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Effect of *Hibiscus Sabdariffa* Extract on Growth of *Fusarium graminearum* and the Expression of *TRI4* and *FG08079* Genes of the Fungus

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Abstract

Introduction: *Fusarium graminearum* produces trichothecenes, such as deoxynivalenol and secondary metabolite butenolide, which cause profound health problems in humans. In this research, the effect of acetone extract of *Hibiscus sabdariffa* is evaluated on growth of *F. graminearum* and expression of *TRI4* and *FG08079* genes, which are involved in deoxynivalenol and butenolide biosynthetic pathways, respectively.

Methods: Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined. The expression of *TRI4* and *FG08079* genes were evaluated by teal-time PCR technique.

Results: The MIC and MFC for acetone extract of *Hibiscus sabdariffa* against *F. graminearum* were 200 mg/mL and 400 mg/mL, respectively. Expression of *TRI4* and *FG08079* genes were significantly decreased by the acetone extract of red tea.

Conclusion: The results showed that acetone extract of *Hibiscus sabdariffa* has inhibitory and fungicidal effects on *F. graminearum* and is effective in reducing the expression of *TRI4* and *FG08079* genes, which play important roles in deoxynivalenol and butenolide production.

Keywords: Butenolide, Deoxynivalenol, *Fusarium graminearum*, Medicinal plant, Real-time PCR.

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Introduction

Fusarium graminearum produces trichothecenes including deoxynivalenol, nivalenol, and 3,7,15-trihydroxy-12,13-epoxytrichothec-9-ene, which cause profound health problems in humans and animals.[1] Structurally, deoxynivalenol is a polar organic compound, and its chemical name is 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-on (Figure 1).[2]

Deoxynivalenol has toxicity on animals and humans and causes vomiting, so it has been given the name vomitoxin.[4,5] Butenolide, also known as 4-hydroxy-2-butenoic acid gamma-lactone (Figure 2), is a secondary metabolite produced by several *Fusarium* species and is co-produced with deoxynivalenol on cereal grains throughout the world.[6] The *Fusarium* secondary metabolite, butenolide, suppressed trichothecene-mediated immune responses in human colon epithelial cells.[6]

The *TRI4*[7] (encodes cytochrome P450 monooxygenase) and *FG08079*[8] (4-acetamido-4-hydroxy-2-butenoic acid gamma-lactone) genes are associated with production of deoxynivalenol and butenolide, respectively.

Hibiscus sabdariffa L. as a medicinal plant has been proved to have antimicrobial activity.[9-16] Sepals of *H. sabdariffa* show the highest levels of water-soluble antioxidants, and its seeds are a good source for lipid-soluble antioxidants, particularly gamma-tocopherol.[17] This study's objectives were to assess the effects of acetone extract of red tea on *F. graminearum* growth, as well as the expression of *TRI4* and *FG08079* genes, which are involved in deoxynivalenol and butenolide biosynthetic pathways, respectively, in this fungus.

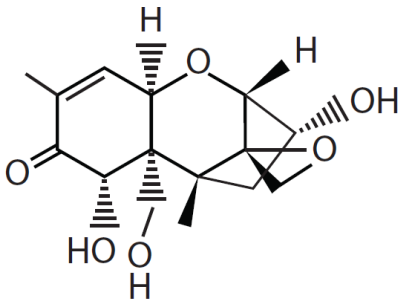

Figure 1. Chemical Structure of Deoxynivalenol.[3]

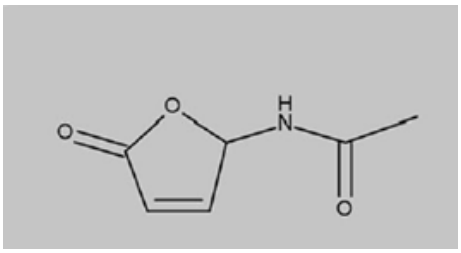

Figure 2. Chemical Structure of Butenolide.[6]

Materials and Methods

Plant Material and Extraction Procedure

Flowers of *Hibiscus sabdariffa* were collected from the Sistan region, Iran. Acetone extract of was prepared using the method described by Jahani et al.[18]

Growth Inhibitory Determination

Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were measured by a microplate method[19,20] and the method described by Espinel-Ingroff et al,[21] respectively. Concentrations tested in MIC were 50, 100, 200, and 400 mg/mL. All the experiments were repeated three times.

Effect of Acetone Extract of Hibiscus sabdariffa on Gene Expression of TRI4 and FG08079

Fusarium graminearum IRAN 124C was cultured in Potato Dextrose Broth medium in the presence of acetone extract of red tea with a concentration of 200 mg/mL and stored for three days at 25°C.[22,23] RNA extraction from mycelia of *F. graminearum* was performed by Ribospin™ plant kit (GeneAll Biotechnology, Seoul, Korea). ExcelRT™ Reverse Transcription Kit (SMOBIO, Taiwan) was used to synthesize the first cDNA string. The desired genes' expressions were measured using Eva Green fluorescent dye (Solis BioDyne, Riia, Estonia) and Real-time PCR technique. Design of primers (Table 1) was carried out by using Primer Express software v3.0 (Applied Biosystems). The polymerase chain reactions included an initial degradation step at 95°C for 15 minutes followed by 40 cycles of 95°C for 20 seconds, 61°C for 20 seconds, and 72°C for 30 seconds. Gene expression experiment was performed for three biological replicates of treated and untreated samples. *EF1A* is used as the reference gene,[24] and gene expression changes were measured by REST software.[25]

Statistical Analysis

The MSTAT-C software, version 1.42 used to analyze the data, followed by utilizing the student t-test to compare the data (mean) ($P < 0.05$).

Results

Effect of Acetone Extract of Hibiscus sabdariffa on Fusarium graminearum Growth

The effects of various concentrations of acetone extract of red tea on Growth of *F. graminearum* after two days of incubation at 25°C have been shown in Figure 3. The growth rate of the fungus decreased with increasing the concentration of acetone extract of, confirming its growth inhibitory effects on *F. graminearum* by the acetone extract. The MICs of acetone extract of was 200 mg/mL. The MFC of the acetone extract of was 400 mg/mL.

Effect of Acetone Extract of Hibiscus sabdariffa on the Expression of TRI4 and FG08079 Genes

Optimal conditions were provided so that no nonspecific amplification could occur during the reaction, which was validated by melt curve analysis (observing a single melt peak) (Figure 4). Amplification curves for the target genes have been shown in Figure 5.

The results of *TIR4* gene expression in the samples treated with acetone extract of *Hibiscus sabdariffa* (200 mg/mL) and untreated (control) *F. graminearum* have been shown in Figure 6. After treatment with acetone extract of *Hibiscus sabdariffa*, a decrease was observed in *TIR4* gene expression. The results showed a significant difference in the expression of the *TIR4* gene between treated and untreated samples at the level of $P < 0.05$.

The results of *FG08079* gene expression in the samples treated with acetone extract of *Hibiscus sabdariffa* (200 mg/mL) and untreated (control) *F. graminearum* have been shown in Figure 6. After treatment with acetone extract of *Hibiscus sabdariffa*, a decrease was observed in

Table 1. Nucleotide Sequences of the Primers Used in the Present Study

Genes	Forward Primer (5'–3')	Reverse Primer (5'–3')	Amplicon Length (bp)	Accession Number	Reference
TRI4	GATCGAGGCACAACAGAAGG	GCTCAATGTCGTAAGCTCGC	146	EF685280	this study
FG08079	TCGCGATACTGACGTGGTTG	TGGTCCACTCTTTGCACGTC	140	CD456573	this study
EF1A	TCACCGACTACCCTCCTCT	TCGACGGCCTTGATGACAC	82		26

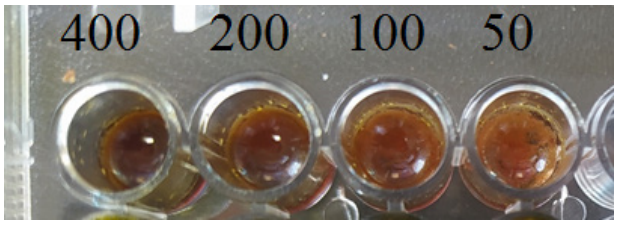

Figure 3. The Level of *Fusarium graminearum* Colonies in Different Concentrations of the Acetone Extract of Red Tea.

FG08079 gene expression. The results showed a significant difference in the expression of the *FG08079* gene between treated and untreated samples at the level of $P < 0.05$.

Discussion

In this research, the antifungal activity of acetone extract of *Hibiscus sabdariffa* investigated on growth of *Fusarium graminearum* and expression of *TRI4* and *FG08079* genes of the fungus. These genes are involved in deoxynivalenol and butenolide biosynthetic pathways respectively. The results of a research showed that extract of *H. sabdariffa* inhibited *in vitro* biofilm formation capacity of *Candida albicans* isolated from patients with recurrent urinary tract infections.[12] Goussous et al[9] investigated the antifungal activity of several medicinal plants against *Alternaria solani*. Their results showed that the extracts of *H. sabdariffa* and *Majorana syriaca* were most effective by completely inhibiting spore germination and mycelial growth at 8–10% concentrations. The extracts of white, green, red, and black teas also inhibited the growth of several bacteria (*Acinetobacter baumannii*, *Enterococcus faecalis*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Serratia marcescens*, *Saprophyticus staphylococcus*, and *Streptococcus pneumonia*).[11] However, different concentrations of *H. sabdariffa* calyx extract showed no significant effect on the growth of *Aspergillus flavus* (SQU 21) and *A. parasiticus* (CBS 921.7) strains, and inhibition of aflatoxin B1 production by *H. sabdariffa* calyx extract ranged between 91.5%–97.9% and 87.1%–93.3% for *A. flavus* and *A. parasiticus* strains, respectively.[10]

In the present study, we observed that the acetone extract of *Hibiscus sabdariffa* reduced the *TRI4* and *FG08079* genes' expressions. The *TRI4* gene of *Fusarium* encodes a key multifunctional cytochrome P450 monooxygenase that participates in four consecutive oxygenation steps in the biosynthesis of trichothecenes.[7] In a research by Jiao et

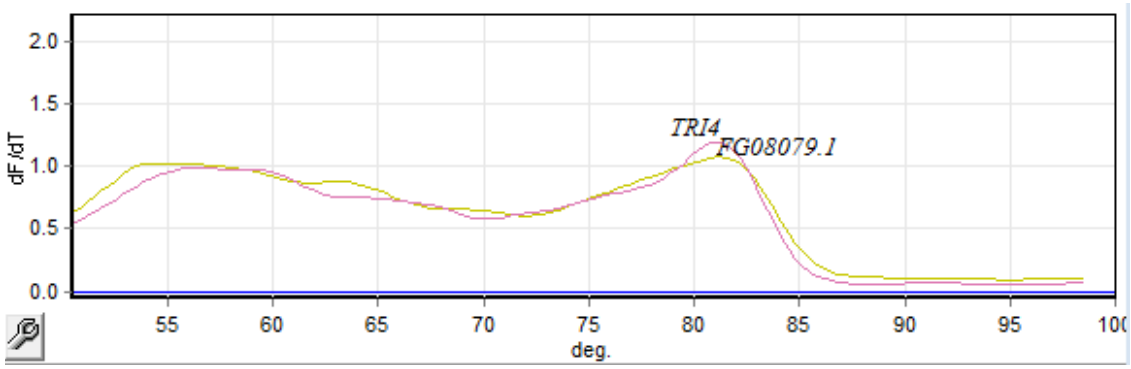

Figure 4. Analysis of the Melting Curve for *TRI4* and *FG08079* Genes. Each peak represents the melting temperature of a PCR product (pink curve: *TRI4*; brown curve: *FG08079*).

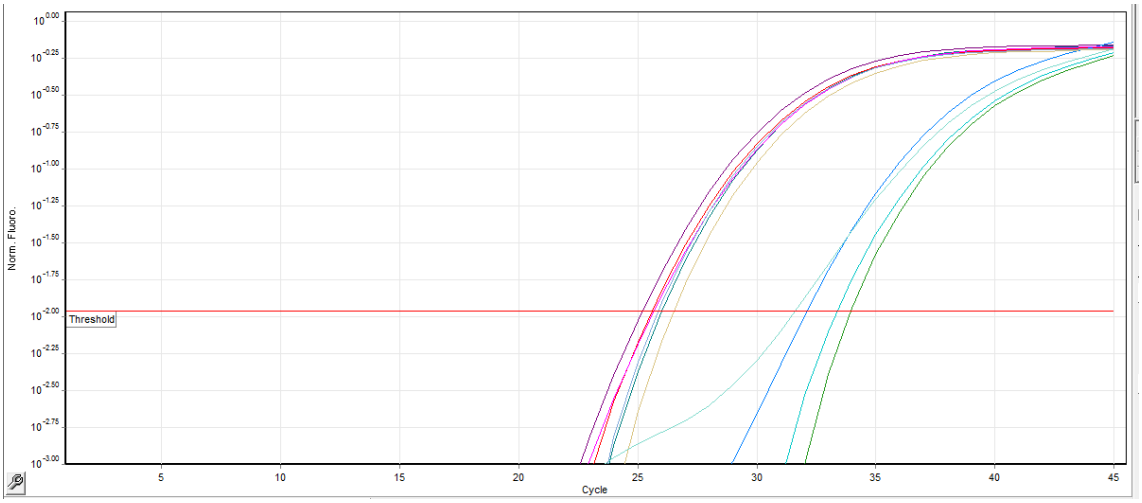

Figure 5. Amplification Curves for the Target Genes.

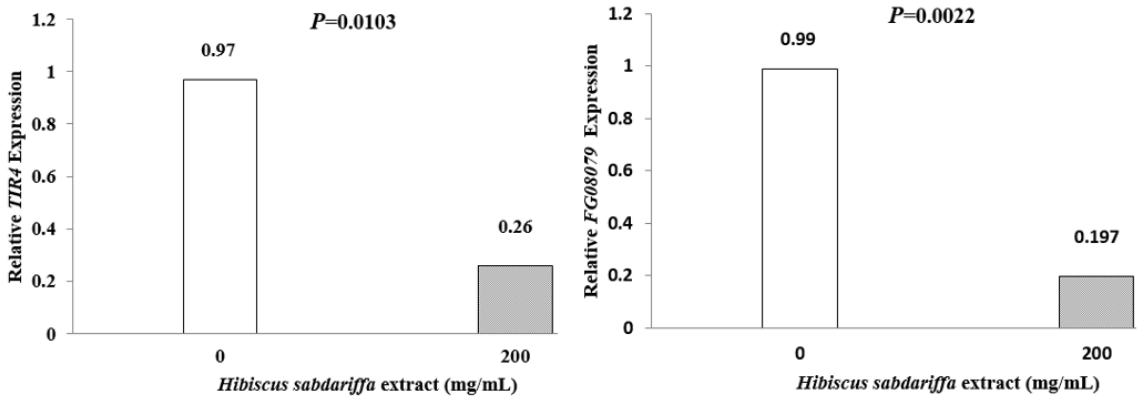

Figure 6. Levels of mRNA Expression of the *TIR4* and *FG02398* Genes in the Treated (200 mg/mL) and Non-Treated (Control) Samples of *Fusarium graminearum*. Each sample is normalized for the amount of the template based on the expression level of *EF1A*. Significant differences were observed for mRNA levels of each of the genes between treated and non-treated fungi ($P<0.05$).

al,[27] expression of *TRI4* and *TRI5* genes were upregulated in the sucrose-containing medium and trichothecene accumulation in this medium was not repressed by the addition of glucose. Yörük et al[28] also showed that the supplementation of H_2O_2 at different pH and temperature values decreased the expression of *TRI4* gene in the *F. culmorum* F15 isolate. Findings of Ponts et al[29] showed that in catalase-treated cultures of *F. graminearum*, deoxynivalenol and 15-acetyldeoxynivalenol accumulation reduced, and *TRI4*, *TRI5*, *TRI6*, *TRI10* and *TRI12* gene expressions significantly down regulated. Therefore, the protein product of *FG08079* is a cytochrome P450 and has low identity with P450, which is involved in trichothecene biosynthesis.[8] This is consistent with the results of Yörük et al,[30] showing that *FG08079* transcripts were present in five *F. graminearum*, five *F. culmorum*, and 10 *Fusarium* isolates that produced butenolide under culture conditions.

Conclusion

In general, the acetone extract of *Hibiscus sabdariffa* can have inhibitory and fungicidal effects on *F. graminearum* and reduce *TRI4* and *FG08079* genes' expression, which are involved in deoxynivalenol and butenolide biosynthetic pathways, respectively. It is suggested to assess the effects of *Hibiscus sabdariffa* extract on other genes involved in deoxynivalenol and butenolide biosynthetic pathways.

Ethical Approval

Not applicable.

Competing Interests

The authors have no conflict of interest to declare.

Authors' contribution

The authors contributed equally to this research.

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