Weakening of Virulence Factors and Biofilm in
Salmonella Typhi by Medicinal Plants Extracts

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(Salmonella typhi), is a serious global health threat because it causes typhoid fever, a severe systemic infection. According to the World Health Organization, millions of cases of typhoid are recorded annually, and thousands of people die from it. To combat this pathogen, new medications are required. The current study aims to study the ability of medicinal plants (thyme and cinnamon) to modulate the properties of Salmonella typhi isolates instead of killing them. The plants were extracted with the help of solvents (ethanol and ethyl acetate) and to find out the minimum inhibitory concentration, the different concentrations were used. The biofilm and expression of genes (invA & fliC) of the bacterium were studied when exposed to sub-inhibitory concentrations of the plant extracts. MIC values ranging between 20-25 mg/ml and 10-15 mg/ml for ethanol and ethyl acetate extracts of Thyme respectively. While the MIC values of cinnamon were 18-25 and 10-15 mg/ml for both ethanol and ethyl acetate extracts respectively. The examinations revealed a significant decrease in the composition of biofilms by isolates when treated with SICs from plant extracts. The transcription expression profile of invasion (invA) and flagellar (fliC) genes were downregulated when treated with the plant extracts. The findings indicate that both thyme and cinnamon extracts may have promising activity against the biofilm and virulence of S. typhi. Thus, they could be used as potential as an antibacterial drug.

Keywords: Biofilm; Cinnamon; Gene expression; Salmonella typhi; Thyme.
(SGIs), and Salmonella pathogenicity islands (SPIs). Salmonella spp. biomarker pathogenicity has been widely researched. The invasion gene (invA), a biomarker for the recognition of Salmonella spp., has been extensively investigated for its capacity to increase pathogenicity, and the fliC gene encodes for the synthesis of H (flagellar) antigen, that serves as the basis for Salmonella classification under the Kauffman-White scheme. However, the ability to treat and even prevent infections due to Salmonella has remained underdeveloped due to antibiotic resistance. Cell-to-cell communication in bacteria known as quorum sensing (QS) system is a mechanism involving various cellular functions, especially those related to bacterial virulence, such as adhesion, invasion, biofilm formation, and bacterial motility. As a result, inhibiting the communication system may be a novel treatment tactic for Salmonella infection that is not dependent on antibiotics. In response, the scientific community is working hard to find natural antimicrobial drug replacement sources that, ideally, do not promote the emergence of resistance. In this perspective, plants are viewed as an essentially unlimited supply of bioactive components, and various methods have been used to utilize their use as antibacterial agents. Amongst these, thyme (Thymus vulgaris) has been well-researched for its antibacterial, antioxidant, and anti-inflammatory effects and is one of the most promising nature-identical substances that has already been approved as a food addition. Additionally, in recent years, cinnamon (Cinnamomum verum) and its compounds, primarily cinnamaldehyde, have been studied for their capacity to inhibit microbial biofilm against a variety of bacteria. It could be used in place of antibiotics to treat infections brought on by biofilms. In this regard, this experimental study aimed to examine the effect of sub-inhibitory concentrations of ethanol and ethyl acetate extracts of thyme and cinnamon on invA and fliC genes expression using Real-time PCR and to study their effect as anti-biofilms on S. typhi strains.

MATERIALS AND METHODS

Plant extraction process
Thymus vulgaris (thyme) leaves were brought from Akre farms in Kurdistan, Iraq, and the Cinnamomum verum (cinnamon) barks were purchased from the market in Erbil city, Iraq. Both plants were identified by the Herbarium of Science, Salahaddin University-Erbil, Iraq. Methods described previously by [13] were used to extract the plant materials. In short, the extracting of the powder of the plant was by the method of maceration with the help of solvents (ethyl acetate and ethanol). The powder of plant (10.0 g) was extracted by stirring three times at regular intervals using 100 mL of the solvents over three days at RT after being filtered through a dual layer of muslin material and filter paper (Whatman no. 1). To obtain the crude material of each fraction vacuum evaporator was used to remove the chemical solvents. The extracted fractions were then kept at -20°C and dissolved in dimethyl sulfoxide (10% DMSO, Merck, Germany) and sterilized by membrane filter (0.45 μm) before use. To prevent their effect, solvents were used as control and blanks in all experiments of this study.

Samples sources and Specimens collection
Five non-duplicate isolates have been collected from S. Typhi from blood samples of patients who suffering from typhoid fever and were transferred to the General Hospital in Iraq. The samples were firstly inoculated onto MacConkey and Salmonella Shigella agar media (acuemedia, Neogen, USA) and incubated at 37 °C overnight. The distinct colonies were identified as S. typhi through various biochemical and conventional diagnostic tests as described by [14]. The VITEK 2 automatic system (Biomerieux, France) was used for further identification of isolates. The susceptibility of the tested bacteria to different antimicrobials (Ceftazidime, Cefepime, Amikacin, Gentamicin, Piperacillin, Pipercillin/Tazobactam, Aztreonam, Ciprofloxacin, Levofloxacin, Imipenem, Meropenem, Netilmicin, Tobramycin, Tigecycline, Tetracycline, Trimethoprim/Sulfamethoxazole) was determined and the most two resistant isolates were selected for the experimentations through the current study. The individual colonies were stored in one mL Tryptic Soy Broth (TSB) (Oxoid) containing 30% glycerol at -70°C for additional study. An ATCC strain of S. typhi (6539) was bought from Medya Diagnostic Center to be used as a control throughout the study.

Minimum Inhibitory Concentrations and Sub
Inhibitory Concentrations (MICs & SICs)

The broth microdilution method was applied to determine the MICs of the plants extracts against multidrug-resistant (MDR) S. typhi isolates. Ten µL of S. typhi cells at stationary phase adjusted to OD550 0.5 and transferred to 100µL NB supplemented with a range (1–30 mg ml⁻¹) of extracts studies in the wells of a polystyrene microtitre plate (MTP). After one day (24 hrs.) of incubation at 37 °C, the MIC was calculated as the lowermost concentration at no observation of growth occurred. The concentration below MICs were considered sub-inhibitory and were used to study the anti-virulence and anti-biofilm activity in the isolated S. typhi strains. Three biological samples were examined separately.

Sub-MIC effect of plant extracts on the biofilm of S. typhi isolates

PCB (Polyvinyl Chloride Biofilm) formation method was used to quantify the biofilm in the bacterial isolates exposed to the SICs of the plant extracts. Overnight cultures of S. typhi were re-suspended in a sterile NB media in the presence and absence of SICs of the studied extracts and incubated at 37 °C in a stationary state for about 24 hours. Then the liquid cultures were removed, and the wells were washed three times with phosphate buffer saline (PBS), dried out and stained with a violet crystal suspension (1%). The excess dye was washed off with distilled water and the amount of dye adherent to the solubility in ethanol was determined (95%). The adhesion ability of the abiotic surface was measured by reading the absorption of the colored suspension by the ELISA reader (Epson, Biotek, UK) with a wavelength of 490 Nm. Separate analyses of three biological samples were conducted, and the standard error was determined.

RNA extraction and quantification of virulence-related genes

Real-time PCR was used to estimate the effect of the plants extracts at SICs value at the level of expression of virulence genes (invA & fliC). Total RNA was extracted from both untreated bacteria which were used as control and bacteria exposed to various plants extracts according to the instructions provided by the manufacturer (total RNA kit, Favorgen Biotech, Taiwan). c-DNA was synthesized through reverse transcription of the isolated RNA using AddScript cDNA synthesis kit afforded by the manufacturer protocol (addbio, Koria). RT-PCR reactions were performed using RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) in the PCRmax Eco 48 RT-PCR system. The primers used for virulence genes quantification were as follows (sense and antisense): fliC d: 5' actcaggcttcccgtaacgc3' & 5'ggctatatgtccttatcgg3'17; and invA, 5' GTGAAATTATCGCCACGTTCGGGCAA3' and 5' TCATCGCACCGTCAAAGGAACC3'18. The candidate genes were analyzed by qPCR and ÄÄCt method19 to calculate the results.

![Fig. 1. Decrease of S. typhi biofilm by SICs of Thymus vulgaris extracts measuring at 490 nm absorbency. Data are expressed as the average±SE. The significant of all data is at P< 0.0001. ET, ethanol extract of thyme; eT, ethyl acetate of thyme](image-url)
Statistical analysis

GraphPad Prism 8.0 software was used to analyze the obtained results. The two-way contrast analysis (ANOVA) method was used for multiple comparisons. Data presented as the mean±SE.

RESULTS

Different concentrations of ethanol and ethyl acetate extracts of both thyme and cinnamon were examined on S. typhi isolates, as shown in Table 1, the MIC for ethanol extracts of thyme was 20 and 25 mg / mL versus different isolates while the MIC for ethyl acetate thyme extracts was 25 mg / mL for the same isolates. The MIC for thyme extracts of cinnamon was 18 and 25 mg / mL and the MIC for cinnamon extracts of ethyl acetate extract was 10 and 14 mg / mL as shown in Table 2. Data below the MICs are considered SICs and used for biofilm and expression experiments.

The plant extracts have a role in decreasing biofilm formation in S. typhi isolates after treating isolates with SIC of thyme ethanol extracts the biofilm formation decreased significantly as shown in Table 1.

Table 1. Minimum Inhibitory Concentrations & Sub-MICs of Thymus vulgaris extracts against MDR S. typhi isolate

<table>
<thead>
<tr>
<th>Bacterial Isolates</th>
<th>MIC (mg/ml)</th>
<th>Sub-MIC (mg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Ethanol</td>
<td>Ethyl Acetate</td>
</tr>
<tr>
<td>ATCC</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>S1</td>
<td>25</td>
<td>14</td>
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<tr>
<td>S2</td>
<td>25</td>
<td>14</td>
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Table 2. Minimum Inhibitory Concentrations & Sub-MICs of Cinnamomum verum extracts against MDR S. typhi isolates

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>MIC (mg/ml)</th>
<th>Sub-MIC (mg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>Ethanol</td>
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<td>S2</td>
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Fig. 2. Decrease of S. typhi biofilm by SICs of Cinnamomum verum extracts measuring at 490 nm absorbency. Data are expressed as the average±SE. The significant of all data is at P<0.0001. EC, ethanol extract of cinnamon; eC, ethyl acetate extract of cinnamon.
shown in figure 1. As shown in figure 2 the biofilm formation decreased significantly by treating the S. typhi isolates with SIC of cinnamon extracts.

The expression of flagellar (fliC) gene of S. typhi isolates were measured by RT_PCR as shown in figure 3 all plant extracts down regulated fliC-d gene in different ratio.

Figure 4 shows the folds of invasion (invA) gene expression change after treatment of S. typhi isolates with plant extracts, the results indicate that all plant extracts have the downregulating effect against S. typhi isolates.

**DISCUSSION**

Infections due to bacteria have been recognized as significant contributors to the aetiology of a variety of human diseases. The advent of MDR organisms has sparked research into quorum-sensing modulation strategies as an alternative to conventional antibiotic therapies for attenuating pathogenicity.

S. typhi infections have grown to be a dangerous problem in hospital-acquired infection, especially in individuals with weakened immune systems. This bacterium is one of the top priority pathogens worldwide according to WHO. Therefore, a broad range of approaches is now being explored in order to generate distinct anti-infective strategies.

This study proves the impact of thyme and cinnamon extracts on the invA and fliC expression and the development of microbial biofilms in S. typhi strains recovered from typhoid fever patients. All of the extracts tested pose a significant antimicrobial activity by retarding or minimizing Salmonella strains biofilm formation by decreasing virulence gene expression when in vitro analyzed. However, the sensitivity of the strains has changed mainly depending on the plant and the type of extracts.
The observed inhibitory activity (Table 1 and Table 2) was indicated by extracts of thyme and cinnamon in different concentrations. The data showed variation in the MIC among plant extracts; the ethanol and ethyl acetate extracts of cinnamon showed the lowest MIC values (18 and 10 mg/ml) respectively against the ATCC strain, together with the ethyl acetate extract of cinnamon (14 mg/ml), against the S1 and S2 strains.

The outcomes are in line with earlier researches which also observed a decline in the bacterial biofilm under the presence of sub-inhibitory concentrations of thyme and cinnamon extracts, the quantitative biofilm measurements were significantly reduced to weak or no biofilm formation.

It has been demonstrated that several plants can effectively stop the development of biofilms in a variety of bacteria, including S. typhi. The present study’s findings suggested that cinnamon ethyl acetate extract may possess

the potential to inhibit the development of biofilms and change their phenotype from moderate to weak and negative biofilms (Fig. 2). Complex mechanisms affect bacteria pathogenic by changing cell wall bacterial permeability, leading to osmotic shock and cytoplasm leakage. The antimicrobial mechanism of extracting thyme and cinnamon, based on the main constituents of essential oils, such as thymol, carvacrol, and cinnamaldehyde, depends on their ability to inhibit bacterial activity by damaging the cell membrane, change the profile of lipids. Inhibition of ATPases, cell division, membrane reservoirs, motility, and biofilm formation, via anti-quorum sensing effects. In particular, these components disintegrate the outer membrane of bacteria (Gram-negative), which release lipopolysaccharides that increase the permeability of the cytoplasmic membrane to ATP12. The bacteria of Gram-negative that still presents a significant human public health and economic problem is Salmonella spp.32,33.

The transcription levels of virulence genes (invA and fliC), under thyme and cinnamon extracts SIC stress were determined by RT-qPCR analysis in the current work. The bacterial strains showed drastically reduced gene expression (Fig. 3 and 4).

Based on the fold change technique, S. typhi strains treated with SICs of thyme and cinnamon extracts showed down-regulation in the fliC gene (involved in the QS path for biofilm development) expression and noticeably inhibited to 9-folds in the cinnamon ethyl acetate extracts, and were blocked at S1 and S2 strains in particular. This emphasized that the extracts reduced the Salmonella virulence by suppressing the QS systems activity. Since host compartment-specific flagellar regulation is important to Salmonella virulence. Our results agree with previous researchers’ conclusions34,35.

On the opposite, invA gene expression was observed differentially among the S. typhi strains after exposure to thyme SIC. The upregulation in ethanolic thyme extract was mainly confirmed on strain S2 followed by thyme ethyl acetate extract. While a significant decrease in regulation has been shown by ethanol cinnamon extracts and ethyl acetate in which some strains have been banned.

The invA gene is needed for full Salmonella virulence because it improves internalization, which is required for deeper tissue invasion36. Thus, thyme and cinnamon can inhibit biofilm formation by affecting gene transcription, implying that these genes are required for S. typhi strains to infect the host37.

Earlier studies and our findings line up with each other. Since the synergistic interactions between an extract’s active ingredients are one of the prime reasons for its ability to preclude the growth of bacteria37, notably Salmonella38,39.

Considering the natural antibacterial agents in thyme and cinnamon combined with their pharmacokinetics such as anti-inflammatory, antioxidant, antitumor, and neuro-protective properties. In addition to their topical applications as a constituent of personal hygiene products, which have no cytotoxicity for human consumption. Regardless, excessive long-term use is not advised because current toxicological data show that undesirable side effects may occur at higher doses of thyme and cinnamon that appear in the studies of pharmacological40.

CONCLUSION

Thyme and cinnamon extracts have shown promising activities against isolates bacteria in both bacterial virulence and the formation of biofilm. The results of the biofilm inhibition examination indicated that the studied plant extracts are able to show anti-biofilm activity against S. typhi. Moreover, we concluded that the study of extracts of plants regulates both the invA and fliC genes. Future analysis could be carried out in order to search for the most effective components of the examined plants. Thus thyme and cinnamon are used as potential antimicrobial drugs.

ACKNOWLEDGMENT

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Conflicts of Interest

No conflict of interests is declared.

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