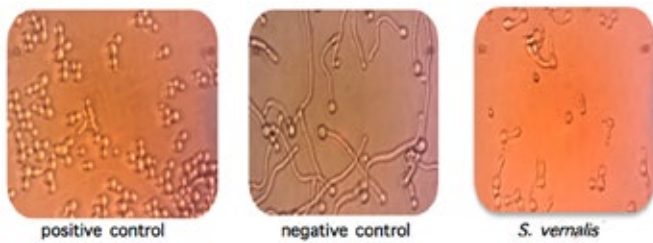


FIGURE 3 - Photomicrography (400x using phase contrast microscopy) of *C.albicans* ATCC 90028 blastospores after 3 h aerobic incubation at 37 °C.

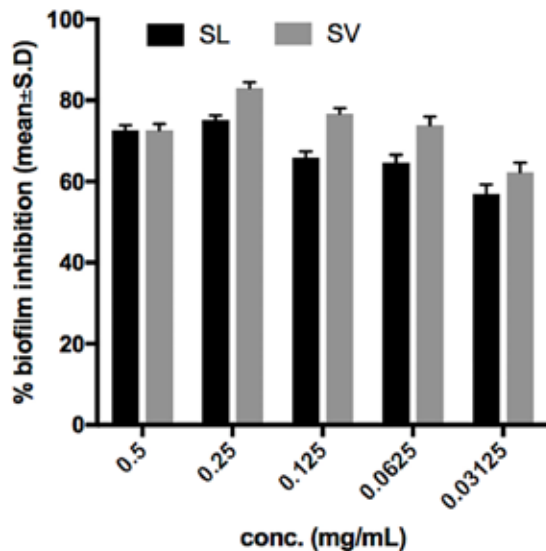


FIGURE 4 - The percentage of biofilm inhibition of *C. albicans* ATCC 90028 by *Sternbergia* spp. Extracts.

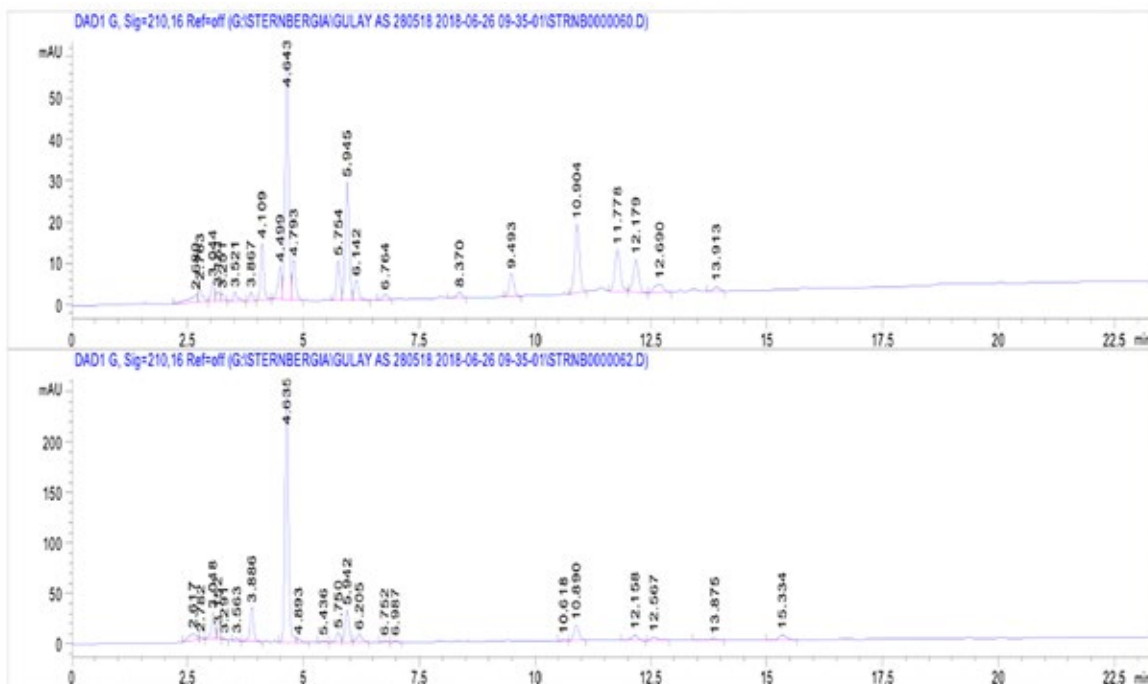


FIGURE 5 - HPLC chromatogram of *S. vernalis* (a), *S. lutea* (b).

CONCLUSION

The present study showed that the extracts of two different *Sternbergia* species with potent antifungal activity also inhibit the germ tube and biofilm formation of *C. albicans* ATCC 90028 strain.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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