Research Article

Convolvulus scammonia Effect on Gene Expression of *Candida tropicalis* Biofilms

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Abstract | *Convolvulus scammonia* is a twining perennial plant with thick, fleshy roots, irregularly arrow-shaped leaves, and flowers resembling those of *Convolvulus arvensis*. The aim of this study was to determine the impact of *C. scammonia* on six genes expression (*i.e., ACT1, BCR1, EFG1, AlS1, AlS3,* and *TEC1*), known to be implicated in the development of *Candida tropicalis* biofilms, and define the minimum inhibitory concentration (MIC) of *C. scammonia* required for this purpose. The 2,3-bis(2-metoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay was used to assess the anti-biofilm activity of *C. scammonia* in order to ascertain the formation of biofilms on *C. tropicalis* isolates obtained from thrush samples and evaluate the MIC of *C. scammonia* that inhibits the biofilms of *Candida tropicalis* during 24 and 48 h. Finally, the impact of *C. scammonia* on *ACT1, AlS1, AlS3, BCR1, TEC1, and EFG1*gene expression in *C. tropicalis* was examined using a real-time polymerase chain reaction (RT-PCR) and compared to the results obtained in the gene expression of the control *C. tropicalis* biofilms untreated with *C. scammonia* during 24 and 48 h. Through comparison, biofilms formation was found to decrease as *C, scammonia* concentration and time increased. Accordingly, the MIC of *C. scammonia* was 20 % w/v, and its minimum fungicidal - concentration (MFC) was 40 % (w/v) in biofilm-forming *C. tropicalis*. Additionally, gene expression level analysis revealed a decrease in *ACT1, AlS1, AlS3, BCR1, EFG1, and TEC1* expression levels on treatment with *C. scammonia* during 24 and 48 h.

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Introduction

There are several species of *Candida* in nature, usually as part of commensal mammalian microbiota (Graf *et al.*, 2019). Nevertheless, the commensal phase may give a way to the pathogenic phase due to modifications in the host environment, such as disruptions in the commensal microbiota (Alves *et al.*, 2020). According to several studies, fungal infections in humans are neglected infectious diseases that haves emerged as a problem in the public health system in recent decades (Kobayashi *et al.*, 2020). Currently, over 200 species of *Candida* have been identified (Pohl, 2022). Non-albicans Candida

(NAC) species are becoming more prevalent in terms of mortality and morbidity, although *C. albicans* remains the most prevalent fungal pathogen (Zhang *et al*., 2020). In addition to *C. albicans*, four other species of NAC have surfaced, mainly *C. tropicalis, C. parapsilosis, C. glabrata,* and *C. krusei* (Atiencia-Carrera *et al*., 2022). Currently regarded as the most significant emerging fungal pathogen among these, *C. tropicalis* has been found to have multiple strains that are resistant to common empirical treatments, including fluconazole (Tsay *et al*., 2020).

It is known that both *C. albicans* and *C. tropicalis* have a wide variety of commensal traits and virulence factors that enable them to colonize and infiltrate the host tissue (de Barros *et al*.,2020).These include phenotypic switching and thigmotropism (contact sensing), production of hydrolytic enzymes, development of biofilms, expression of adhesins and invasins on the cell surface, and capacity to damage host cells (Flores-Vargas *et al*., 2021). Although biofilms are the most prevalent way for microorganisms to grow and the formation of these biofilms among *Candida* species confers significant resistance to antifungal therapy, little is known about the life cycle of *C. tropicalis* biofilms (Atriwal *et al*., 2021). In 2024, the efficacy of liposomal amphotericin B antifungal and the development of *C. tropicalis* biofilms were evaluated through time-lapse imaging, demonstrating that *C. tropicalis* is a fast-growing species capable of forming aggressive biofilms (Omelchuk *et al*., 2024).

Wild plants have been used extensively by Aboriginal people to prevent nutritional and pathogen-related illnesses since the dawn of human history (Bourhia *et al*., 2019). Since the beginning of time, people have utilized plant-based products in a wide range of settings. Researchers have been examining the deliberate use of natural resources for more than a millennium (Sharma and Gupta, 2015). The medicinal plants have long been used to treat a variety of ailments, and they are still used as emerging new tools to combat these diseases (Bourhia *et al*., 2019).

Because of their bio-availability, biological properties, safety, and clinical efficacy, *C. scammonia* plant extracts and essential oils have drawn more attention (Chen *et al*., 2018). The abundance of the following compounds was observed in the genus *Convolvulus*: fatty acids, alkaloids, lipids, coumarins, lignans, alkaloids, terpenoids, steroids, flavonoids, amino acids, carbohydrates, anthraquinones, anthocyanidins, phenylpropanoids, and resins (Al-Rifai *et al*., 2017; Salehi *et al*., 2020). *Convolvulus* species have been found to possess medicinal properties as the ability to lessen or completely eradicate the symptoms of several severe illnesses, including e fever, heart disease, sleeplessness, memory loss, and hair loss, in addition to urogenital disorders, severe purgatives, diarrhea, gastrointestinal irritation, animal stings; congestion, edema, gaseous distended intestine, and organ hemorrhages (Salehi *et al*., 2020).

The objective of this study was to detect the effect of *C. scammonia* on the expression of several genes (*ACT1, AlS1, AlS3, BCR1, EFG1, and TEC1*) that are involved in *C. tropicalis* biofilm formation, in addition to the minimum inhibitory concentration of *C. scammonia* needed to accomplish this.

Materials and Methods

Candida tropicalis isolation

Candida tropicalis was isolated from thrush samples at the Samawah Teaching Hospital for pediatrics and Gynecology in AL-Muthanna governorates, Iraq, during the period from December 2021 to June 2023. Fifty oral swabs were obtained from children suffering from oral candidiasis and septicemia with oral thrush. Specimens were taken using sterile swabs, and then transported to the microbiology laboratory for diagnosis. *C. tropicalis* was isolated and identified using Sabouraud dextrose agar (SDA) and CHROM agar.

Analytical Profile Index (API) (HIMDIA/India) was employed as a biochemical assay for detection of *C. tropicalis* and to validate the morphological, cultural, and biochemical characteristics required to differentiate between *Candida* species, and demonstrate that *Candida* can utilize different types of sugars (Calderón-Hernández *et al*., 2024).

Convolvulus scammonia collection

The plnats samples (leaves, stems, and flowers) used in these experiments were gathered from general local gardens in Samawah city. Crude alkaloids extraction was conducted on the leafs of these plants as described by Hussein (2024).

Minimum inhibitory concentration (MIC) determination

The microdilution broth method was used to calculate the MICs of natural *C. scammonia* against the biofilms

of *C. tropicalis*. *C. scammonia* was diluted in Yeast extract Peptone Dextrose (YPD) broth in steps of 5 % w/v, 10 % w/v, 20 % w/v, 40 % w/v, and 80 % w/v. Under vigorous shaking (120 rpm), the cultures were incubated at 35 ˚C for 36 h. After incubation, 1 ml of a broth (10^3 cfu/ ml) was aseptically injected into Petri plates of SDA. Following a 48-h of incubation, the resulting colonies' growth was evaluated by ELISA, and the MIC of *C. scammonia* (w/v) was taken as the dilution that prevented *C. tropicalis* from making a significant growth (Agarwal *et al*. 2010).

Formation of biofilms

The biofilms were prepared in 96-well flat-bottomed polystyrene microtiter plates and their growth was evaluated using the XTT (2,3-bis(2-metoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt) reduction assay (Lal *et al*., 2010). The kinetics of biofilm inhibition were studied using various MIC dilutions of *C. scammonia* in YPD broth (5 % w/v, 10 % w/v, 20 % w/v, 40 % w/v, and 80 % w/v) in order to ascertain whether *C. scammonia* could prevent the formation of *Candida* biofilms and find the minimum concentration of *C. scammonia* that could do so. Every MIC dilution in a microtiter plate was analyzed in at least 7 wells. Each dilution (5 % w/v, 10 % w/v, 20 % w/v, 40 % w/v, and 80 % w/v) was added to aliquots that held 190 ml in the microtiter plate wells. A culture of 48 h of *C. tropicalis* was cultivated in 10 ml of YPD broth with 5×10^8 cfu/ ml. On an orbital shaker running at 75 rpm, 10 µl of this 48-h culture were applied to each well and incubated for 1.5 h at 37 ˚C to guarantee even distribution and adherence to the well surfaces. In order to remove non-adherent cells, the adherent cells were carefully washed twice in sterile phosphate buffered saline (PBS) with a pH of 7.4, being cautious not to disturb the adherent cells. Following washing, 200 ml of a second aliquot of the same *C. scammonia* dilution in sterile YPD broth was added to each plate well. Afterward, the plates were kept in the same incubator for 48 h so that the biofilms could settle in and grow. As a control, 200 ml of an autoclaved YPD broth was added to each of the seven micro-titer plate wells. The broth could be positive (containing *Candida*) or negative (not containing *Candida*). The plate was incubated for 48 h at 37 ˚C (Poon and Hui, 2023).

Biofilm evaluation using the XTT reduction assay

The *C. tropicalis* cell concentrations in the biofilms were measured using the XTT assay following *C. scammonia* treatment. A filter of a pore size 0.22 mm was used to filter and sterilize the cells after making the XTT solution (1 mg/ml in PBS). Prior to the commencement of every assay, a menadione solution and XTT solution were mixed at a 5:1 (v/v). To stop the adherent biofilms from growing further, 2.5 % glutaraldehyde was applied for 5 min. to the microtiter plate wells after three PBS washings. Following the removal of the fixative, two PBS washes were applied to the wells. In all but the control well, which had no biofilm, 1 ml of PBS was added to the XTT-menadione solution. After that, the Metyrapones (MTPs) were maintained in the darkness for 2 h at 37 ˚C. After incubation, 75 ml of XTT-menadione solution were added to each well on a fresh microtiter plate, and spectrophotometry was used to measure the absorbance at 492 nm (Poon and Hui, 2023).

RNA isolation and RT-PCR

The PrestoTM Mini gDNA Yeast Kit was used to extract the total RNA from the *C. tropicalis* culture cells and co-culture them with *C. scammonia* in accordance with the manufacturer's instructions. The concentration and degree of RNA presence were determined using a Nanodrop-1000 UV-VIS spectrophotometer. For the agarose gel electrophoresis, RNA templates were employed. The cDNA synthesis SuperMix Kit and EasyScript One-Step gDNA were used to perform the reverse transcription PCR cDNA synthesis. One cycle at 42 ˚C for 30 min. and 85 ˚C for 5 sec was the RT-PCR setup (de Barros *et al*., 2020).

The mRNA transcripts were detected using real-time PCR with the Mx3000p (Agilent Technologies, USA). 10 mM primers were used to prepare the PCR components, which were then amplified by the genes presented in Table 1 (de Barros *et al*., 2020). In this experiment, the housekeeping gene beta-actin was amplified in the forward direction 5'-CGTCGGTAGACCAAGACACC-3' and the opposite direction 5'-CCCAGTTGGAGACAATACCGT-3', A final volume of 20 µl was achieved by adding nuclease-free water, 200 ng of cDNA, and 10 µl of Luna universal qPCR master mix. The following parameters were used to run the PCR: 30 sec at 94 ˚C, 45 cycles of 94 ˚C for 5 sec, and 30 sec at 60 ˚C. Then, a dissociation curve was employed, with a single cycle lasting 1.0 min. at 95 ˚C, 30 sec at 55 ˚C, and 30 sec at 95 ˚C (de Barros *et al*., 2020).

Table 1: The nucleotide sequence of the primers for real-time PCR amplification.

Genes	Primer sequence (5`→3`)	GenBank accession no.	Product size (bp)
ACT1	Forward TACGCTGGTTTCTCCTTGCC Reverse GCGGTGGTGGAGAAAGTGT	XM_002549283.1	116
ALS1	Forward TCTGTTGCCATTCCTGTCGAA Reverse AAACCAACCCAAGCAAGATCG	MK182724.1	70
AlS3	ForwardTGACTCCTAAGGAAGTATCGGGA Reverese GCCAAGCTGGATTAGCAGGA	MH753521.1	102
BCR1	Forward ATCCTTTACCTGCGTTGCGT Reverese GGACGAAGCTACTGACTCGG	XM_002545781.1	146
EFG1	Forward TCCTGCAGCTCCACCTATACC Reverese TTGGCTGGGTTTAACGTGTCT	KP314278.1	99
TEC1	Forward TTCAAGCAGTGCTCCAAGTTC Reverese AGGTGCAGAATGGAAACCAGT	XM_002547951.1	103

Statistical analysis

The analysis of variance (ANOVA) and t test were used to examine the various means of biofilm biomass (absorbance). GraphPad was used to analyze the data.

Results

Minimum inhibitory concentration determination

The growth of *C. tropicalis* was inhibited by *C. scammonia* in a concentration-dependent manner. The minimum fungicidal concentration (MFC) of *C. scammonia* on biofilm-forming *C. tropicalis* was 40 % (w/v), while its MIC was 20 % (w/v). Approximately, 99.9 % of the inoculum must be removed by the MFC. The value of MFC was typically higher than the MIC. Compared to cell growth without *C. scammonia*, the curves of growth for the *C. tropicalis* when exposed to 20 % (w/v) of the *C. scammonia* for 24 h showed a decrease in both the overall cells number and the growth rate (Figures 1 and 2). The treatment group and the control group differed significantly (p <0.05) according to the results of the one-way ANOVA test.

Effect of Convolvulus scammonia on biofilm formation

Convolvulus scammonia was added to YPD broth at several concentrations (5 % w/v, 10 % w/v, 20 % w/v, 40 % w/v, and 80 % w/v). It was shown that the amount of *C. scammonia* present affected the suppression of biofilm formation. Figure 2 shows that the formation of biofilm was neither disrupted nor stimulated by *C. scammonia* concentrations below 10 % w/v. However, biofilm development was greatly inhibited at concentrations greater than 10 % w/v. There was a significant difference between the control and treatment groups (p <0.05) according to the one-way ANOVA test analysis.

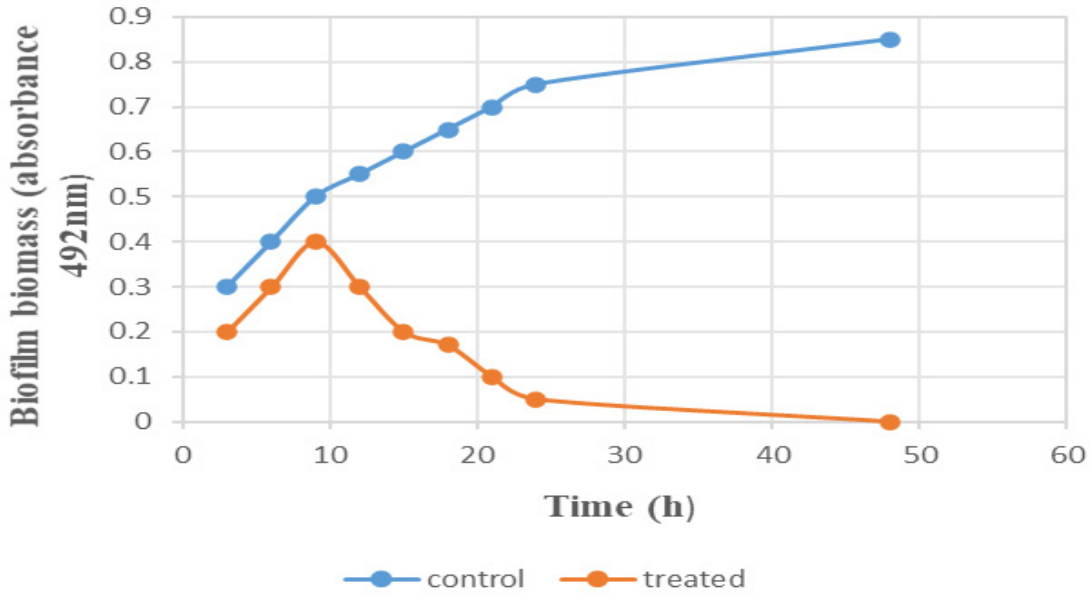

Figure 1: *Growth evaluations of Convolvulus scammonia-treatment on established Candida tropicalis biofilms during 24 to 48 h (F=30.65941, p < 0.05).*

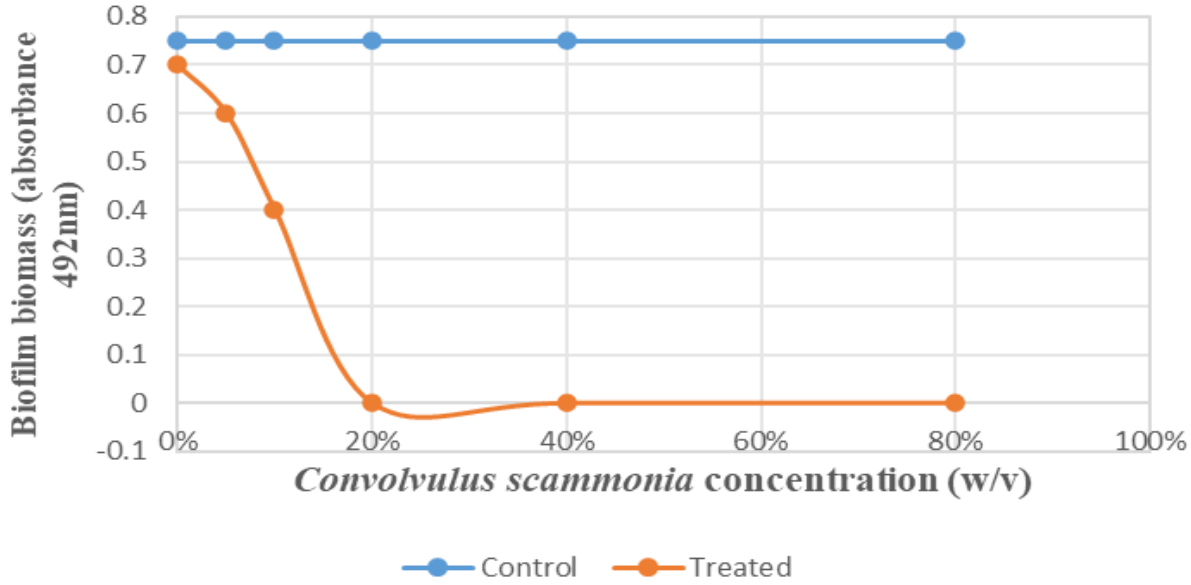

Figure 2: *Convolvulus scammonia's impact on the development of Candida tropicalis biofilms. Convolvulus scammonia's MIC was 20 % (w/v), and its MFC was 40 % (w/v) in biofilm-forming Candida tropicalis during 24 to 48 h (F=12.36593, p < 0.05).*

Gene expression analysis via RT-qPCR

The implicated genes (*ACT1, AlS1, AlS3, BCR1, EFG1,* and *TEC1*) were measured for relative expression in *C. tropicalis* over the course of the biofilm experiment for 24 and 48 h using RT-PCR. In contrast to the genes encoding the biofilm treated with *C. scammonia*, the expression of the biofilm genes (*ACT1, AlS1, AlS3, BCR1, EFG1,* and *TEC1*) increased significantly over time in the absence of *C. scammonia* treatment. This is regarded as a measre of control (Figure 3). A p <0.05 for the expression of the biofilm genes was deemed statistically significant based on the t-test analysis.

Candida tropicalis cells exposed to *C. scammonia* had less biofilm formation during 24 h, according to results from a RT-PCR using the specific primers of *ACT1, AlS1, AlS3, BCR1, EFG1,* and *TEC1* genes. Figure 4 shows the *ACT1* (0.5), *AIS1* (1), *AIS3* (0.8), *BCR1* (0.7), *EFG1* (0.6), and *TEC1* (0.8) genes that were treated with *C. scammonia*, respectively. This indicates the inhibitory role of *C. scammonia* on the *C. tropicalis* biofilm formation within 24 h compared to the control gene expression. According to the t-test analysis, the expression of the biofilm genes was p <0.05, which is considered statistically significant.

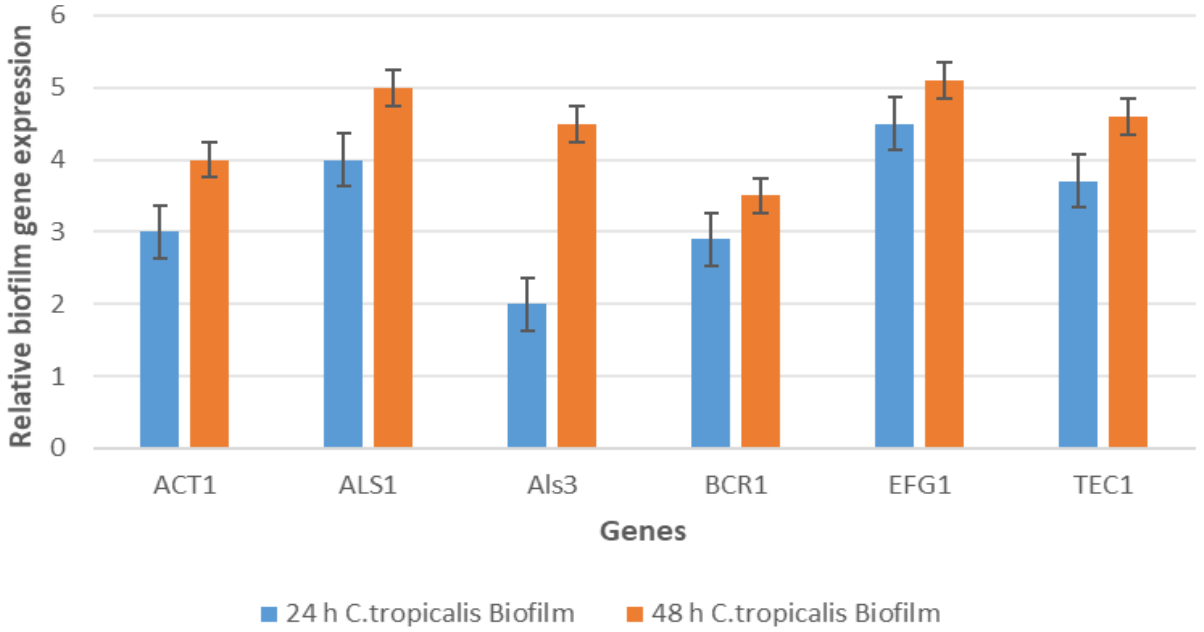

Figure 3: *Relative expression of the implicated biofilm forming genes (ACT1, ALS1, AlS3, BCR1, EFG1, and TEC1) in Candida tropicalis during the biofilm experiment without treated of Convolvulus scammonia as a control for 24 and 48 h using reverse transcriptase real-time PCR. (t= 2.4868, df =10, standard error of difference = 0.442). p < 0.05 was considered statistically significant.*

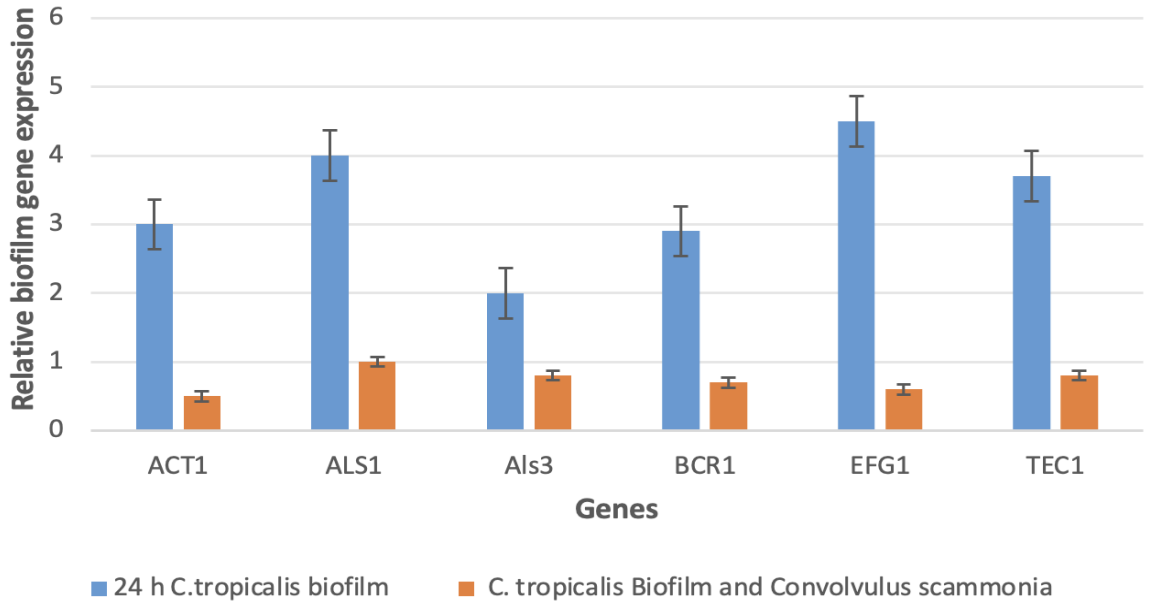

Figure 4: *Relative expression of the implicated biofilm forming genes (ACT1, AlS1, AlS3, BCR1, EFG1, and TEC1) in Candida tropicalis during the biofilm experiment for 24 h and Candida tropicalis biofilm with Convolvulus scammonia using reverse transcriptase real-time PCR. (t =7.0198, df =10, standard error of difference= 0.373). p <0.05 was considered statistically significant.*

Results from RT-PCR using the particular primers *ACT1*, *AlS1*, *AlS3*, *BCR1*, *EFG1*, and *TEC1* showed that *C. tropicalis* cells exposed to *C. scammonia* had less biofilm formation during 48 h. Figure 5 illustrates that *C. scammonia* was applied to the *ACT1* (1), *AlS1* (1.5), *AlS3* (0.5), *BCR1* (0.3), *EFG1* (0.7), and *TEC1* (0.9) genes, respectively. These results are conclusive evidence that *C. scammonia* is a highly efficient inhibitor of biofilm formation by *C. tropicalis*. It can be used as therapeutic alternatives for many diseases caused by *C. tropicalis*. These results display a positive impact of *C. scammonia* on the genes responsible for the formation of *C. tropicalis* biofilms (Figure 5). According to the t-test analysis, the expression of the biofilm genes was $p < 0.05$, which is considered statistically significant.

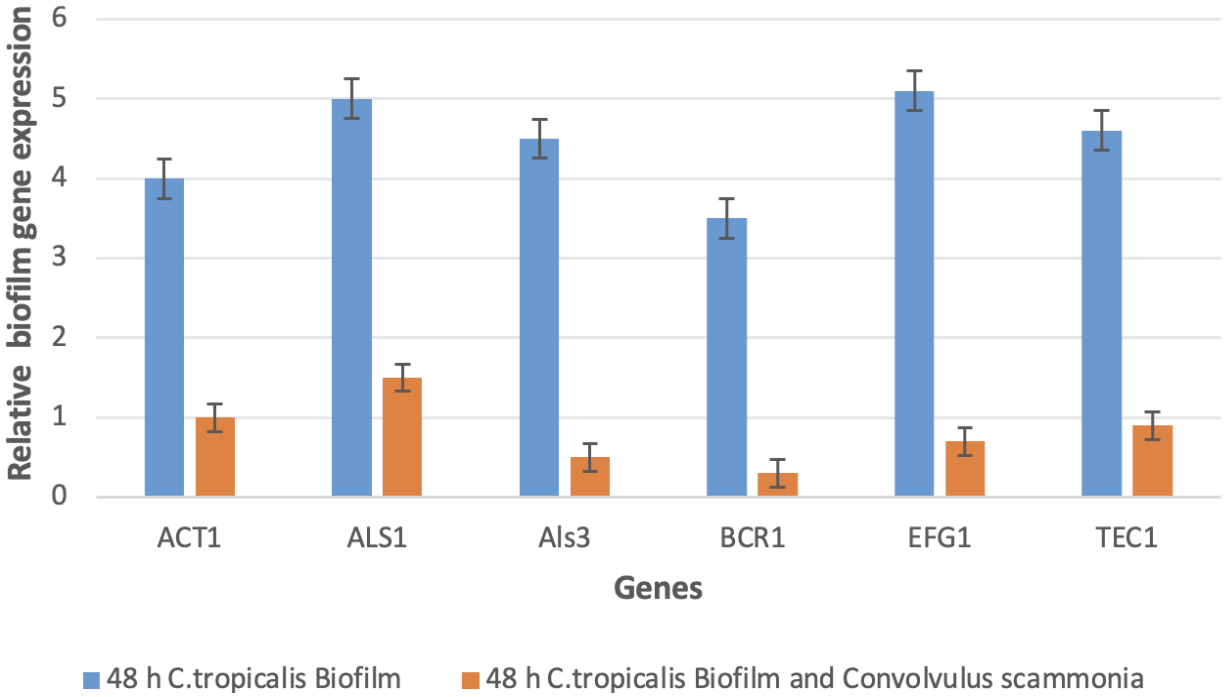

Figure 5: *Relative expression of the implicated biofilm forming genes (ACT1, AlS1, AlS3, BCR1, EFG1, and TEC1) in Candida tropicalis during the biofilm experiment for 48 h and Candida tropicalis biofilm with Convolvulus scammonia using reverse transcription real-time PCR. (t= 12.0151, df = 10, standard error of difference= 0.302), p < 0.05 was considered statistically significant.*

Discussion

Some intrinsic biofilm-associated factors, including immune system avoidance, antibiotic resistance, and mechanisms of horizontal gene transfer in multispecies biofilms, make biofilm formation a crucial component of pathogenicity in bacterial and fungal diseases (Eix and Nett, 2020; Vitális *et al*., 2020; Pinto *et al*., 2021). *Candida* biofilms have been found to form on a variety of surfaces, including blood, mucosal surfaces, and majority of medical devices, which are inanimate objects that come into contact with patients' bodies (Salehi *et al*., 2020; Pinto *et al*., 2021; Ponde *et al*., 2021). These indicate that *C. tropicalis* is the most common microbial species forming biofilms even more than *C. albicans*.

Thus, the purpose of this study was to determine *C. scammonia* effect on the expression of several genes (*ACT1, AlS1, AlS3, BCR1, EFG1, and TEC1*) involved in the formation of *C. tropicalis* biofilms. This experiment also aimed to determine the MIC of *C. scammonia* required to inhibit the growth of *C. tropicalis* forming biofilms. To the authors' knowledge, this study is the first to employ these techniques to examine the biofilms of this *Candida* species throughout time, evaluate the precision of the biofilm formation evaluation, and determine the activity of *C. scammonia* affecting it.

These findings partially corroborate with previous studies showing that *C. arvensis* contains cadinene, carvacrol, and O-cymene (Salamatullah, 2022). Additionally, the current results support the literature's assertion that the *Convolvulus* contains oxygenated monoterpenes, oxygenated sesquiterpenes, and sesquiterpene hydrocarbons. It's interesting to note that numerous authors have examined the phytochemical makeup of *C. arvensis* and found that the main constituents were lipids, sugar derivatives of quercetin and kaempferol, coumarins, saponins, flavonoids, phenolic acids, tannins, alkaloids, lactones, and steroids or terpenoids (Salehi *et al*., 2020).

Importantly, *C. arvensis* organic extracts showed antifungal efficacy against *A. fumigatus*, *C. albicans*, *C. tropicalis*, *Geotrichum candidum*, *Microsporum canis*, and *Trichophyton mentagrophytes*, with MIC values ranging from 0.001 to 0.156 mg/ ml (Martins *et al*., 2014; Hrichi *et al*., 2022). Additionally, the antifungal results of this study are in line with those that reported that the extracts of *C. althaeoides* L. leaves with increasing polarity were effective against several dermatophytes (*i.e*., *Microsporum canis*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes*), with 10 % inhibiting percentages at 50 mg/ml. Notably, *C. althaeoides* L. extracts significantly inhibited *C. albicans* (Klotoe *et al*., 2021), which is inconsistent with the current findings. Additionally, the antifungal findings in this study were consistent with earlier research on the antifungal effects of volatile fractions of *C. althaeoides* L. root (Al-Rifai *et al*., 2017). Furthermore, numerous studies have shown that essential oils and extracts from *Convolvulus* plants significantly suppress the growth of a variety of fungal pathogens such as *Candida* species (Gupta and Fernandes, 2019).

The glycoproteins Als1 and Als3 of the cell wall of *C. tropicalis* are encoded by six biofilm-associated genes belonging to *ALS* gene family: *ACT1*, *BCR1*, *ALS1*, *ALS3*, *TEC1*, and *EFG1*. *ALS1* expression can be found in both yeast and hyphae, whereas *ALS3* transcription occurs only in germ tubes and hyphae (Galán-Ladero *et al*., 2019). Sessile cells in *C. tropicalis* exhibited higher levels of expression for *ALS1, ALS2,* and *ALS3* like genes, indicating their potential roles in biofilm formation (Galán-Ladero *et al*., 2019). In *C. tropicalis*, the *EFG1* ortholog showed a conserved effect on fungal filaments and biofilm formation (Mancera *et al*., 2015). According to *C. tropicalis* deletion mutants of *BRG1, TEC1, BCR1, EFG1*, or NDT80 homologs have significantly reduced biofilm biomass, indicating that these genes have conserved functions in biofilm formation (Tseng *et al*., 2020). *C. albicans*'s hyphae-specific gene expression is either fully or partially controlled by UME6, a common downstream target of the regulators *Efg1, Chp1*, and *Ras1* (Poon and Hui, 2023).

The results of various analyses techniques of profile metabolites such as hydrogen peroxide activity, protease stability, thermal stability, and using liquid chromatography-mass spectrometry to might assist on identifying the active components and elucidate their mechanisms of action. An additional constraint pertains to the fact that we chose only a small number of target genes for the gene expression analysis out of the hundreds of genes and numerous signaling pathways that comprise the *Candida* biofilm regulatory network (Finkel and Mitchell, 2011). Other well-known genes linked to biofilms were investigated by Nobile *et al*.,

(2006), including HWP1, which encodes adhesin in fungal cell wall necessary for the formation of biofilm and the negative regulator of filamentation NRG1.

Genomic-wide transcriptional profiling employing some technologies such as RNAsequence is a more efficient way to identify the elusive processes of *C. scammonia's* activity (Chong *et al*., 2018).

Conclusions and Recommendations

The current results showed that *C. scammonia* could inhibit the expression of several genes in biofilm-forming *C. tropicalis*, including *ACT1, BCR1, AlS1, AlS3, EFG1*, and *TEC1*. Its MIC was 20 % (w/v) and its MFC was 40 % (w/v). Strong antifungal efficacies have been demonstrated for *C. scammonia*, suggesting that it contains compounds that could be useful in treating *Candida* infections. If these findings may be used to the treatment of biofilm-associated candidiasis; however, more *in vivo* studies are highly recommended.

Novelty Statement

This study enhances our understanding to determine the impact of *C. scammonia* on six gene expressions (*i.e*., *ACT1, BCR1, EFG1, AlS1, AlS3,* and *TEC1*); known to be implicated in the development of *C. tropicalis* biofilms, through the analysis of fifty oral swabs obtained from children suffering from oral candidiasis and septicemia with oral thrush. These findings offer valuable insights into the genetic dynamics of *C. tropicalis* biofilms, underscoring the necessity for continuous monitoring to address the *C. scammonia* impact.

Author's Contribution

Mouna Akeel Hamed Al-Oebady: Investigation, validation of results, writing original draft, writing review & editing
Wafaa Ayad Al-Nuaimy: Investigation, writing original draft, writing review & editing
Noor Sami Al-Lebawy: Validation of results, writing original draft

Ethical approval
This study was approved by the ethical committee of Al-Muthanna University, Iraq, and the ethical approval code is 3102R5A5A2A11021350. Written consents of the participants were provided.

Funding source
None to declare.

Conflict of interests
The author have declared no conflicts of interest.

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