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*Original Research*

# The Lebanese *Trifolium purpureum*: An Effective Source to Fight *Escherichia coli* and Associated Biofilms

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**Abstract**

**Background:** This study aimed to investigate the antibacterial and antibiofilm activities of *Trifolium purpureum* against *Escherichia coli*. **Methods:** *T. purpureum* Loisel. was collected from the mountains of western Lebanon. The plant parts were then extracted by maceration in 70% ethanol and water. The plant fractions were screened for secondary metabolite classes. The antibacterial activity of the extracts was determined using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Antibiofilm activity was assessed using a colorimetric method, and the effects of *T. purpureum* extracts on the expression of genes involved in biofilm formation in *E. coli* were assessed by real-time quantitative polymerase chain reaction (qPCR). **Results:** Coumarins, tannins, volatile oils, and phenols were absent in *T. purpureum*; resins were detected only in the flowers, alkaloids were detected in all plant parts except the stems, and saponins, terpenoids, and flavonoids were found in all plant parts. Regarding antibacterial activity, although all extracts had measurable MIC values, only the whole-plant water extract exhibited a bactericidal effect. Furthermore, the water and ethanol extracts exhibited variable biofilm-prevention activity, with minimum biofilm prevention concentration (MBPC) values ranging from 30 to 68.9 mg/mL. Biofilm eradication activity also varied, with minimum biofilm eradication concentration (MBEC) values ranging from 0.13 to 49.65 mg/mL corresponding to 47.3–66.8% eradication. Finally, the whole-plant water extract suppressed the expression of *fimA*, *fimH*, and *flu* in both planktonic cells and cells within established *E. coli* biofilms. **Conclusions:** Our results demonstrate that *T. purpureum* is rich in secondary metabolites and has notable antibacterial and antibiofilm potential against *E. coli*, especially at the molecular level. These findings suggest that *T. purpureum* may be a promising candidate for use as an alternative antibacterial and antibiofilm agent.

**Keywords:** *Trifolium*; *Escherichia coli*; anti-bacterial agents; biofilms; microbial sensitivity tests; plant extracts; secondary metabolites

## 1. Introduction

Every disease has a cure; however, the question is where and how to find it. Infectious diseases are increasing and are responsible for 50,000 deaths worldwide each day. Moreover, infectious diseases account for one-third of all deaths [1,2].

Nosocomial infections are the most dangerous. This is due to two main reasons: (i) the association of these infections with resistance to antibiotics [3,4,5] and (ii) the ability of the causative agents to form biofilms, which confer "infinite" resistance to antimicrobials [6,7,8,9,10,11,12]. Despite the seriousness of drug resistance, the world is now facing the emergence and spread of an even more serious problem that constitutes a global public health threat: multidrug resistance (MDR), which is defined as resistance to more than one antimicrobial [13,14,15].

Many factors may contribute to the development and spread of MDR, including (i) the overuse or misuse of antibiotics [2,3], (ii) the transfer of patients infected with MDR bacteria [4], (iii) the exchange of MDR bacteria between public health establishments [16,17], (iv) mutations or genetic recombination of resistance genes [5], and (v) the biofilm lifestyle of bacteria [18,19].

Microorganisms tend to live in biofilms. Bacteria form biofilms after adhering to a surface and producing extracellular polymeric substances that encase the cells and protect them against antimicrobials. Biofilm resistance to antibiotics, which is a multifactorial process, contributes to the persistence of infections, 65% of which are associated with indwelling medical devices [20,21,22].

Regarding the impact of MDR and biofilms on human health and treatment costs, it is estimated that the spread of MDR will lead to 10 million deaths per year and reduce global production by USD 100 trillion by 2050 [23,24,25]. Therefore, finding solutions to manage MDR and biofilm-associated infections through the discovery and characterization of new compounds as alternatives to antibiotics and antibiofilm agents is an urgent task [26,27,28].

Since ancient times, herbs, natural products, and plants have been widely used to treat different infections

and disorders. This is due to the associated high efficacy, low cost, lack of side effects, and wide availability worldwide [29,30]. Furthermore, a significant portion of the global population and many medical products depend on these natural products, particularly medicinal plants [20]. Medicinal plants are rich in secondary metabolites that underlie the associated biological activities, including antimicrobial and antibiofilm properties [31,32].

*Trifolium* species are used in Turkish folk medicine as agents for dermal wound healing and as expectorants, antiseptics, sedatives, and remedies to reduce rheumatic pain [29].

The flowering plants of the genus Trifolium are commonly known as clover. Clover species include red clover (*Trifolium pratense L.*), white clover (*T. repens*), and purple clover (*T. purpureum*).

Among Arabs in the Middle East, traditional herbalists use the leaves and roots of *T. purpureum* as tranquilizers and antispasmodics to treat emotional tension and strain. The pharmacological activities attributed to *T. purpureum* include estrogenic, chemoprotective, and cardiotonic effects, as well as potential activity against prostate cancer (PC).

*Escherichia coli* is one of the five main pathogens that cause nosocomial infections, especially those associated with medical devices. *E. coli* is a major cause of urinary tract infections (UTIs), accounting for more than 80% of all UTIs worldwide [31,33,34]. Therefore, this study aimed to investigate the antibacterial and antibiofilm activities of the Lebanese plant *Trifolium purpureum* against *E. coli*.

## 2. Materials and Methods

### *2.1 Plant Profile*

*Trifolium purpureum* Loisel. – Purple clover.

### *2.2 Taxonomic Classification*

Full lineage: cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; Mesangiospermae; eudicotyledons; Gunneridae; Pentapetalae; Rosids; Fabids; Fabales; Fabaceae; Papilionoideae; Trifolieae; *Trifolium* [35].

### *2.3 Collection of Specimens*

The plant material of *T. purpureum* Loisel. was collected from Chkara in the Western Lebanon mountain range (1350 m a.s.l.).

### *2.4 Preparation of the Plant Material*

Before the extraction step, the collected plant material was rinsed with tap water, shade-dried, ground to a fine texture using a mechanical grinder, and sieved.

### *2.5 Plant Extracts*

*Trifolium purpureum* was divided into 4 parts: whole plant, flowers, leaves, and stems. Ground plant material from each part was then extracted separately with 70% ethanol and distilled water.

### *2.6 Preparation of the Crude Extracts by Maceration*

The powdered plant material was sequentially extracted by maceration in sufficient volumes of 70% ethanol and water for extended periods. First, 50–100 g of each ground plant part was soaked successively in sufficient volumes of the aforementioned solvents, or in a volume reaching 2 cm above the powder level in a beaker (1000 mL). Following the addition of each solvent, the containers were covered with aluminum foil to protect the light-sensitive compounds, and the mixtures were placed on an agitator at room temperature and then at 37 °C for 12 h. After the release of the solvent-soluble medicinal constituents from the plant, the solvent extracts were recovered by filtering the resulting liquefied mixtures. The extracts were concentrated to dryness using a rotavapor at 40 °C under reduced pressure, lyophilized (Christ Alpha 1-4 LD plus, Martin Christ, Germany) to a powder, and redissolved in water to achieve the desired concentrations. The residual mass was then pressed, dried, and weighed [36].

### *2.7 Phytochemical Screening*

For this test, aliquots from each fraction of the ethanolic and aqueous extracts of the plant were prepared and screened for common plant secondary metabolites, including tannins, resins, coumarins, saponins, alkaloids, phenols, terpenoids, volatile oils, and flavonoids, using qualitative phytochemical tests. The results were evaluated based on the response of the extract to these tests, primarily color change and/or precipitate formation [37].

### *2.8 Bacterial Strains, Media, and Reagents*

*Escherichia coli* (ATCC 35218), American Type Culture Collection (ATCC), Virginia, USA, was used in this study. *Escherichia coli* was preserved in glycerol at –80 °C and used as needed. Mueller Hinton broth (MHB) and tryptic soy agar (TSA) were prepared and autoclaved according to the manufacturer's instructions.

### *2.9 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Assays*

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined under sterile conditions using the methodology described by Sweidan et al. (2020) [38], with minor modifications. The MIC was defined as the minimum concentration required to produce no observable growth [38]. A 96-well microplate was used to prepare two-fold dilutions of the extracts in MHB. Each well was inoculated with a bacterial suspension to achieve a final concentration of 5

× $10^5$ CFU/mL. Positive controls consisted of wells containing no products. Wells without bacterial inoculum were used as negative controls. All plates were incubated at 37 °C for 24 h. The contents of wells, 100 µL aliquots, with no visible growth were plated onto TSA, and colony counts were performed after overnight incubation at 37 °C. The MBC was defined as the lowest concentration required to kill >99.9% of the bacteria (<5 CFU per plate).

### *2.10 Biofilm Formation Assay*

*E. coli* biofilms were formed using a conventional process developed by Wiegand et al. [39], with minor modifications. A bacterial suspension at 5 × $10^7$ CFU/mL in 100 µL of MHB was added to each well of a 96-well microplate, which was then incubated at 37 °C for 24 h. Wells lacking any extract served as positive controls for biofilm formation, and wells without bacterial inoculum served as negative controls. After incubation, the microplate contents were discarded, and the remaining attached biofilm matrix was fixed by heating at 80 °C for 1 hour. The microplates were then allowed to cool to room temperature. Afterward, 100 µL of 0.1% crystal violet was added to each well for staining, and the wells were rinsed after 10 min. After adding 100 µL of ethanol, the optical density (OD) was measured at 570 nm using a microplate reader (Tristar2 S, LB 942, Berthold, Germany). For all anti-biofilm assays, planktonic cell density was measured simultaneously. Biofilm mass was normalized to total bacterial growth to confirm that the observed reduction in biofilm formation was a specific effect on the biofilm lifestyle rather than a secondary result of growth inhibition.

### *2.11 Biofilm Prevention Activity and Determination of the Minimum Biofilm Prevention Concentration*

A total of 100 µL of MHB and 100 µL of extracts were added to the first well of the first column of a 96-well microplate, followed by 50% serial dilutions up to the tenth well. Each well then received a bacterial suspension to achieve a final concentration of 5 × $10^7$ CFU/mL, while wells without extract served as positive controls for biofilm formation. Wells without microorganisms were utilized as negative controls. The subsequent fixation, staining, and washing steps were performed as previously described in the biofilm formation assay section, and the optical density at 570 nm was measured. The minimum biofilm prevention concentration (MBPC) was defined as the lowest concentration that produced the greatest significant reduction in biofilm formation.

### *2.12 Biofilm Eradication Activity and Determination of MBEC*

Biofilm formation was conducted as described in the previous section of the biofilm formation experiment. After biofilm formation was fixed, successive dilutions of the extracts were prepared, and the plates were incubated at 37 °C for 18 h. Wells without product were used as positive controls for biofilm formation, while wells without microorganisms were used as negative controls. After incubation, the contents were discarded, and the wells were stained and washed before measuring the optical density at 570 nm. The minimum biofilm eradication concentration (MBEC) was defined as the lowest concentration required to achieve the greatest eradication [36,37]. To evaluate transcriptional changes before complete bacterial killing, RNA was isolated at an early exponential/growth phase (10 h incubation). Additionally, to rule out generalized transcriptional suppression caused by growth inhibition or bactericidal effects, gene expression was normalized to the internal reference gene 16S rRNA. The sub-MIC concentration (MBPC/2 = 16.05 mg/mL), which yielded minimal biofilm prevention (~13%), was included specifically to demonstrate selective gene repression independent of cell death.

### *2.13 Isolation of RNA and Real-Time Quantitative polymerase chain reaction (PCR)*

After 10 h of *E. coli* culture, with or without extracts, planktonic bacteria or bacteria embedded in biofilms were centrifuged at 5000 ×g for 5 min. The cells were washed once, and the cell pellet was incubated with 200 µL of lysis buffer (10 mM Tris, 1mM EDTA, 0.6% SDS) and proteinase K (1 mg/mL) at 37 °C for 30 min. A total of 1 mL of QIAzol lysis reagent was then added to the lysate, and the mixture was incubated at room temperature for 5 min. The subsequent steps of total RNA extraction were performed according to the QIAzol lysis reagent protocol. Extracted RNA was treated with RNase-free DNase reagent (Qiagen) to eliminate DNA contamination. Reverse transcription was conducted using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions (Bio-Rad Laboratories, Inc, Hercules, CA, USA). The primer sequences were designed using Primer3 software (Whitehead Institute, Cambridge, MA, USA). The primer sequences are shown in Table 1. Primer efficiencies for all target and reference genes were confirmed to be between 90% and 110%, and reaction specificity was verified by melt-curve analysis, which showed a single, sharp peak for each product. Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFX96 thermal cycler with an annealing and extension temperature of 60 °C (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The mRNA expression levels of target genes were normalized to the housekeeping gene *16S rRNA*. The $2^{-\Delta Ct}$ values for each gene were calculated and presented in the graph. All experiments were performed four times in triplicate. Results are reported as the mean of relative expression ± standard deviation. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA), with a one-way analysis of variance (ANOVA) followed by Dunnett's test.

**Table 1. Primer sequences of the amplified genes.**

| Primer | Sequence |
|---|---|
| *csgD* Forward | 5′-TGGACGATATCTCTTCAGGCTCT-3′ |
| *csgD* Reverse | 5′-CGCGGTACGGGTAATCTTCAGC-3′ |
| *pgaB* Forward | 5′-AATTCGACAAAACCCGGAGC-3′ |
| *pgaB* Reverse | 5′-TTCTGTGCAAACCAGGCTTC-3′ |
| *fimA* Forward | 5′-TAATGGTGGGACCGTTCACT-3′ |
| *fimA* Reverse | 5′-AAAACCGACAGCAGAACTGG-3′ |
| *fimH* Forward | 5′-CTTATGGCGGCGTGTTATCT-3′ |
| *fimH* Reverse | 5′-CTGCTCACAGGCGTCAAATA-3′ |
| *flu* Forward | 5′-GGAAGACGGTGAACAACGAT-3′ |
| *flu* Reverse | 5′-TGAGTGTGGTGTTGCTGACA-3′ |
| 16S rRNA Forward | 5′-GGGCTACACACGTGCTACAA-3′ |
| 16S rRNA Reverse | 5′-GTACAAGACCCGGGAACGTA-3′ |

### *2.14 Statistical Analysis*

Data on antibiofilm activity are presented as the mean ± standard deviation (SD) values of optical density. Each experiment was performed in quadruplicate. Statistical analyses were conducted using Prism 5.0. Comparisons with the positive control were made using a one-way ANOVA, followed by Dunnett's post hoc test. Statistical significance was defined at $p \leq 0.05$.

## 3. Results

### *3.1 T. purpureum Loisel. – Phytochemical Profile*

According to preliminary qualitative screening tests, coumarins, tannins, volatile oils, and phenols were absent from all plant parts. However, resins were detected only in the flowers. Alkaloids were present in all plant parts except the stems, whereas saponins, terpenoids, and flavonoids were detected in all plant parts. Simply, the whole plant, leaves, and flowers were found to contain saponins, alkaloids, terpenoids, and flavonoids, while the flowers of *T. purpureum,* in addition to containing these phytochemicals, also contained resins (Table 2).

Flavonoids, saponins, and terpenoids were detected in all eight fractions, indicating that these components were extracted from all of the parts of *T. purpureum* by both solvents used. Alkaloids were found in three fractions, whereas resins, ranking last, were present in only two of the eight fractions (Table 2).

### *3.2 Antibacterial Activity*

To assess antibacterial activity, the MIC and MBC of each extract were determined. The nature of the antibacterial effect was then classified based on the MBC/MIC ratio, which distinguishes bacteriostatic from bactericidal activity. An MBC/MIC ratio of ≤4 indicates bactericidal activity, whereas a ratio of >4 indicates bacteriostatic activity. For extracts whose MBC values could not be determined, the nature of their antibacterial activity was classified as undetermined. Among all extracts, only the whole distilled water extract exhibited a bactericidal effect against *E. coli* (Table 3). The activities of the other extracts remained undetermined because their MBC values could not be established.

### *3.3 Anti-Biofilm Activity*

#### 3.3.1 Prevention of Biofilm Formation

The preliminary results of the anti-biofilm prevention assay are shown in Figs. 1,2. Based on the associated efficacy, the extracts that showed significant biofilm prevention or eradication were classified into four groups: strong activity (above 80%), intermediate activity (between 60% and 80%), low activity (between 50% and 60%), and weak activity (below 50%). The prevention percentage was calculated as follows:

$$\%prevention = \left[\frac{OD\ (positivecontrol) - OD\ (treatedwell)}{OD\ (positivecontrol) - OD\ (negativecontrol)}\right] \times 100$$

For each extract, we defined the MBPC as the lowest concentration that produced maximal inhibition of biofilm formation. Aqueous and ethanolic extracts of *T. purpureum* showed varying degrees of activity against *E. coli* biofilm formation. Strong activity: aqueous flower extract (MBPC 68.9 mg/mL, 80% inhibition), and the aqueous stem extract (MBPC 41.9 mg/mL, 86.7% inhibition). Intermediate activity: aqueous whole plant extract (MBPC 32.1 mg/mL, 78.7% inhibition), aqueous leaf extract (MBPC 35.15 mg/mL, 69% inhibition), and the ethanolic whole plant extract (MBPC 41.7 mg/mL, 60% inhibition). Weak activity: ethanolic flower extract (MBPC 57.85 mg/mL, 39% inhibition), the ethanolic leaf extract (MBPC 30 mg/mL, 41% inhibition), and the ethanolic stem extract (MBPC 32.45 mg/mL, 49.8% inhibition) (Table 4; Figs. 1,2).

#### 3.3.2 Eradication of Biofilm

Considering our results on *E. coli* biofilm eradication, *T. purpureum* extracts showed intermediate activity for the aqueous stem and leaf extracts, low activity for the aqueous flower extract and the ethanolic whole plant, flower, leaf, and stem extracts, and weak activity for the aqueous whole plant extract. The corresponding MBECs and eradication percentages were 0.18 mg/mL and 47.3%; 0.19 mg/mL and 57.2%; 0.2 mg/mL and 62.5%; 49.65 mg/mL and 66.8% for aqueous whole-plant, flower, leaf, and stem extracts, respectively. Meanwhile, the MBEC and eradication percentages for the ethanolic extracts were 0.16 mg/mL and 51.3%; 0.27 mg/mL and 50.5%; 0.13 mg/mL and 59.5%; 0.13 mg/mL and 55% for the whole-plant, flower, leaf, and stem extracts, respectively (Table 4; Figs. 3,4).

**Table 2. Detection of different classes of phytochemicals in aqueous and ethanolic extracts from various parts of *T. purpureum*.**

| | Extract | Tannins | Resins | Coumarins | Saponins | Alkaloids | Phenols | Terpenoids | Volatile oils | Flavonoids |
|---|---|---|---|---|---|---|---|---|---|---|
| Whole plant | Distilled water | - | - | - | + | + | - | + | - | + |
| | Ethanol | - | - | - | + | - | - | + | - | + |
| Leaves | Distilled water | - | - | - | + | + | - | + | - | + |
| | Ethanol | - | - | - | + | - | - | + | - | + |
| Flowers | Distilled water | - | + | - | + | + | - | + | - | + |
| | Ethanol | - | + | - | + | - | - | + | - | + |
| Stems | Distilled water | - | - | - | + | - | - | + | - | + |
| | Ethanol | - | - | - | + | - | - | + | - | + |

"–" = not detected / "+" = detected.

**Table 3. MIC and MBC results for *Trifolium purpureum* extracts against *E. coli*.**

| Plant extract | Solvent | MBC mg/mL | MIC mg/mL | MBC/MIC | Nature of activity |
|---|---|---|---|---|---|
| Whole plant | Distilled water | 32.1 | 16.05 | 2 | Bactericidal |
| | Ethanol | >41.78* | 41.7 | N/A | ND |
| Flower | Distilled water | >68.95* | 68.95 | N/A | ND |
| | Ethanol | >57.85* | 57.85 | N/A | ND |
| Leaves | Distilled water | >35.15* | 35.15 | N/A | ND |
| | Ethanol | >30* | 30 | N/A | ND |
| Stem | Distilled water | >41.9* | 41.9 | N/A | ND |
| | Ethanol | >32.45* | 32.45 | N/A | ND |

MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; N/A, not applicable; ND, not determined because MBCs are unknown; *, MBC > (highest tested concentration) of the extract powder, indicating no bactericidal activity was observed at the maximum concentration tested. Therefore, numerical MBC/MIC ratios and the specific nature of activity (ND) could not be determined for these fractions.

**Table 4. MBPC and MBEC results for *Trifolium purpureum* extracts and the associated percentages against *E. coli*.**

| Plant extract | Solvent | MBPC (mg/mL) | Percentage of prevention (%) | MBEC (mg/mL) | Percentage of eradication (%) |
|---|---|---|---|---|---|
| Whole plant | Distilled water | 32.1 | 78.7 | 0.18 | 47.3 |
| | Ethanol | 41.7 | 60 | 0.16 | 51.3 |
| Flower | Distilled water | 68.9 | 80 | 0.19 | 57.2 |
| | Ethanol | 57.85 | 39 | 0.27 | 50.5 |
| Leaves | Distilled water | 35.15 | 69 | 0.2 | 62.5 |
| | Ethanol | 30 | 41 | 0.13 | 59.5 |
| Stem | Distilled water | 41.9 | 86.7 | 49.65 | 66.8 |
| | Ethanol | 32.45 | 49.8 | 0.13 | 55 |

Note: The MBEC for the stem water extract (49.65 mg/mL) was confirmed across multiple replicates and consistently required a higher concentration to eradicate biofilm than other fractions. MBEC, minimum biofilm eradication concentration.

#### 3.3.3 Effect of T. purpureum Extracts on the Expression of E. coli Biofilm-Forming Genes

After confirming the ability of *T. purpureum* to prevent *E. coli* biofilm formation using a colorimetric method, we investigated the effects of selected extracts, specifically those with the strongest preventive activity, on the expression of five genes involved in different stages of biofilm formation. For this purpose, we separated the planktonic and biofilm populations and measured gene expression in both after 10 hours of culture at two extract concentrations: MBPC and MBPC/2.

Our results clearly show that the aqueous whole plant extract represses the *pgaB*, *csgD*, *fimA*, *fimH*, and *flu* genes in the planktonic population (Fig. 5A). In the biofilm population, *fimA*, *fimH*, and *flu* genes were also repressed after biofilm establishment (Fig. 5B).

At the MBPC concentration, the aqueous whole plant extract of *T. purpureum* reduced the expression of the *pgaB* and *csgD* genes by 5-fold and that of the *fimA*, *fimH*, and *flu* genes by 10-, 4-, and 2-fold, respectively, in the planktonic population (Fig. 5A). Within the biofilm population, *fimA*, *fimH*, and *flu* were repressed by 9-, 3-, and 4-fold, re-

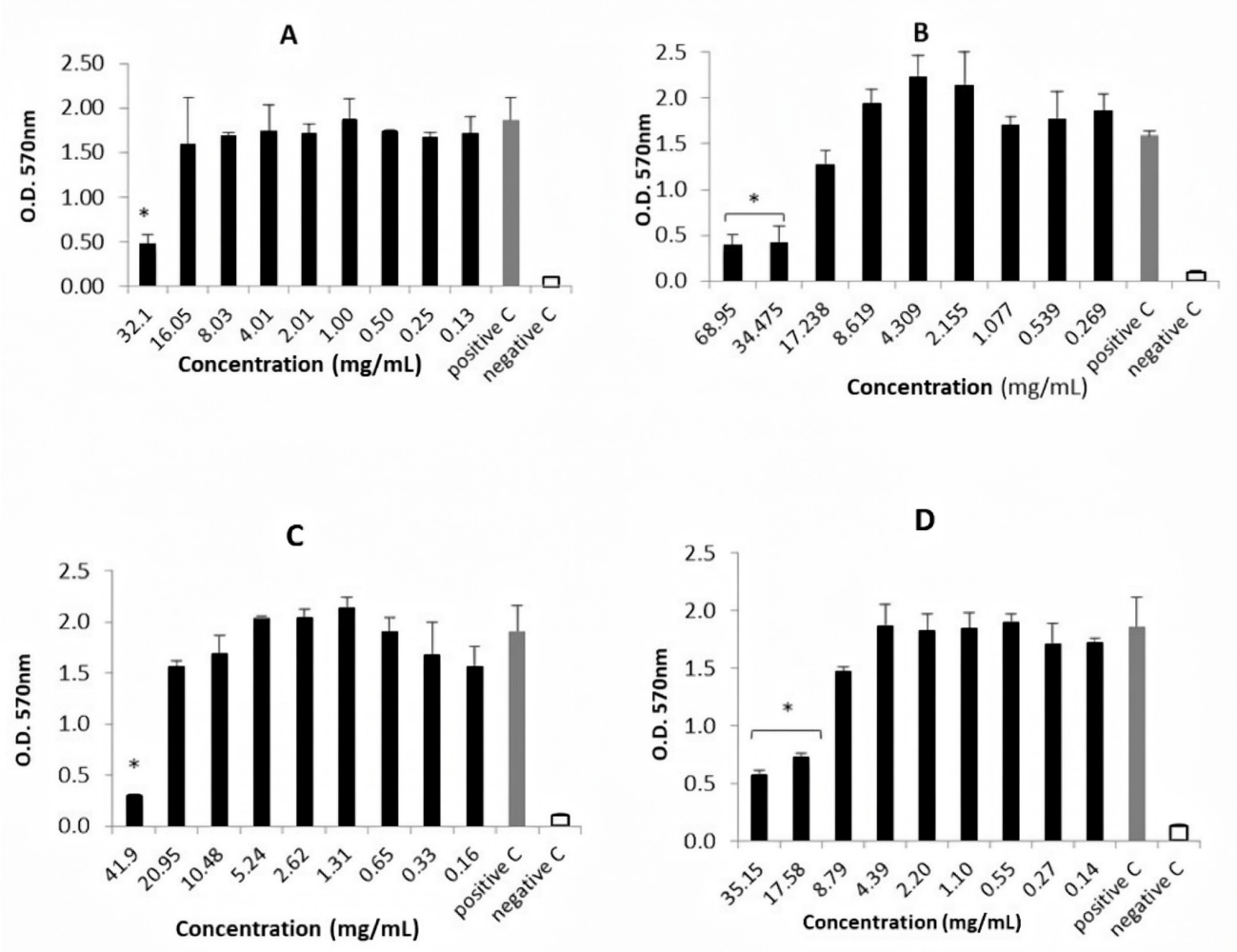


**Fig. 1. Antibiofilm preventive activity of aqueous *T. purpureum* extracts against *E. coli*.** The negative control was Mueller-Hinton broth (MHB), and the positive control was the biofilm formed by *E. coli* under the same conditions but without the extracts. (A) Whole plant extract: minimum biofilm prevention concentration (MBPC) = 32.1 mg/mL, resulting in 78.7% biofilm inhibition. (B) Flower extract: MBPC = 68.9 mg/mL, resulting in 80% biofilm inhibition. (C) Stem extract: MBPC = 41.9 mg/mL, resulting in 86.7% biofilm inhibition. (D) Leaf extract: MBPC = 35.15 mg/mL, resulting in 69% biofilm inhibition. The data are presented as mean values from four independent experiments, with two wells per plate. The bars represent the mean ± standard deviation (SD). * indicates a statistically significant difference compared with the positive control ($p < 0.05$). The bars with the lowest optical density (OD) values in each fraction correspond to the MBPCs.

spectively. Interestingly, at half the MBPC of the aqueous whole plant extract, corresponding to a 13% biofilm prevention rate, the extract still strongly inhibited gene expression in the biofilm population and significantly repressed expression in the planktonic population (Fig. 5B).

The aqueous flower extract and the aqueous whole plant extract of *T. purpureum* showed similar gene repression patterns. At the MBPC, the extract repressed the expression of *pgaB* by 13-fold, *csgD* by 9-fold, *fimA* by 8-fold, *fimH* and *flu* by 5-fold relative to the control in the planktonic population (Fig. 5C). In the biofilm population, repression at the same concentration was 5-fold for *pgaB* and 3.5-fold for the other genes (Fig. 5D). At half the MBPC, a similar level of repression was observed in both populations, except for *fimA* (Fig. 5C,D). These findings indicate that both aqueous whole plant and flower extracts efficiently repress genes involved in *E. coli* biofilm attachment and maturation.

## 4. Discussion

Plants are a rich source of antimicrobial agents and have been used for therapeutic purposes since ancient times. As documented in the literature, the phytochemicals detected in *T. purpureum* extracts are biologically active. Alkaloids are best known for their antimicrobial properties, including antibacterial and antifungal activities [40]. Flavonoids exhibit antidiarrheal, antimicrobial [41], anticancer, anthelmintic, and antioxidant effects [42]. Finally,

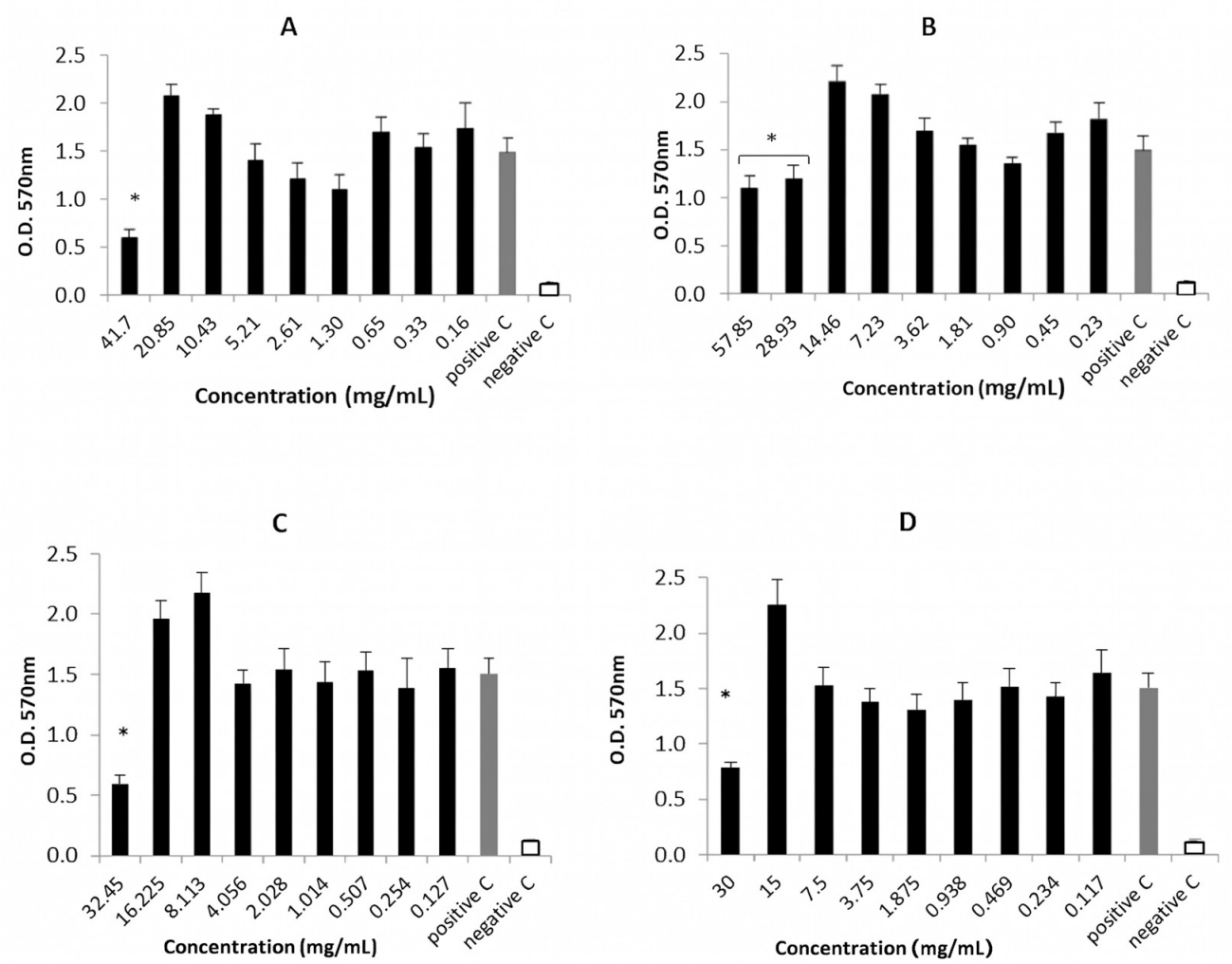


**Fig. 2. Antibiofilm preventive activity of ethanolic *T. purpureum* extracts against *E. coli*.** The negative control was MHB, and the positive control was the biofilm formed by *E. coli* under the same conditions but without the extracts. (A) Whole plant extract: MBPC = 41.7 mg/mL, resulting in 60% biofilm inhibition. (B) Flower extract: MBPC = 57.85 mg/mL, resulting in 39% biofilm inhibition. (C) Stem extract: MBPC = 32.45 mg/mL, resulting in 49.8% biofilm inhibition. (D) Leaf extract: MBPC = 30 mg/mL, resulting in 41% biofilm inhibition. Data are the mean values from four independent experiments, with two wells per plate. Bars represent means ± SD. * indicates a statistically significant difference compared with the positive control ($p < 0.05$). The bars with the lowest ODs in each fraction correspond to the MBPCs.

resins are well documented for their antiprotozoal effects [43].

The genus *Tephrosia*, particularly *Tephrosia purpurea* (Linn.) Pers., has been extensively investigated and documented for its wide range of biological and pharmacological activities, including antibacterial, antimicrobial, antioxidant, and anti-inflammatory properties. In contrast, the biological potential and specific antibacterial and antibiofilm mechanisms of *Trifolium purpureum* LOIS. remain significantly less explored, warranting detailed investigation [44,45]. In this study, extracts from *T. purpureum* were used for phytochemical screening and for assessing their antimicrobial and antibiofilm potential against *E. coli*. It is worth noting that although the MIC values (16–69 mg/mL) are high compared with those of clinical antibiotics (μg/mL), these ranges are typical for crude plant extracts and serve as a baseline for the future isolation of more potent, purified fractions.

Both aqueous and ethanolic extracts of *T. purpureum* derived from leaves, stems, flowers, and the whole plant efficiently inhibited the growth of *E. coli*. Furthermore, the extracts prevented biofilm formation and eradicated preformed biofilms. Aqueous extracts often showed higher activity than ethanolic ones. This likely results from the higher concentration of polar bioactive compounds, which are more efficiently extracted by water and may possess more potent antibacterial properties in this species. These findings are consistent with existing studies on antimicrobial activity in *Trifolium* spp [46,47].

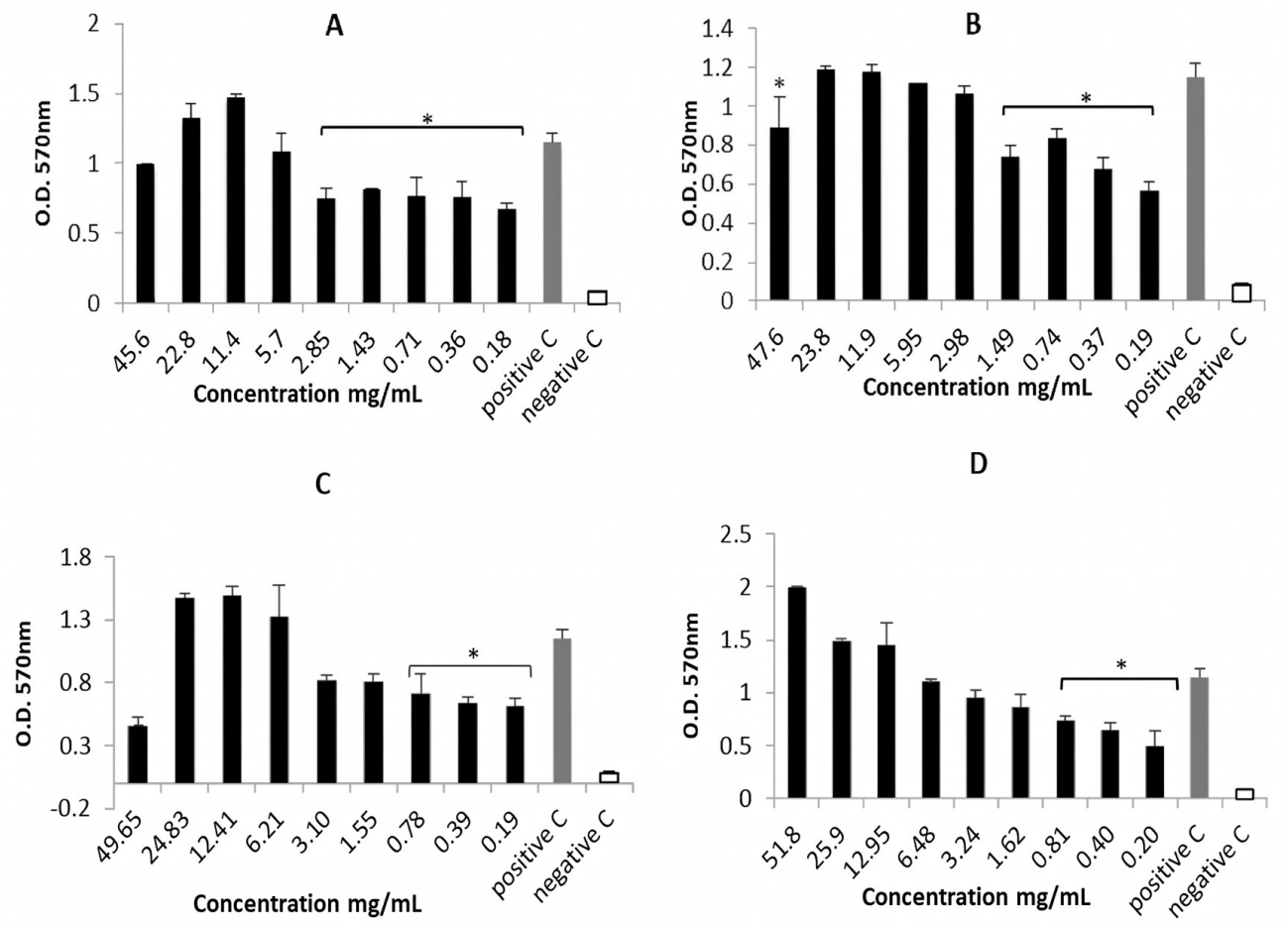


**Fig. 3. Antibiofilm eradication activity of aqueous *T. purpureum* extracts against *E. coli*.** The negative control was MHB, and the positive control was the biofilm formed by *E. coli* under the same conditions but without the extracts. (A) Whole plant extract: MBEC = 0.18 mg/mL, resulting in 47.3% biofilm eradication. (B) Flower extract: MBEC = 0.19 mg/mL, resulting in 57.2% biofilm eradication. (C) Stem extract: MBEC = 49.65 mg/mL, resulting in 66.8% biofilm eradication. (D) Leaf extract: MBEC = 0.2 mg/mL, resulting in 62.5% biofilm eradication. The data are presented as the mean values from four independent experiments, with two wells per plate. The bars represent means ± SD. * indicates a statistically significant difference compared with the positive control ($p < 0.05$). The bars with the lowest OD values in each fraction correspond to the MBECs.

*Trifolium hybridum* (alsike clover) has been reported to exhibit dose-dependent antimicrobial activity against bacteria (*Escherichia coli*, *Salmonella typhi*, *Bacillus aureus*, and *Staphylococcus aureus*), yeast (*C. albicans*), and fungi (*Aspergillus flavus*) [48].

Similarly, an aqueous solution of Egyptian clover honey demonstrated inhibitory effects against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [49].

Moreover, many plant extracts have shown successful antibacterial and antibiofilm activity against *E. coli*. Antibacterial effects have been reported for extracts of several species, including *Myroxylon peruiferum* [50], *Dioclea grandiflora* [50], *Origanum marjorana* [17], *Thymus zygis* [17], *Rosmarinus officinalis* [17], *Eugenia brasiliensis* [51], *Carum copticum* [52], condensed tannins derived from yew leaves, tilia flowers, and black locust leaves [53], *Rosmarinus officinalis* L. [54], *Asphodelus microcarpus* [55], *Punica granatum* L. [56], *Rhus coriaria* L. [57], and *Lagerstroemia speciosa* [58].

Our phytochemical screening revealed that *T. purpureum* contains flavonoids, saponins, and terpenoids across all plant parts; alkaloids are present throughout the plant, especially in the leaves and flowers; resins were detected only in the flowers. These phytocompounds, particularly alkaloids, flavonoids, saponins, and terpenoids, likely contribute synergistically to the antimicrobial and, notably, antibiofilm activity of *T. purpureum*.

Accordingly, Lee et al. [58] showed that harmaline, one of the β-carboline alkaloids found in *Peganum harmala* [59], was effective against the biofilms of many human pathogens, including *E. coli* O157:H7. Phakellin-based alkaloids [60], as well as alkaloid-rich plants such as *Hymenocallis littoralis* [61], have also shown effective an-

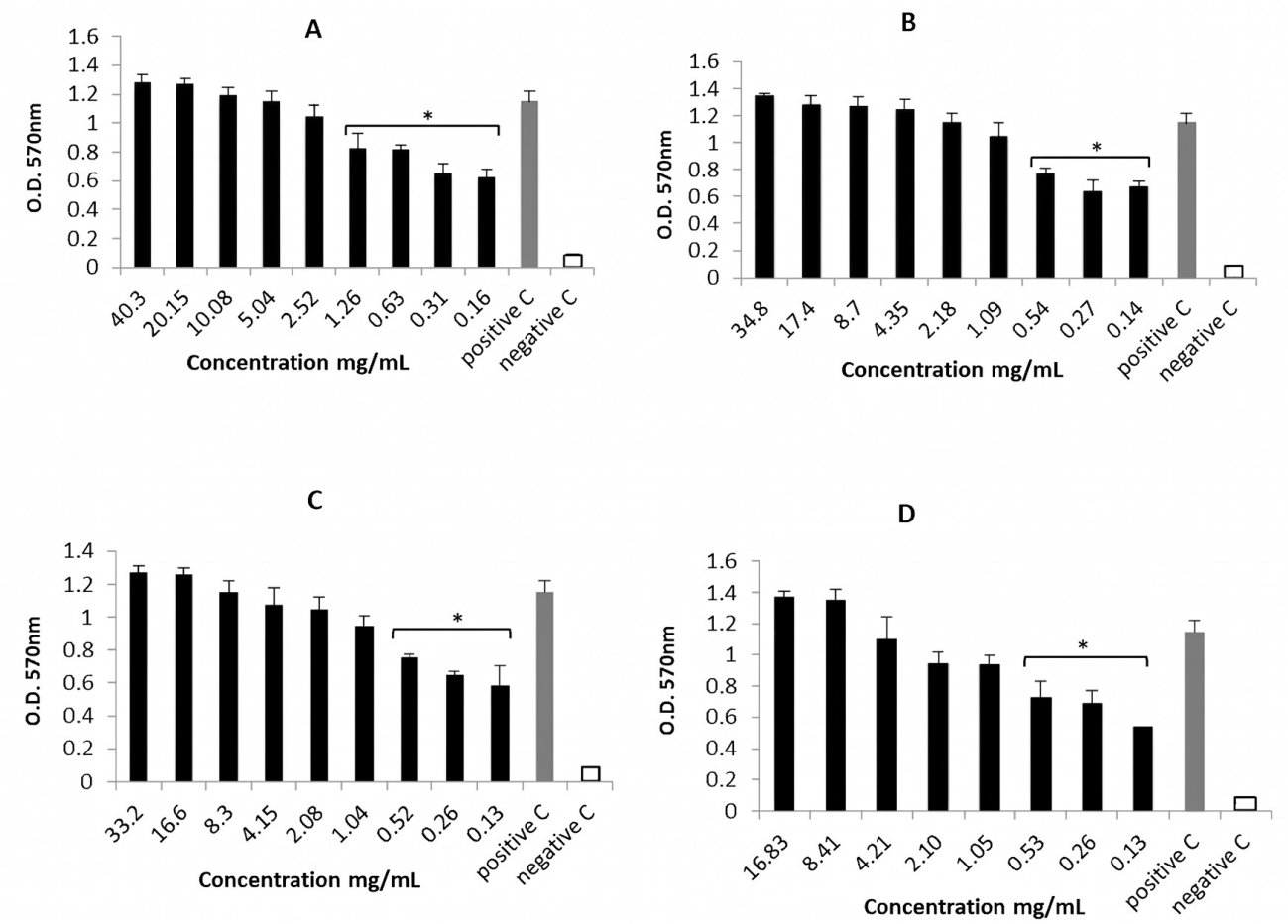


**Fig. 4. Antibiofilm eradication activity of ethanolic *T. purpureum* extracts against *E. coli*.** The negative control was MHB, and the positive control was the biofilm formed by *E. coli* under the same conditions but without the extracts. (A) Whole plant extract: MBEC = 0.16 mg/mL, resulting in 51.3% biofilm eradication. (B) Flower extract: MBEC = 0.27 mg/mL, resulting in 50.5% biofilm eradication. (C) Stem extract: MBEC = 0.13 mg/mL, resulting in 55% biofilm eradication. (D) Leaf extract: MBEC = 0.13 mg/mL, resulting in 59.5% biofilm eradication. Data are presented as the mean values from four independent experiments, with two wells per plate. Bars represent means ± SD. * indicates a statistically significant difference compared with the positive control ($p < 0.05$). The bars with the lowest OD values in each fraction correspond to the MBECs.

tibiofilm activity against *E. coli* and many other bacterial species.

Alternatively, flavonoids extracted from *Moringa oleifera* [62] and other plants have exhibited strong antibiofilm activity against *E. coli* and both Gram-positive and Gram-negative bacteria. Likewise, terpenoids from a variety of species, including Lippia *alba* [63], *Vitex gardneriana* [64], *Eucalyptus globulus*, *Thymus mastichina*, *Mentha pulegium*, *Rosmarinus officinalis*, *Calamintha nepeta* ssp. *nepeta*, *Cistus ladanifer*, *Foeniculum vulgare*, *Dittrichia viscosa* ssp. *viscosa* [65], and *Camellia oleifera* [66] have shown potent antibiofilm activity. Moreover, *Propolis* resins [67,68,69,70,71] collected from regions worldwide have demonstrated strong antibiofilm activity against a variety of bacterial pathogens.

Our molecular analyses of *E. coli* biofilm formation identified numerous genes involved in various stages of biofilm development, particularly during attachment and maturation. These include *csgD*, *pga* genes (*pgaB*), *fim genes* (*fimA*, *fimH*), and the *flu* locus [72,73,74,75,76]. These genes represent critical stages of biofilm development. *csgD* is the master regulator of curli fimbriae and cellulose production; *pgaB* is involved in the synthesis of PGA, a crucial matrix polysaccharide for structural integrity; *fimA/fimH* are essential for type 1 fimbriae-mediated initial attachment; and *flu* encodes antigen 43, which mediates autoaggregation and interbacterial adhesion.

In the present study, we showed that aqueous extracts from *Trifolium purpureum* flowers and the whole plant significantly repressed the expression of *csgD*, *pgaB*, *fimA*, *fimH*, and *flu*, thereby effectively impairing the ability of *E. coli* to initiate and develop biofilms. *pgaB* and *csgD* were repressed primarily in planktonic cells. These

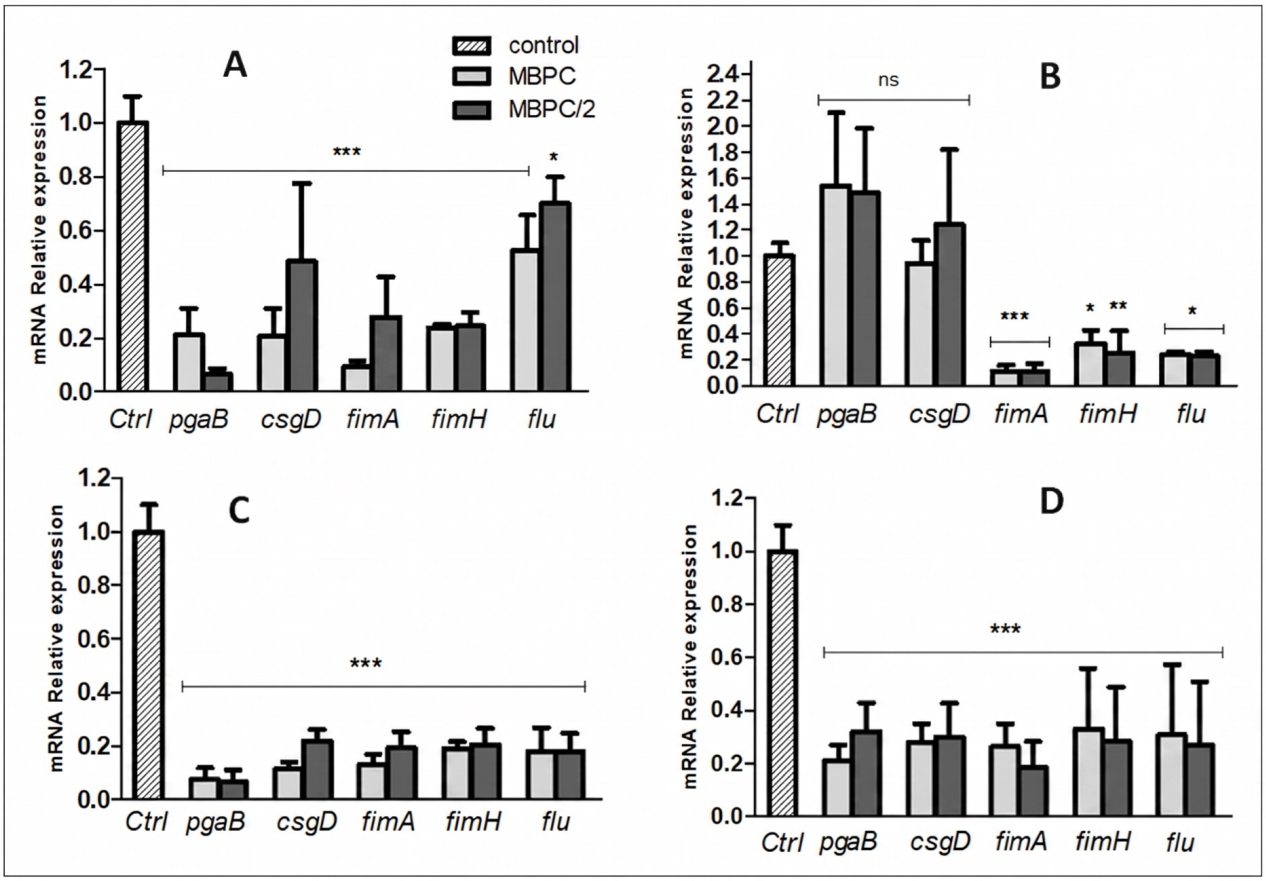


**Fig. 5. Relative mRNA expression of the biofilm formation genes (*pgaB*, *csgD*, *fimA*, *fimH*, and *flu*) at two extract concentrations: one equal to MBPC and the other to MBPC/2.** (A,B) show the relative mRNA expression of genes in planktonic (A) and biofilm (B) bacterial populations treated with the aqueous whole-plant extract of *T. purpureum*. (C,D) show the relative mRNA expression of genes in the planktonic (C) and biofilm (D) bacterial populations treated with the aqueous flower extract. Bars represent means ± SD. * indicates a statistically significant difference compared with the positive control ($p < 0.05$), ** (Very Significant): $p < 0.01$, *** (Extremely Significant): $p < 0.001$ and ns (Not Significant): $p > 0.05$.

genes are early-stage regulators and matrix synthesizers. Their repression during the planktonic phase prevents the switch to a sessile lifestyle, which may explain why our extracts were more effective at preventing biofilm formation than at eradicating established biofilms. Selective repression of *pgaB/csgD* in planktonic cells suggests interference with early biofilm signaling rather than with mature matrix degradation.

Ren et al. [75] found that plant-derived ursolic acid inhibited *E. coli* biofilm formation, likely by downregulating sulfur metabolism genes and upregulating motility and stress-response genes. Moreover, leaf extracts of *Orthosiphon stamineus* demonstrated a strong anti-adhesive effect against *E. coli* by downregulating the expression of curli and fimbriae genes [76].

Coddens et al. [77] found that cranberry extracts effectively inhibit the *in vitro* adhesion of F4 and F18 strains. Moreover, Deipenbrock and Hensel [78] showed that *Orthosiphon stamineus* leaves reduce the adherence of uropathogenic *E. coli* (UPEC) to bladder cells by reducing the expression of *fimH*, *fimC*, *fimD*, *csgA*, and *focG*, all of which are involved in the formation of adhesins essential for adhesion, a key step in *E. coli* biofilm formation. Other authors have also highlighted the significant anti-adhesive potential of cranberries against UPEC [79,80]. Furthermore, Yang et al. [81] found that pomegranate rind extracts inhibited biofilm formation in *E. coli* by repressing the expression of *csgB, csgD*, and *fimH*.

## 5. Conclusions

In conclusion, *Trifolium purpureum* showed significant therapeutic potential as a source of bioactive secondary metabolites, especially saponins, terpenoids, and flavonoids across all plant tissues. While various tested extracts showed inhibitory activity, the whole-plant water extract emerged as the most potent, exhibiting definitive potential antibacterial and antibiofilm activity of *T. purpureum* against *E. coli* colonies. Most notably, the ability

of the plant to downregulate key virulence genes, such as *pgaB*, *csgD*, and *fimA*, in both planktonic and established biofilm cells underscores its promise as a molecular-level alternative to traditional antibacterial agents.

## 6. Limitations and Future Perspectives

This study used qualitative screening to identify major metabolite classes; quantitative profiling (*e.g.*, high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS), and total phenolic content) is planned for future bioassay-guided fractionation to identify the specific potent compounds.

## Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding authors on reasonable request.

## Author Contributions

AC designed the research study. FM, RB, NB and ZA performed the research. AC and NA analyzed the data. FM and RB wrote the manuscript. ZE searched references, reviewed and edited the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

According to Lebanese local regulations and policies, this study does not involve human or animal subjects; therefore, ethical committee approval is not necessary. Plant material of *T. purpureum* LOIS. was collected from Chkara in the Western Lebanon mountain range (1,350 m above sea level). Plant collection was carried out in compliance with local institutional guidelines and relevant national regulations governing wild plant sampling. As *Trifolium purpureum* is an uncultivated, non-endangered, and non-threatened wild plant species in Lebanon, no specific permits or specialized licenses were required for collection for academic research purposes under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) or IUCN policies. The plant species was taxonomically identified and authenticated according to standard botanical keys for Lebanese flora. A representative has been deposited and preserved in the Research Laboratory of Microbiology (RLM), Department of Life and Earth Sciences, Faculty of Sciences I, Lebanese University (Hadat Campus, Beirut, Lebanon) for future reference.


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## Conflicts of Interest

The authors declare no conflicts of interest.

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