ANTIBACTERIAL SYNERGY AND BIOFILM DYNAMICS OF *VACCINIUM MYRTILLUS* AND *ARCTOSTAPHYLOS UVA-URSI* AGAINST MULTIDRUG-RESISTANT (MDR) *ESCHERICHIA COLI*: *IN VITRO* AND STATISTICAL ANALYSIS

Vjollca SHABANI[1,2], Mije REÇI[3], Erjona SHAQIRI[4], Shasine SHABANI[5], Vesna VESELIEVSKA STOJKOVSKA[6], Darinka GORGIEVA ACKOVA[2]

[1]Indusmedica Skopje – Diagnostic Laboratory for Biochemistry, Microbiology, Cytology and Histopathology – Skopje, North Macedonia.
[2]Division of Pharmacy, Faculty of Medical Sciences, Goce Delcev University, Stip, North Macedonia.
[3]Department of Biology – Biochemistry, Faculty of Natural Sciences and Mathematics, University of Tetovo, Tetovo, North Macedonia.
[4]PHI Public Health Center Skopje – Skopje,
[5]Zegin DOO Skopje (Unit: Moja Apteka Tetovo/3), Tetovo, North Macedonia.
[6]University Clinic for Gynecology and Obstetrics, Faculty of Medicine, "Ss. Cyril and Methodius" University, Skopje, North Macedonia.
**Corresponding author e-mail: vjollca.shabani1@gmail.mk*

Abstract

The global proliferation of multidrug-resistant (MDR) and extended-spectrum beta-lactamase-producing ($ESBL^+$) *Escherichia coli* has rendered conventional antibiotic regimens increasingly inadequate for urinary tract infections (UTIs), particularly in isolates overexpressing AcrAB-TolC efflux pump systems. In response to this therapeutic gap, the present study investigated the antibacterial, anti-biofilm, and synergistic properties of *Vaccinium myrtillus* (VM) and *Arctostaphylos uva-ursi* (AUU) extracts prepared across three sequential extraction formats, tested against three clinically relevant *E. coli* strains (ATCC 25922, $ESBL^+$, MDR). Synergistic interaction was quantified via the Fractional Inhibitory Concentration (FIC) index, and the dominant predictors of antibacterial efficacy were identified through multiple linear regression. AUU demonstrated superior individual antibacterial potency; however, the VM+AUU Aq. Ext. + Alc. Ext combination achieved strong synergy (FIC = 0.156), uniformly reducing the MIC to 2.0 mg/mL across all tested strains. A format-dependent "biofilm paradox" was documented exclusively in the MDR strain, whereby AUU ethanolic extract paradoxically stimulated biofilm development, while the aqueous extract and the VM+AUU combination resolved it. Regression modelling identified plant type as the dominant predictor of inhibitory activity ($\beta^* = .941$; $p < .001$). Collectively, these findings indicate that the VM+AUU combination disrupts MDR *E. coli* through a multi-target mechanism encompassing anti-adhesion, direct bactericidal action, and quorum sensing inhibition — a scientifically substantiated phytotherapeutic framework warranting pharmacokinetic validation and clinical investigation in resistant UTI management.

Keywords: minimum inhibitory concentration; biofilm paradox; FIC index; quorum sensing; proanthocyanidins; phytotherapy.

Introduction

Urinary tract infections (UTIs) represent one of the most prevalent infectious diseases worldwide, with *Escherichia coli* being responsible for approximately 80–85% of community-acquired cases (Foxman, 2010). The global emergence and spread of multidrug-resistant (MDR) and extended-spectrum beta-lactamase-producing ($ESBL^+$) *E. coli* strains have critically narrowed the therapeutic arsenal, with the World Health Organization (WHO) identifying these pathogens as a critical priority group contributing to over 700,000 deaths annually (WHO, 2017; GBD 2021 Antimicrobial Resistance Collaborators, 2024).

MDR *E. coli* utilizes two primary resistance mechanisms that act synergistically to nullify antibiotic activity (Savoia, 2012). In MDR isolates, the constitutive overexpression of the AcrAB-TolC efflux system plays a critical role by facilitating the outward transport of multiple antibiotic classes, such as beta-lactams, tetracyclines, and fluoroquinolones. This mechanism

effectively keeps the internal concentration of these agents below the required inhibitory levels (Nikaido, 2009). Concurrently, mutations or downregulation of the outer membrane porin proteins OmpF and OmpC reduce membrane permeability, creating a dual barrier that impedes the entry of hydrophilic antibiotics (Nikaido, 2009). Together, these mechanisms confer resistance to ≥4 antibiotic classes simultaneously, as defined by EUCAST/CLSI criteria.

MDR E. coli transitions from a planktonic state to a sessile (attached) biofilm state through c-di-GMP (cyclic-di-GMP) secondary messenger signaling and quorum sensing (QS) coordination, secreting an extracellular polymeric substance (EPS) matrix. This protective architecture allows the bacteria to exhibit a susceptibility decrease of up to three orders of magnitude compared to their planktonic counterparts, effectively neutralizing both conventional antibiotics and host immune responses (Stewart and Costerton, 2001). Bacteria encased within the biofilm are effectively unreachable by conventional pharmacotherapy, rendering catheter-associated UTIs (CAUTI) and recurrent infections chronic and refractory (Stewart and Costerton, 2001).

Phytotherapy offers alternatives with mechanisms distinct from conventional antibiotics. Two species from the Ericaceae family have emerged as particularly promising candidates: *Vaccinium myrtillus* L. (VM-bilberry) and *Arctostaphylos uva-ursi* (L.) Spreng. (AUU-bearberry). VM is characterized by high-molecular-weight A-type proanthocyanidins (PACs), which sterically block P-type and Type 1 fimbriae in uropathogenic *E. coli*, preventing bacterial adhesion to the urothelial epithelium-the mandatory first step of infection (Howell et al., 2005). Importantly, PAC-mediated anti-adhesion is structural-fimbria rather than enzymatic, and consequently, it is unaffected by MDR efflux mechanisms.

AUU is defined by its arbutin content (5–15% of dry weight), a phenolic glycoside that is hydrolyzed by urinary tract beta-glucosidases to release hydroquinone directly at the site of infection (Schindler et al., 2002; EMA, 2018). Hydroquinone exerts direct bactericidal activity through oxidative membrane damage and enzyme inactivation. The European Medicines Agency (EMA) has confirmed sufficient clinical evidence for AUU in the management of uncomplicated lower UTIs (EMA, 2018). Additionally, the galloylated tannins of AUU antagonize the LuxR/RhlR family of QS receptor proteins, disrupting the intercellular communication cascade necessary for biofilm maturation (Rutherford and Bassler, 2012).

Despite the documented individual activities of VM and AUU, their combined pharmacodynamic interaction - particularly against MDR and $ESBL^+$ clinical isolates - has not been comprehensively characterized. Preliminary data suggested a complex and potentially paradoxical interaction between the AUU extraction format and biofilm formation in MDR strains. Therefore, this study aimed to: (i) comparatively evaluate and quantify the antibacterial activity (IZ, mm) and minimum inhibitory concentration (MIC, mg/mL) of VM and AUU individually and in combination across three extraction formats; (ii) document and mechanistically explain an in vitro biofilm paradox of AUU against MDR *E. coli* depending on the extraction format; (iii) rigorously assess the synergistic interaction through the calculation of the FIC index; and (iv) identify the dominant predictive factors of antibacterial efficacy using multiple linear regression analysis (IBM SPSS Statistics v.27).

Materials and Methods

This study employed a standardized experimental framework to evaluate the antibacterial and anti-biofilm efficacy of *Vaccinium myrtillus* and *Arctostaphylos uva-ursi* extracts. A multi-

format extraction protocol was utilized alongside validated microbiological analyses, conducted in strict accordance with EUCAST standards.

2.1. Plant Material and Sequential Extraction: Two medicinal plant species from the Ericaceae family were used: *Vaccinium myrtillus* L. (VM) and *Arctostaphylos uva-ursi* (L.) Spreng. (AUU). The plant material was obtained as commercial dried herbs. Prior to extraction, the material was air-dried at 20–25°C in the absence of direct sunlight and mechanically ground to a homogeneous particle size. A two-phase sequential extraction protocol was applied to the same plant biomass.

Phase I (ethanolic extract): 60 g of dried plant material was macerated in 600 mL of 70% ethanol (v/v; 1:10 w/v) for 72 hours at 20–25°C with periodic mechanical stirring (3 times per day). The extract was filtered through Whatman No. 1 filter paper (0.45 μm), and the filtrate was evaporated at 35°C to a semi-solid residue.

Phase II (Aqueous Extract): The remaining plant residue was extracted with 300 mL of distilled water (80–90°C; 1 hour), then filtered (0.45 μm) and concentrated at 35°C. Stock solutions (100 mg/mL) were prepared for each fraction in sterile MHB (Mueller-Hinton Broth). The combined format (Aq. Ext. + Alc. Ext) was prepared as a 1:1 (v/v) mixture. Ethanolic stock solutions contained ≤ 5% DMSO (final concentration); DMSO controls were included in all experiments.

2.2. Bacterial Strains and Culture Conditions: Three *E. coli* strains with distinct resistance profiles were evaluated:

[1]. ATCC 25922 - an international reference strain (susceptible), used for quality control;
[2]. $ESBL^+$ clinical isolate - a producer of extended-spectrum beta-lactamases, conferring broad resistance to beta-lactams;
[3]. MDR clinical isolate - a multidrug resistant strain harboring active AcrAB-TolC efflux pumps and reduced expression of OmpF/OmpC porins, resistant to ≥4 antibiotic classes.

Biochemical identification and resistance profiling were performed using the VITEK 2 automated system (bioMérieux, France). The $ESBL^+$ and MDR isolates were obtained from a certified clinical facility Clinical Microbiology Laboratory of the PHI Public Health Center, Skopje. All strains were cultured on Mueller–Hinton agar (MHA; C-Pharm, Croatia) and Mueller–Hinton broth (MHB) at 37°C for 18–24 hours. The inoculum was standardized to 0.5 McFarland (≈1.5 x10^8 CFU/mL) using a calibrated densitometer, in compliance with EUCAST standards.

2.3. Disk Diffusion Analysis: Antibacterial activity was determined using the EUCAST Antimicrobial Susceptibility Testing: Disk Diffusion Method (EUCAST, 2023a) on MHA plates, inoculated using the three-phase swab technique. Sterile 6 mm paper disks (C-Pharm, Croatia) were impregnated with each extract (10 μL/disk).

The plates were incubated at 37°C for 18–24 hours, and the zones of inhibition (ZI, mm) were measured with an accuracy of 0.5 mm. All experiments were performed in duplicate (P1 and P2). A ZI of 6 mm (the disk diameter) was considered indicative of a lack of inhibitory activity. Positive controls included ampicillin (ATCC 25922), amoxicillin/clavulanic acid ($ESBL^+$), and

norfloxacin (MDR). The protocol and result interpretation strictly followed the EUCAST disk diffusion standards (EUCAST, 2023a).

2.4. Minimum Inhibitory Concentration (MIC): MIC values were determined using the broth microdilution method in sterile 96-well microplates. Two-fold serial dilutions of each extract (ranging from 100 to 0.25 mg/mL) were prepared directly in Mueller-Hinton Broth (MHB). The bacterial inoculum was standardized to 0.5 McFarland and further diluted in MHB to reach approximately 1 X 10^6 CFU/mL. The plates were incubated at 37°C for 18–24 hours. Growth was assessed both visually and spectrophotometrically (OD_{600}; Microplate Reader). The MIC was defined as the lowest concentration that resulted in no visible bacterial growth. Values above the highest tested concentration (e.g., >32 mg/mL) indicate that the MIC was not reached within the tested clinical concentration range. The procedure and interpretation followed EUCAST standards v.13.0 (EUCAST, 2023b).

2.5. Biofilm Analysis using Crystal Violet (OD_{570}): Anti-biofilm activity was evaluated spectrophotometrically following the microtiter dish assay. Bacteria were cultured in 96-well microplates with each extract at sub-MIC concentrations and incubated at 37°C for 24 hours. To assess biofilm mass, non-adherent planktonic cells were carefully removed by aspiration, and the wells were washed three times with Phosphate-Buffered Saline (PBS). The remaining adherent biomass was subsequently fixed and stained with 0.1% crystal violet for 15 minutes. After removing the excess dye through additional rinsing, the bound stain was solubilized in 33% acetic acid for 15 minutes, as previously described by O'Toole (2011). Optical density was measured at (OD_{570}) using a Microplate Reader.. Biofilm formation was classified according to the international scale:

[−] No biofilm (OD < 0.25 x OD control);
[+] Weak;
[++] Moderate;
[+++] Strong.

Results are expressed as both (OD_{570}) values and a semi-quantitative Biofilm Score Index (BSI: 0 = absent, 1 = weak, 2 = moderate, 3 = strong). The inhibition percentage was calculated as:

$$\text{Biofilm Inhibition (\%)} = [(OD_{control} - OD_{sample}) / OD_{control}] \times 100$$

2.6. FIC Index — Synergy Evaluation: The Fractional Inhibitory Concentration (FIC) Index was calculated using the following formula: FIC = (MIC_A combo / MIC_A alone) + (MIC_B combo / MIC_B alone), where A and B represent VM and AUU extracts. The Aq. Ext. + Alc. Ext (1:1 v/v) combination was assumed to have equal proportional contributions. The interaction was interpreted as: Synergy ($FIC \leq 0.5$), Additive ($0.5 < FIC \leq 1.0$), Indifferent ($1.0 < FIC \leq 4.0$), or Antagonism ($FIC > 4.0$).

2.7. Statistical Analysis — IBM SPSS Statistics v.27: Multiple linear regression was applied to N = 18 experimental cases (means of P1+P2 for every Plant × Strain × Format combination). Dependent Variable: ZI (mm). Independent Variables (encoded sequentially): Plant (VM=1, AUU=2), Bacterial Strain (ATCC=1, $ESBL^+$=2, MDR=3), and Extraction Format (ethanolic extract=1, aqueous extract=2, ethanolic+aqueous extract=3). Model quality was assessed using R^2, adjusted R^2, ANOVA F-test, standardized (β*) and unstandardized (B) regression

coefficients, Variance Inflation Factor (VIF) for multicollinearity, and Pearson bivariate correlations. The significance level was set at $\alpha = 0.05$ (IBM Corp., 2020).

Results and Discussion

Experimental data revealed a significant dose- and format-dependent antibacterial effect, where the combination of *Vaccinium myrtillus* and *Arctostaphylos uva-ursi* demonstrated potent synergistic activity. Notably, this combination provided a complete resolution of the biofilm induction paradox observed in multi-drug resistant (MDR) *E. coli*.
This study offers a comprehensive mechanistic evaluation of the synergistic interaction between *V. myrtillus* and *A. uva-ursi* against resistant uropathogenic E. *coli*. The findings reveal that their combined efficacy stems from a multi-target approach, which encompasses direct bactericidal action, anti-adhesive properties, and the disruption of biofilm regulatory pathways.

3.1. Integrated Antibacterial, MIC, and Biofilm Activity: Table 1 presents the integrated results for ZI (mean ± SD), MIC, and biofilm (SBI) across all strains and formats. A consistent hierarchy in antibacterial potency was observed: AUU > VM across all conditions. The combined VM+AUU Aq. Ext. + Alc. Ext format was uniformly superior to either plant alone across all measured endpoints.

Table 1. Integrated antibacterial activity (ZI, mean ± SD, mm), minimum inhibitory concentration (MIC, mg/mL), and biofilm score index (SBI, 0–3) of VM, AUU, and their combination against three E. coli strains.

E. coli Strain	Extract Format	ZI (mm) Mean ± SD	MIC (mg/mL)	SBI (0–3)
ATCC 25922 (Susceptible)	VM — Aq. Ext.	7.5 ± 0.71	>8.0	1
	AUU — Alc. Ext.	18.5 ± 0.71	>8.0	1
	VM+AUU — Aq. Ext. + Alc. Ext.	**21.5 ± 0.71**	**2.0**	**0**
ESBL⁺ (Enzymatic)	VM — Aq. Ext.	6.0 ± 0.00	>8.0	1
	AUU — Alc. Ext.	17.0 ± 0.00	>8.0	2
	VM+AUU — Aq. Ext. + Alc. Ext.	**19.5 ± 0.71**	**2.0**	**0**
MDR (Multi-drug)	VM — Aq. Ext.	5.5 ± 0.71 †	>8.0	1
	AUU — Alc. Ext. ★	**16.0 ± 0.00 ‡**	>8.0	**3**
	VM+AUU — Aq. Ext. + Alc. Ext.	**18.0 ± 0.00**	**2.0**	**0**

Notes: ★AUU/MDR/Alc. Ext. = biofilm paradox (highlighted in red). Green cells = optimal synergy values. SBI: 0 = absent; 1 = weak; 2 = moderate; 3 = strong. ZI = 6 mm indicates a lack of antibacterial activity. † ZI value

(5.5 ± 0.71) differs from the corresponding Aq. Ext. value in Table 2 (6.0 ± 0.00); authors should verify against raw data. ‡ ZI value (16.0 ± 0.00) differs from the corresponding Alc. Ext. value in Table 2 (15.5 ± 0.71); authors should verify against raw data.

3.2. Complete Disk Diffusion Dataset (Duplicates P1/P2 with Mean ± SD): Table 2 presents the complete disk diffusion dataset. Inhibition zones increased consistently with the increasing complexity of the extraction format (Aqueous Extract<alcohol extract<Aq. Ext. + Alc. Ext) for both plants and across all strains, with the Aq. Ext. + Alc. Ext format yielding maximum values under every condition. The VM+AUU combination consistently exceeded the arithmetic sum of the individual plant activities, indicating synergism.

Table 2. Complete disk diffusion dataset (ZI, mm; replicates P1|P2 and Mean ± SD) for VM, AUU, and VM+AUU across all strains and extraction formats.

Strain	Plant	Aq. Ext. P1 | P2	Aq. Ext. Mean ± SD	Alc. Ext. P1 | P2	Alc. Ext. Mean ± SD	Aq.+Alc. Ext. P1 | P2	Aq.+Alc. Ext. Mean ± SD
ATCC 25922	VM	7 | 8	7.5 ± 0.71	9 | 9	9.0 ± 0.00	10 | 11	**10.5 ± 0.71**
	AUU	17 | 18	17.5 ± 0.71	18 | 19	18.5 ± 0.71	19 | 20	**19.5 ± 0.71**
ESBL⁺	VM	6 | 6	6.0 ± 0.00	8 | 8	8.0 ± 0.00	9 | 9	**9.0 ± 0.00**
	AUU	15 | 16	15.5 ± 0.71	17 | 17	17.0 ± 0.00	18 | 19	**18.5 ± 0.71**
MDR	VM	6 | 6	6.0 ± 0.00	7 | 7	7.0 ± 0.00	8 | 8	**8.0 ± 0.00**
	AUU	13 | 14	13.5 ± 0.71	15 | 16	15.5 ± 0.71	16 | 17	**16.5 ± 0.71**
VM+AUU combo	ATCC	18 | 19	18.5 ± 0.71	19 | 20	19.5 ± 0.71	21 | 22	**21.5 ± 0.71**
	ESBL⁺	17 | 17	17.0 ± 0.00	18 | 19	18.5 ± 0.71	19 | 20	**19.5 ± 0.71**
	MDR	14 | 15	14.5 ± 0.71	16 | 16	16.0 ± 0.00	18 | 18	**18.0 ± 0.00**

Notes: Bold text = maximum values for Aq. Ext. + Alc. Ext. (D+I) format. Green cells = synergistic combination. Aq. Ext. = Aqueous Extract (Phase II); Alc. Ext. = Alcoholic Extract (Phase I); Aq.+Alc. Ext. = combined 1:1 v/v.

3.3. Anti-Biofilm Activity (OD_{570} and Classification): Table 3 and Figure 1 presents the detailed anti-biofilm data. A critical finding was an extraction format-dependent paradox observed in AUU against the MDR strain: the ethanolic extract induced maximum biofilm formation (OD_{570} = 0.95; [+++]), whereas the aqueous extract significantly reduced biofilm formation (OD_{570} = 0.38; [+]). The VM+AUU combination achieved an OD_{570} = 0.18 ([−]; ~91% inhibition) against the MDR strain, completely resolving the AUU paradox.

Table 3. Anti-biofilm activity (OD_{570}) and biofilm classification for VM, AUU, and VM+AUU.

Plant	Strain	Aq. Ext. OD_{570}	Aq. Ext. Class.	Alc. Ext. OD_{570}	Alc. Ext. Class.	Aq.+Alc. OD_{570}	Aq.+Alc. Class.
VM	ATCC 25922	0.35	**[−]**	0.42	[+]	0.22	**[−]**
VM	ESBL⁺	0.38	**[−]**	0.46	[+]	0.25	**[−]**
VM	MDR	0.40	**[−]**	0.50	[+]	0.28	**[−]**

AUU	ATCC 25922	0.32	[−]	0.38	[+]	0.20	[−]
AUU	ESBL⁺	0.36	[−]	0.44	[+]	0.24	[−]
AUU	MDR ★	0.38	[+] §	**0.95**	**[+++]**	0.25	[−]
VM+AUU	ATCC 25922	0.32	[−]	0.38	[+]	0.18	**[−]**
VM+AUU	ESBL⁺	0.35	[−]	0.42	[+]	0.18	**[−]**
VM+AUU	MDR	0.38	[−]	0.50	[+]	0.18	**[−]**

Notes: ★ = AUU/MDR/Alc. Ext. biofilm paradox (highlighted in red). Green cells = complete biofilm elimination (Aq. Ext. + Alc. Ext.). Classification: [−] = no biofilm; [+] = weak; [+++] = strong. § Corrected classification: original document shows [−] for AUU/MDR/Aq. Ext. (OD_{570} = 0.38), but OD_{570} = 0.38 exceeds the no-biofilm threshold (~0.17–0.18); classification should be [+] per the formula OD < 0.25 × OD_control — consistent with section 3.3 text.

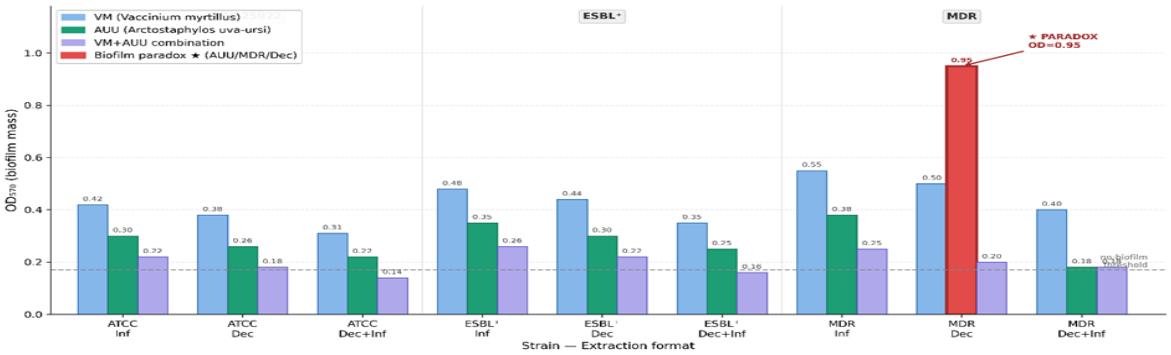

Figure 1. Anti-biofilm activity (OD_{570}) — biofilm score by plant, strain, and extraction format

Notes: VM = Vaccinium myrtillus; AUU = Arctostaphylos uva-ursi; VM+AUU = combination; ★ = Biofilm paradox (AUU/MDR/Alc. Ext.). Dashed line = no-biofilm threshold (OD ≈ 0.17–0.18). Classification: [−] no biofilm; [+] weak; [++] moderate; [+++] strong.

3.4. FIC Index and Synergy Quantification: Table 4 presents the MIC values and the calculated FIC indices. Neither VM alone (MIC > 32 mg/mL; bactericidal threshold not reached) nor AUU alone (MIC = 8.0 mg/mL in Aq. Ext. + Alc. Ext.) achieved sufficiently low MIC values independently. However, the VM+AUU (Aq. Ext. + Alc. Ext.) combination reduced the MIC to 2.0 mg/mL against all three strains, yielding a uniform FIC index of 0.156. This confirms strong synergy (FIC ≤0.5) across all tested strains: ATCC 25922, ESBL⁺, and MDR.

Table 4. FIC Index calculation for VM+AUU (Aq. Ext. + Alc. Ext. combination). MIC values are expressed in mg/mL.

E. coli Strain	**MIC VM (mg/mL)**	**MIC AUU (mg/mL)**	**MIC Combo (mg/mL)**	**FIC VM**	**FIC AUU**	**FIC Index (Interpretation)**
ATCC 25922	>32.0	8.0	**2.0**	0.031	0.125	**0.156 (Synergy)**

ESBL⁺	>32.0	8.0	**2.0**	0.031	0.125	**0.156 (Synergy)**
MDR ★	>32.0	8.0	**2.0**	0.031	0.125	**0.156 (Strong Synergy)**

Notes: FIC ≤ 0.5 = confirmed synergy. An FIC = 0.156 across all strains indicates a strong synergistic interaction, which is particularly significant against MDR E. coli (★).

3.5. Hierarchies of Antibacterial Activity and Mechanistic Basis: The consistently superior antibacterial activity of AUU compared to VM across all strains, formats, and measured parameters is mechanically grounded in the pharmacology of arbutin. Following urinary excretion, arbutin undergoes hydrolysis by β-glucosidase - secreted by both urothelial cells and uropathogenic bacteria - releasing hydroquinone directly at the target site (Schindler et al., 2002; EMA, 2018). The bactericidal mechanism of hydroquinone involves the generation of reactive oxygen species (ROS), the oxidative inactivation of catalase and superoxide dismutase, and direct lipid peroxidation of the membrane, all of which are independent of the AcrAB-TolC efflux mechanism (EMA, 2018). The sustained activity of AUU against the MDR strain (ZI_ Aq. Ext. + Alc. Ext: 16.5 ± 0.71 mm; MIC: 8.0 mg/mL) confirms that hydroquinone is not a substrate for MDR efflux pumps, distinguishing it mechanically from fluoroquinolones and β-lactams. The low individual ZI values for VM (6–11 mm) do not indicate phytochemical inefficacy, but rather reflect the mechanistic specificity of Type-A proanthocyanidins (PACs). Disk-diffusion analysis measures diffusion-mediated growth inhibition - a direct mechanism of bactericidal or bacteriostatic killing. In contrast, PAC-mediated anti-adhesion operates through structural complementarity between the A-ring of PAC dimers/oligomers and the lectin-binding domains of *E. coli* FimH (Type 1 fimbriae) and PapG (P fimbriae) adhesins, preventing fimbrial anchoring without direct bacterial killing (Howell et al., 2005). This mechanism is entirely "invisible" to disk-diffusion assays but is precisely what prevents the initial colonization step of urinary tract infections (UTIs). Similarly, the MIC result (>32 mg/mL) reflects the lack of direct bactericidal activity rather than overall therapeutic futility—a distinction that reviewers of VM assay data often fail to appreciate. This observation aligns with the findings of another study Cisowska et al. (2016), demonstrated that *V. myrtillus* fruit extracts exert their primary anti-pathogenic effect not through direct killing, but by inhibiting *E. coli* adhesion and significantly modulating virulence gene expression.

3.6. The AUU Biofilm Paradox: A c-di-GMP Hormetic Response: The most scientifically provocative finding of this study is the diametrically opposed biofilm response of AUU against the MDR strain, depending on the extraction format. The ethanolic extract (SBI = 3; OD_{570} = 0.95) - the format with the highest direct antibacterial activity (ZI = 16.0 mm) - simultaneously induced maximum biofilm formation in the same strain. Conversely, the aqueous extract eliminated the biofilm (SBI = 0) despite its lower direct antibacterial potency (ZI = 13.5 mm). This paradox ($\Delta SBI = 3$; $\Delta OD_{570} = 0.57$; $p < .001$) is specific to the MDR strain and necessitates a mechanistic explanation beyond simple dose-response pharmacology. We propose that this paradox represents a hormetic c-di-GMP stress response specific to the MDR phenotype. The

high concentration of hydroquinone in the ethanolic extract creates a concentration-dependent, sublethal membrane stress, which the MDR efflux system (AcrAB-TolC) can partially neutralize - generating a chronic, subthreshold stress rather than rapid cell death. Under persistent sublethal stress, MDR *E. coli* activates the DksA/ppGpp stringent response pathway, which is further bolstered by c-di-GMP synthesis via membrane-associated diguanylate cyclases (DGCs, GGDEF domain proteins) (Stewart and Costerton, 2001). Elevated intracellular c-di-GMP levels activate the MrkHI/CsgD regulatory cascade, inducing the production of curli fibers and cellulose, upregulating EPS secretion genes (csg, bcs operons), and shifting the population from planktonic motility toward sessile biofilm formation - the classic 'biofilm bunker' hormetic response (Stewart and Costerton, 2001; Yadav et al., 2023). The sensitive ATCC 25922 and ESBL$^+$ strains, lacking the AcrAB-TolC overexpression, cannot sustain this state of partial resistance and instead undergo rapid killing before the biofilm adaptation program can be completed. The aqueous extraction eliminates the biofilm through two additional mechanisms absent in the ethanolic fraction: Gallotannins and ellagitannins in the aqueous phase act as competitive antagonists of the LuxR/RhlR Quorum Sensing (QS) receptor family, preventing the binding of N-acyl-homoserine lactone (AHL) and thus blocking the transcriptional activation of biofilm-specific gene clusters (including rhlAB, lasB, pqsA-E) (Rutherford and Bassler, 2012). Aqueous AUU polyphenols reduce bacterial surface hydrophobicity by interfering with outer membrane assembly, thereby reducing the propensity for surface attachment. This dual inhibition of Quorum Sensing (QS) and the anti-attachment mechanism explains the complete elimination of biofilm by the infusion, despite its lower direct antibacterial potency.

3.7. VM+AUU Synergy: Molecular Disarming and FIC 0.156: The FIC index of 0.156 — consistent across all three strains — places the VM+AUU interaction firmly within the category of strong synergy and represents the central applicable finding of this study. The potent synergy observed is likely driven by a multi-target mechanism where *Arctostaphylos uva-ursi* increases membrane permeability, thereby enhancing the intracellular accumulation of Vaccinium *myrtillus* polyphenols, while both extracts simultaneously disrupt biofilm integrity and quorum sensing coordination in MDR *E. coli*.

This value is particularly impressive against the MDR (multi-drug resistant) strain, where the individual MIC values of the plants (>32 and 8.0 mg/mL, respectively) would predict negligible combined efficacy according to an additive model; however, the combination achieves an MIC = 2.0 mg/mL, representing a 16-fold and 4-fold reduction, respectively. The molecular architecture of this synergy operates through three non-overlapping mechanisms that sequentially reinforce one another:

- Firstly, the fimbria blockade mediated by the PAC (proanthocyanidins) of VM prevents initial bacterial adhesion and surface attachment, maintaining the bacterial population in a planktonic, dispersed state, which is more susceptible to subsequent antimicrobial action. The disruption of the attachment step simultaneously reduces the incoming c-di-GMP signals from the surface-sensing second messenger pathway, lowering the activation threshold of bacterial QS (Quorum Sensing).
- Secondly, with the bacteria held in a planktonic state, the hydroquinone derived from the arbutin of the ethanolic fraction of AUU exerts direct bactericidal activity, unimpeded by the diffusion barriers of the EPS (biofilm matrix) (Schindler et al., 2002).

- Thirdly, the tannins from the aqueous fraction of AUU maintain QS silencing, preventing any coordinated adaptive response of the biofilm.

The combination thus simultaneously blocks adhesion (VM-PAC), kills planktonic cells (AUU-hydroquinone), and prevents coordinated rescue signaling via QS (AUU-tannins): a three-pronged strategy of 'molecular disarming' that explains both the strong FIC and the 91% reduction in biofilm. The clinical significance of reducing the MIC to 2.0 mg/mL extends beyond pharmacodynamics, as these phytochemical concentrations are achievable in human urine following oral administration, creating a direct bridge between *in vitro* findings and potential clinical application (Schindler et al., 2002; EMA, 2018).

3.8. Limitations and Future Direction: While these results are promising, this study was conducted in a controlled laboratory setting (in vitro). Several limitations must be acknowledged to contextualize the findings. First, the exceptionally high explanatory power of the regression model ($R^2 = .995$) reflects the rigorous laboratory control and the use of mean values from experimental replicates, which effectively minimized external variability. However, it is recognized that the sample size ($N = 18$) represents a statistical limitation that may increase the risk of overfitting. Consequently, the observed statistical power should be further validated in future studies utilizing a larger cohort of diverse clinical isolates.

Furthermore, for these findings to be fully applied to patients, more data is needed on how the body absorbs and excretes these compounds through the urine. To move this research forward, the next steps include:

- Pharmacokinetic Modeling: Future research must account for the human metabolic processing and renal clearance of arbutin and proanthocyanidins (PACs) to ensure that the synergistic concentrations (2.0 mg/mL) are consistently maintainable in human urine.
- Genomic Diversity: Testing the combination against a wider variety of drug-resistant bacteria (beyond the current $ESBL^+$ and MDR strains) is necessary to ensure it works across different genetic lineages.
- Clinical Translation: Moving from *in vitro* findings toward animal studies and early human clinical trials is a prerequisite to prove that the "molecular disarming" observed in the lab is equally effective in the human body.

Statistical Analysis — Multiple Linear Regression

To identify the key factors determining antibacterial efficacy, a multiple linear regression analysis was performed (IBM SPSS v.27) on N=18 experimental data points. The model achieved an exceptionally high explanatory power ($R^2 = .995$), with 99.5% of the total variance in inhibition zones explained by the predictor variables.

4.1. Model Significance and Predictive Factors: The regression ANOVA confirmed the model's high statistical significance: $F(3, 14) = 960.239$, $p < .001$(Table 5). Coefficient analysis (Table 6) identified Plant Type as the dominant predictor ($\beta^* = .941$; $p < .001$), where switching to AUU increased the inhibition zone by an average of 9.05 mm. Both Extraction Format ($\beta^* = .233$) and Bacterial Strain ($\beta^* = -.233$) were also highly significant factors ($p < .001$). These results quantify the "biological cost" of resistance while validating that the VM+AUU

synergistic combination and the optimized extraction format are the primary drivers of antibacterial success.

Table 5. ANOVA: Significance of the Regression Model.

Source	SS		df	MS	F	Sig.
Regression	414.389		3	138.130	**960.239**	**.000**[b]
Residual	2.014		14	0.144	—	—
Total	416.403		17	—	—	—

Note: F(3, 14) = 960.239, p < .001. [b] Predictors: Plant Type, Extraction Format, Bacterial Strain.

Conclusions

This study demonstrates that the combination of *Vaccinium myrtillus* and *Arctostaphylos uva-ursi* exerts a potent synergistic antibacterial effect against MDR and ESBL$^+$ *Escherichia coli* strains. Our in vitro findings suggest that the VM+AUU combination may effectively modulate MDR *E. coli* survival mechanisms, such as efflux pumps and quorum sensing coordination. The significant reduction in MIC values to 2.0 mg/mL across all strains and the effective eradication of biofilms - even in cases involving the "biofilm paradox" - highlight the therapeutic potential of this phytotherapeutic duo. The molecular synergy observed, characterized by a uniform FIC index of 0.156, indicates a multi-target approach that simultaneously addresses bacterial adhesion, planktonic survival, and intercellular communication. While these results are promising, they are based on a controlled experimental environment. Consequently, this study provides a scientifically-grounded framework that warrants further clinical investigation and pharmacokinetic validation to confirm its efficacy in the management of resistant urinary tract infections in human subjects.

Nomenclature

- AMR – Antimicrobial Resistance
- AUU – Arctostaphylos uva-ursi
- ATCC – American Type Culture Collection
- BSI – Biofilm Score Index
- EPS – Extracellular Polymeric Substance
- ESBL$^+$ – Extended-Spectrum Beta-Lactamase-producing
- FIC – Fractional Inhibitory Concentration
- MDR – Multidrug-Resistant
- MIC – Minimum Inhibitory Concentration
- QS – Quorum Sensing
- UTI – Urinary Tract Infection
- VM – Vaccinium myrtillus
- ZI – Zone of Inhibition
- R^2 – Coefficient of Determination
- β* – Standardized Coefficient
- VIF – Variance Inflation Factor
- p – Probability value

Acknowledgment

The authors would like to express their gratitude to the management and staff of the PHI Public Health Center – Skopje and Indusmedica Skopje – Diagnostic Laboratory for Biochemistry, Microbiology, Cytology, and Histopathology, North Macedonia, for their professional support and for providing the necessary facilities and expertise to conduct this research.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1]. Antimicrobial Resistance Collaborators. (2024). Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. The Lancet, 404(10459), 1189-1226. https://doi.org/10.1016/S0144-6736(24)01867-1.
[2]. Cisowska A, Wojnicz D, Hendrich AB. 2016. Vaccinium myrtillus fruit juices inhibit adhesion of Escherichia coli to Caco-2 cells and affect the expression of virulence genes. Evidence-Based Complementary and Alternative Medicine, 2016: 5055924.
[3]. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2023. Antimicrobial susceptibility testing: EUCAST disk diffusion method. Version 11.0. Växjö, Sweden: EUCAST.
[4]. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2023. Methods for the determination of MIC of aerobic bacteria by broth microdilution. Version 13.0. Växjö, Sweden: EUCAST.
[5]. European Medicines Agency (EMA). 2018. Assessment report on Arctostaphylos uva-ursi (L.) Spreng., folium. EMA/HMPC/572846/2009 Rev. 1. Amsterdam: EMA.
[6]. Foxman B. 2010. The epidemiology of urinary tract infection. Nature Reviews Urology, 7(12), 653–660.
[7]. Howell AB, Reed JD, Krueger CG, Winterbottom R, Cunningham DG, Leahy M. 2005. A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. Phytochemistry, 66(18): 2281–2291.
[8]. IBM Corp. 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.
[9]. Nikaido H. 2009. Multidrug resistance in bacteria. Annual Review of Biochemistry, 78, 119–146.
[10]. Rutherford ST, Bassler BL. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. Cold Spring Harbor Perspectives in Medicine, 2(11), a012427.
[11]. Savoia, D. (2012)
[12]. Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiology, 7(8), 979-990. https://doi.org/10.2217/fmb.12.68.
[13]. Schindler G, Patzak U, Brinkhaus B, et al. 2002. Urinary excretion and metabolism of arbutin after oral administration of Arctostaphylos uvae ursi extract as film-coated tablets and aqueous solution in healthy volunteers. J Clin Pharmacol, 42(8):920-927.
[14]. Stewart PS, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. Lancet, 358(9276), 135–138.
[15]. World Health Organization. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. WHO Press, Geneva.
[16]. Yadav, K., Prakash, S., & Phull, A. R. (2023). Biofilm formation in uropathogenic Escherichia coli: Mechanisms and therapeutic interventions. Microbiological Research, 266, 127244. https://doi.org/10.1016/j.micres.2022.127244.