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Phytochemical analysis and antimicrobial activity of blackberry (*Rubus fruticosus*) fruit extract against Gram-negative multidrug-resistant bacteria isolated from clinical samples

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ABSTRACT

While antibiotics remain foundational in treating microbial infections, their overuse has accelerated the emergence and global spread of multidrug-resistant (MDR) bacterial strains. This study aimed to characterize the phytochemical composition of a concentrated blackberry (*Rubus fruticosus*) extract and evaluate its antimicrobial potential against clinical MDR strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. Phytochemical quantification was performed using spectrophotometric, titrimetric, and HPLC analyses, while antimicrobial activity was determined via the well diffusion method and minimum inhibitory concentration (MIC) assays. The blackberry extract contained 0.54% total phenolic compounds and 4.60% organic acids; in comparison, a green tea leaf extract exhibited higher phenolic content (10.10%) but lower organic acids (1.60%). The total anthocyanin content of the blackberry extract was 159.81 mg/100 g, with cyanidin-3-O-glucoside (134.56 ± 0.10 mg/100 g) identified as the predominant constituent.

In silico molecular docking studies indicated that neither individual antibiotics nor isolated anthocyanins could effectively inhibit the full array of resistance mechanisms in Gram-negative bacteria. In contrast, *in vitro* assays demonstrated that the whole blackberry extract significantly inhibited the growth of all tested MDR strains. These integrated findings suggest that combating complex MDR pathogens requires a multi-target strategy. The broad-spectrum efficacy of the crude extract, likely arising from synergistic interactions among its constituents, underscores the potential of complex phytochemical preparations. Such natural formulations represent a promising avenue for the development of novel antimicrobial agents and may offer a viable strategy to restore the efficacy of existing antibiotics through adjunctive therapy.

INTRODUCTION

The rise of antimicrobial resistance (AMR) poses a paramount threat to global public health. Historical precedents from the conflicts in Iraq and Afghanistan first highlighted the emergence of resistant bacterial infections in combat zones [1]. This pattern persists; data from the conflict in Ukraine (2014-2020) demonstrated a substantially higher burden of multidrug-resistant (MDR) organisms in war-related wounds compared to general hospital settings [2].

In these environments, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* have been identified as the predominant pathogens. Most alarmingly, 71.3% of these Gram-negative isolates demonstrated resistance to carbapenems, antibiotics of last resort [3]. The severity of this threat was formally recognized in a March 2022 alert from the European Centre for Disease Prevention and Control (ECDC), which cautioned that patients with traumatic wounds could be carriers of resistant *A. baumannii* and *K. pneumoniae*, advising stringent isolation and screening protocols [4]. This warning was corroborated by clinical

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data from Germany, where 17% of 103 wounded patients admitted between March and June 2022 harbored resistant Gram-negative infections [5]. The rapid dissemination of these resistant strains underscores a critical imperative to develop novel antimicrobial agents.

In the search for alternative solutions, scientific attention is increasingly returning to traditional phytomedicines. Prior to the antibiotic era, plants such as raspberries, blackberries, and lingonberries were employed for centuries to manage infectious diseases [6]. These species accumulate bioactive compounds differentially: while leaves and shoots are rich in tannins, flavonoids, and hydroxycinnamic acids, the fruits are particularly abundant in anthocyanins and organic acids [7]. Notably, anthocyanins isolated from blackberry fruits have demonstrated promising antimicrobial activity against Gram-negative pathogens [8].

Consequently, the aim of this study is to characterize the phytochemical profile of a concentrated blackberry fruit extract and to evaluate its *in vitro* and *in silico* antimicrobial efficacy against clinical, multidrug-resistant strains of *A. baumannii*, *P. aeruginosa*, *K. pneumoniae*, and *E. cloacae*.

MATERIALS AND METHODS

Plant material

The study utilized the fruits of the common blackberry (*Rubus fruticosus*). The plant material was harvested from its natural habitat near the village of Ternova, Kharkiv region, Ukraine (coordinates: 50.193116, 36.669353). Collection was conducted during the 2021 fruiting season to ensure optimal ripeness and peak phytochemical content.

Reagents

All solvents and analytical standards were of analytical grade. Acetonitrile, acetic acid, and phosphoric acid were sourced from Allchem (Kharkiv, Ukraine). High-purity anthocyanin reference standards (purity ≥ 98.0%), including cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, cyanidin-3-O-malonyl glycoside, cyanidin-3-O-xyloside, and cyanidin-3,3'-diglucoside, were obtained from Sigma-Aldrich (Lublin, Poland).

Phytochemical analysis

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method [9], while the concentration of total catechins was measured via the vanillin assay [10]. Total anthocyanin content was assessed by molecular absorption spectrophotometry, with absorbance measured at 546 nm [11]. The total organic acid content was quantified through potentiometric acid-base titration, ensuring precise detection of the equivalence point [12].

Extraction method

Fresh *R. fruticosus* fruits (100.0 g) were pressed to obtain a pulp, which was subsequently subjected to maceration with a threefold volume of 96% (v/v) ethanol. Following filtration, the resulting filtrate was concentrated via rotary evaporation under reduced pressure at 50-60°C. The process was continued until a viscous crude extract with a residual moisture content of 25% was achieved.

HPLC analysis

Anthocyanin content was quantified using high-performance liquid chromatography. [8]

Test organisms

Clinical multidrug-resistant (MDR) isolates, specifically *Acinetobacter baumannii 150*, *Klebsiella pneumonia 18, Pseudomonas aeruginosa 18* and *Enterococcus cloacae 17*, were selected for investigation. These strains were isolated from respiratory specimens, including tracheal aspirates and bronchoalveolar lavage (BAL), and were provided by the Mechnikov Institute of Microbiology and Immunology.

Antimicrobial activity of extract

Antimicrobial activity was evaluated using the agar well diffusion method [13].

MIC assay

Minimum Inhibitory Concentrations (MICs), defined as the lowest concentration of the extract required to achieve complete inhibition of visible bacterial growth, were determined using the broth microdilution technique [14].

Molecular docking

Molecular docking simulations were performed using AutoDockTools 1.5.6. The three-dimensional crystal structures of six bacterial target proteins (resolution ≤ 2.40 Å) were retrieved from the Protein Data Bank (PDB) [15]: DNA gyrase (PDB ID: 1KIJ), dihydrofolate reductase (DHFR; 1RX3), LpxC deacetylase (3UHM), acyl-homoserine lactone synthases LasI (1RO5) and RhlI (1KZF), and diguanylate cyclase (3BRE). The ligand structures of the relevant anthocyanins were downloaded from PubChem [16] using their respective compound identifiers (CIDs). The protein active sites were identified using the CASTp server [17].

RESULTS

Analysis of phytochemical constituents

As shown in Table 1, the green tea leaf extract had a significantly higher total phenolic content (10.10 ± 0.25%) than the *R. fruticosus* (blackberry) thick fruit extract (0.52 ± 0.02%).

Conversely, anthocyanins were detected exclusively in the *R. fruticosus* extract at a concentration of 0.16 ± 0.002%, constituting approximately 30% of its total phenolic content. No anthocyanins were identified in the green tea leaf extract.

Analysis of organic acids revealed that the *R. fruticosus* extract contained a substantially higher concentration 4.60 ± 0.50%, which is approximately three times greater than that observed in the green tea extract (1.60 ± 0.10%). Consequently, the ratio of total organic acids to total phenolics differed markedly between the two preparations. In the *R. fruticosus* extract, organic acids were 8.8 times more abundant than phenolics. In contrast, the green tea extract exhibited a phenolic content 6.3 times higher than its organic acid content (Table 1).

Table 1. Total content of some biologically active compounds in the thick fruit extract of *R. fruticosus*

Analyzed Sample	Sum of polyphenols %±SD	Sum of anthocyanins %±SD	Sum of catechins %±SD	Sum of organic acids %±SD
R. fruticosus fruit thick extract	0.54±0.02	0.16±0.002	–	4.60±0.50
Green tea leaf extract	**10.10±0.25**	–	**10.47±0.25**	**1.60±0.10**

Note: Data are presented as mean ± SD (n = 3)

High-performance liquid chromatography (HPLC) was employed for the qualitative and quantitative determination of anthocyanins in the *R. fruticosus* fruit extract. The chromatogram (Figure 1) confirmed the presence of five anthocyanin compounds. Their quantitative distribution is summarized in Table 2.

Cyanidin-3-O-glucoside was identified as the predominant compound, accounting for 84.10% of the total anthocyanin content. This was followed by cyanidin-3,3′-diglucoside (7.38%) and cyanidin-3-O-malonyl glycoside (6.30%). Cyanidin-3-O-rutinoside was present in the lowest proportion (0.60%).

Table 2. Anthocyanin composition in the thick fruit extract of *R. fruticosus* as determined by HPLC analysis

№	Compounds	Rt, min	Identified compounds in extract, mg/100 g of extract ±SD	% out of total antocyanins
1	Cyanidin-3-O-glycoside	8.010	134.56 ±2.68	84.10
2	Cyanidin-3-O-rutinoside	9.560	0.96 ±0.20	0.60
3	Cyanidin-3-O-malonyl glycoside	14.723	10.08±0.20	6.30
4	Cyanidin-3-O-xyloside	15.110	2.40±0.05	1.50
5	Cyanidin-3,3′-diglucoside	16.450	11.81±0.26	7.38
The Total Antocyanins		**159.81**		

Notes: Data are presented as mean ± SD (n = 3)., n=3, Rt – retention time

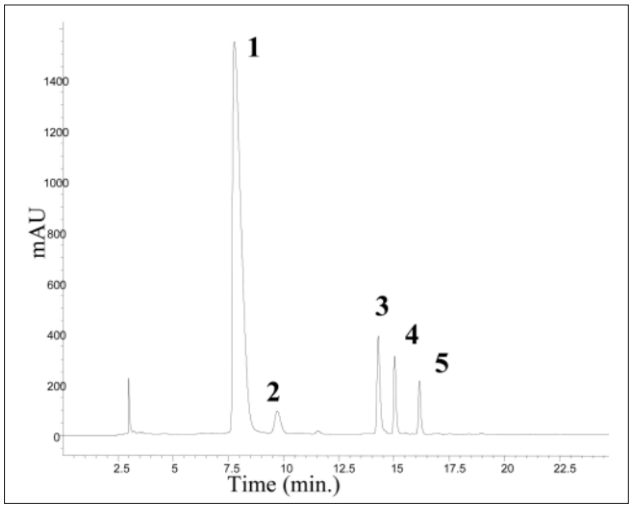

Figure 1. HPLC chromatogram of the thick fruit extract from *R. fruticosus*

Molecular docking analysis

To theoretically assess the antimicrobial potential of the identified anthocyanins, molecular docking studies were conducted against six key bacterial enzymes: DNA gyrase, dihydrofolate reductase (DHFR), deacetylase, acyl-homoserine lactone synthases (LasI and RhI), and diguanylate cyclase. Representative agents from six main antimicrobial classes were chosen as reference standards for comparative purposes: tetracyclines (doxycycline), aminoglycosides (gentamicin and netilmicin), fluoroquinolones (moxifloxacin and levofloxacin), β-lactams (cefepime, ceftazidime, and ceftriaxone), penicillins (amoxicillin), and amphenicols (chloramphenicol).

Although molecular docking is widely used in pharmacological research, many published studies lack a standardized framework for interpreting binding affinity results. Comparing ligands to a single reference compound is common practice, but it can be problematic because the choice of standard may vary and lead to inconsistent conclusions. To address this issue, we developed a conditional rating classification system based solely on calculated binding energy (ΔG) values from our study. The classification system categorizes affinity as follows: low affinity (-4.5 kcal/mol), medium affinity (-4.5 to -9.5 kcal/mol), and high affinity (-9.5 kcal/mol).

Molecular docking against dihydrofolate reductase (DHFR), with an active site characterized by interactions with NADP+, Asp30, Ile8, Phe34, Arg55, Arg60, Ile104, and Tyr110, identified several compounds with high binding affinity. Notable ligands included doxycycline, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, moxifloxacin, cyanidin-3,3’-diglucoside, and netilmicin (Table 3).

Subsequent modeling with the deacetylase enzyme (active site: Thr190, Lys238, Gly92, Phe191, Leu18, Ala206) also revealed high-affinity interactions for multiple ligands. These included cyanidin-3,3’-diglucoside, netilmicin, doxycycline, ceftazidime, moxifloxacin, cyanidin-3-O-glucoside, and cyanidin-3-O-rutinoside (Table 3).

Subsequently, docking studies were performed against acyl-homoserine lactone synthase LasI (AHS LasI). The defined active site (residues: Thr142, Thr144, Val143, Phe27, Arg30, Arg104, Met79, Leu102, Phe106, and Ser103) exhibited a high predicted binding affinity for only one compound: chloramphenicol. In contrast, levofloxacin exhibited the weakest binding affinity. Several compounds, including cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, cyanidin-3-O-malonyl glycoside, cyanidin-3-O-xyloside, gentamicin, ceftazidime, cefepime, and netilmicin, showed no significant interaction with the AHS LasI active site (Table 4).

For the related AHL synthase, RhlI (active site: Asp48, Tyr54, Met42, Leu63, and Leu56), high-affinity binding was predicted for cyanidin-3-O-rutinoside, doxycycline, and cyanidin-3-O-xyloside. Conversely, ceftriaxone demonstrated the weakest binding affinity. Notably, cyanidin-3-O-glucoside and gentamicin showed no significant interaction with this protein target (Table 4).

Finally, interactions with diguanylate cyclase (active site: Glu254, Glu253, Glu252, Lys327, Arg331, Thr262, Arg198, and Arg194) were evaluated. Based on our scoring classification, no compound achieved a high-affinity score for this enzyme. All tested ligands, with the exceptions of gentamicin and cefepime, were categorized within the medium-affinity range (Table 4).

To make the docking results easier to interpret, the compounds were categorized based on their predicted binding affinities. Category I comprised ligands exhibiting high affinity for the target enzyme's active site, while Category

Table 3. Molecular docking results of HPLC-identified compounds from *R. fruticosus* thick extract and reference antimicrobial drugs with DNA gyrase, DHFR, and deacetylase

№	Ligand	DNA-gyrase	Ligand	DHFR	Ligand	Deacytelese
	ΔGbind[a] (kcal/mol)					
1	**"Doxycycline"**	**-11.59**	**Doxycycline**	**-11.59**	Cyanidin-3.3'-diglucoside	-11.18
2	"Cyanidin-3-O-rutinoside"	-11.41	Cyanidin-3-O-glycoside	-11.42	**Netilmycine**	**-11.09**
3	"Cyanidin-3.3'-diglucoside"	-11.28	Cyanidin-3-O-rutinoside	-11.16	**Doxycycline**	**-11.03**
4	**"Moxifloxacine"**	**-10.29**	**Moxifloxacine**	**-10.89**	**Ceftazidime**	**-10.37**
5	"Cyanidin-3-O-glycoside"	-9.69	Cyanidin-3.3'-diglucoside	-10.79	**Moxifloxacine**	**-9.78**
6	**"Netilmycine"**	-9	**Netilmycine**	**-10.7**	Cyanidin-3-O-glycoside	-9.74
7	**"Levofloxacin"**	**-8.69**	**Ceftazidime**	**-9.49**	Cyanidin-3-O-rutinoside	-9.61
8	**"Cefepime"**	**-8.27**	**Levofloxacin**	**-8.98**	**Cefepime**	**-8.77**
9	"Cyanidin-3-O-xyloside"	-8.04	**Cefepime**	**-8.37**	**Levofloxacin**	**-8.34**
10	"Cyanidin-3-O-malonyl glycoside"	-7.46	Cyanidin-3-O-xyloside	-8.18	Cyanidin-3-O-xyloside	-8.28
11	**"Amoxicillin"**	**-7.24**	**Chloramphenicol**	**-7.97**	**Gentamycin**	**-7.45**
12	**"Ceftazidime"**	**-6.48**	Cyanidin-3-O-malonyl glycoside	-7.88	**Chloramphenicol**	**-7.19**
13	**"Chloramphenicol"**	**-6.38**	**Amoxicillin**	**-7.87**	Cyanidin-3-O-malonyl glycoside	-6.67
14	**"Ceftriaxone"**	**-4.61**	**Gentamycin**	**-6.78**	**Amoxicillin**	**-6.64**
15	**"Gentamycin"**	**-4.08**	**Ceftriaxone**	**-6.36**	**Ceftriaxone**	**-6.09**

Note: The predicted binding affinity (ΔG) is categorized as follows: low (ΔG > -4.5 kcal/mol; *red*), medium (-4.5 ≤ ΔG ≤ -9.5 kcal/mol; *orange*), and high (ΔG < -9.5 kcal/mol; *green*)

Table 4. Molecular docking results of HPLC-identified compounds from *R. fruticosus* thick extract and reference antimicrobial drugs with quorum-sensing (AHS LasI, AHS RhI) and biofilm-related (diguanylate cyclase) targets

№	Ligand	AHS LasI	Ligand	AHS RhI	Ligand	Diguanylate cyclase
	ΔGbinda (kcal/mol)					
1	**Chloramphenicol**	**-10.76**	Cyanidin-3-O-rutinoside	-12.94	**Doxycycline**	**-9.14**
2	**Ceftriaxone**	**-6.56**	**Doxycycline**	**-10.99**	**Ceftazidime**	**-8.06**
3	**Amoxicillin**	**-6.55**	Cyanidin-3-O-xyloside	-10.1	Cyanidin-3-O-rutinoside	-7.66
4	**Moxifloxacine**	**-6.34**	Cyanidin-3-O-malonyl glycoside	-9.37	Cyanidin-3-O-glycoside	-7.63
5	**Doxycycline**	**-4.99**	Cyanidin-3.3'-diglucoside	-8.95	**Chloramphenicol**	**-6.59**
6	**Levofloxacin**	**-4.11**	**Netilmycine**	**-8.36**	Cyanidin-3.3'-diglucoside	-6.49
7	Cyanidin-3-O-glycoside	—	**Moxifloxacine**	**-8.27**	Cyanidin-3-O-xyloside	-6.32
8	Cyanidin-3-O-rutinoside	—	**Amoxicillin**	**-7.41**	**Moxifloxacine**	**-6.3**
9	Cyanidin-3-O-malonyl glycoside	—	**Levofloxacin**	**-6.62**	**Netilmycine**	**-6.06**
10	Cyanidin-3-O-xyloside	—	**Ceftazidime**	**-6.43**	**Amoxicillin**	**-5.89**
11	Cyanidin-3.3'-diglucoside	—	**Chloramphenicol**	**-5.88**	**Levofloxacin**	**-5.32**
12	**Gentamycin**	—	**Cefepime**	**-5.05**	Cyanidin-3-O-malonyl glycoside	-5.26
13	**Ceftazidime**	—	**Ceftriaxone**	**-4.48**	**Ceftriaxone**	**-5.19**
14	**Cefepime**	—	Cyanidin-3-O-glycoside	—	**Gentamycin**	**-4.49**
15	**Netilmycine**	—	**Gentamycin**	—	**Cefepime**	**-4.40**

Note: *red* colour – low level of affinity ΔG <4.5 kcal/mol; *orange* colour – medium level of affinity 4.5<ΔG<9.5 kcal/mol; *green* colour – high level of of affinity ΔG>9.5 kcal/mol

II included those with moderate to low binding energies. This classification framework highlights the compounds with the highest therapeutic potential to disrupt specific bacterial targets.

To facilitate interpretation, the compounds were categorized based on their predicted binding affinity. Category I comprised ligands with high affinity for a given enzyme's active site, while Category II included those with medium or low binding energies. This classification identifies compounds that have the greatest potential to disrupt specific antimicrobial targets.

As summarized in Table 5, doxycycline demonstrated the broadest high-affinity profile among the antibiotics. It effectively targeted all primary defense enzymes ("first line of defense") and one biofilm-related mechanism (AHS LasI). In contrast, amoxicillin, cefepime, ceftriaxone, gentamicin, and levofloxacin did not demonstrate high-affinity binding to any of the evaluated targets.

Table 5. Categorization of reference antimicrobial drugs and identified anthocyanins based on predicted binding affinity

№	Compound	DNA-gyrase	DHFR	Deacytelese	AHS LasI	AHS RhI	Diguanylate cyclase	№ of inhibition enzymes of "First line of protection"	№ of inhibition enzymes of "Biofilm"
Antimicrobial drug standards									
1	**"Chloramphenicol"**							0	1
2	**"Levofloxacin"**							0	0
3	**"Gentamycin"**							0	0
4	**"Doxycycline"**							3	1
5	**"Ceftriaxone"**							0	0
6	**"Ceftazidime"**							1	0
7	**"Cefepime"**							0	0
8	**"Moxifloxacine"**							3	0
9	**"Netilmycine"**							2	0
10	**"Amoxicillin"**							0	0
Identified anthocyanins									
11	"Cyanidin-3-O-glycoside"							3	0
12	"Cyanidin-3-O-rutinoside"							3	1
13	"Cyanidin-3-O-malonyl glycoside"							0	0
14	"Cyanidin-3-O-xyloside"							0	1
15	"Cyanidin-3,3′-diglucoside"							3	0

Note: green colour – high level of inhibition; red colour – lower and medium of inhibition

Among the anthocyanins, cyanidin-3-O-rutinoside exhibited the most promising activity, with high-affinity predictions for the primary bacterial defense enzymes and the AHS LasI biofilm target. Conversely, cyanidin-3-O-malonyl glycoside did not demonstrate high-affinity binding to any of the studied mechanisms.

Antimicrobial and anti-fungi activity

This study evaluated the antimicrobial activity of a thick fruit extract from Rubus fruticosus (blackberry) against multidrug-resistant strains of *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*. The extract exhibited significant inhibitory effects against all tested pathogens.

The extract was most potent against *E. cloacae* and least inhibitory against *K. pneumoniae*, though it remained active against *A. baumannii* and *P. aeruginosa*. Notably, standard antibiotic susceptibility testing revealed that the *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* strains were resistant to all tested reference drugs. Only three reference antibiotics showed inhibitory activity against *E. cloacae*, while all others were ineffective (Table 6).

Minimum Inhibitory Concentration (MIC)

The *R. fruticosus* fruit extract significantly inhibited the growth of all tested antibiotic-resistant strains, as determined by its MIC. In this assay, the extract demonstrated potent and broad-spectrum activity. As shown in Table 7, the extract exhibited strong efficacy with an MIC of 1.13 µM against *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae*.

The MIC of the *R. fruticosus* fruit extract was determined and found to significantly inhibit the growth of all tested antibiotic-resistant strains. In this assay, the extract demonstrated potent, broad-spectrum activity. As shown in Table 7, the extract exhibited strong efficacy against *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae* with an MIC of 1.13 µM.

Table 6. Inhibition zone diameters (mm) for *Rubus fruticosus* thick extract and reference antibiotics against multidrug-resistant strains of *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae* (well diffusion assay)

Sample	Concentration mM	Inhibition zone diameter (mm) ± SD			
		A. baumannii 150	*K. pneumoniae 18*	*P. aeruginosa 18*	*E. cloacae 17*
Blackberry thick extract	0.009a	16.5 ±0.5	16.0 ±0.2	16.50 ±0.1	17.0 ±0.2
Chloramphenicol	1.86	#	#	#	#
Levofloxacin	0.28	#	#	#	#
Gentamycin	0.42	#	#	#	**12.0 ±0.1**
Doxycycline	1.35	#	#	#	**12.0 ±0.2**
Ceftriaxone	1.08	#	#	#	#
Ceftazidime	0.94	#	#	#	#
Cefepime	1.25	#	#	#	#
Moxifloxacine	0.23	#	#	#	#
Netilmycine	0.42	#	#	#	**11.0 ±0.3**
Amoxicillin	1.64	#	#	#	#

Notes: Data are presented as mean ± SD (n=3). The total phenolic content (a) is expressed as molar concentration in gallic acid equivalents; the symbol (#) indicates bacterial growth

Table 7. MIC of the *R. fructicocus* thick extract against the Gram-negative resistance strains

Analyzed Sample	MIC, µM			
	A. baumannii 150	*K. pneumoniae* 18	*P. aeruginosa* 18	*E. cloacae* 17
Blackberry thick extract	1.13	1.13	1.13	1.13

DISCUSSION

Analysis of Phytochemical Constituents

Biologically active substances in the *Rubus fruticosus* and green tea leaf extracts were characterized and quantified via spectrophotometric, titrimetric, and HPLC analyses. A comparative assessment revealed that the *R. fruticosus* fruit extract contained a substantially greater concentration of organic acids than the green tea extract. This difference likely reflects their distinct physiological roles: in fruits, organic acids serve as precursors for sugar biosynthesis, while in leaves, their primary function is related to photosynthesis, necessitating lower levels of accumulation [18].

The anthocyanin profile was determined by HPLC. For comparison, Fan-Chiang *et al*. [19] reported the following concentrations in a 70% acetone extract of *R. fruticosus* fruit: cyanidin-3-O-glucoside (80 mg/100 g), cyanidin-3-O-rutinoside (1 mg/100 g), and cyanidin-3-O-xyloside (6.5 mg/100 g). While the absolute quantities in our study were lower, cyanidin-3-O-glucoside was similarly the predominant anthocyanin in both investigations. These variations in phytochemical composition are expected and can be

attributed to factors such as the developmental stage and ripening status of the fruit, as well as differences in the cultivar.

Molecular docking

The global rise of multidrug-resistant (MDR) bacteria, or "superbugs," represents a critical threat to public health, creating an urgent need for novel antimicrobial agents. Natural compounds, including anthocyanins and catechins, have emerged as promising candidates. Gram-negative pathogens such as *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae* are particularly concerning because they frequently resist last-line antibiotics, including carbapenems [20,24].

Bacterial survival and resistance are mediated by multiple mechanisms. Effective inhibition requires targeting three primary pathways: (1) DNA replication via DNA gyrase, (2) metabolism via dihydrofolate reductase (DHFR), which is essential for folate synthesis [21], and (3) cell integrity via enzymes such as UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase, which contributes to the synthesis of unique Gram-negative outer membrane lipopolysaccharides [22]. Biofilm formation adds a major layer of defense by physically limiting antibiotic penetration. This process relies on quorum sensing mediated by acyl-homoserine lactone synthases (LasI and RhI) and cellular adhesion regulated by cyclic di-GMP, synthesized by diguanylate cyclase [20,23]. Therefore, a comprehensive antimicrobial strategy must address six key targets: DNA gyrase, DHFR, deacetylase, LasI, RhI, and diguanylate cyclase [25].

Our *in silico* molecular docking analysis revealed that no single antibiotic or isolated anthocyanin exhibited high-affinity binding across all six targets. Specific antibiotics showed selective profiles: chloramphenicol targeted only LasI, doxycycline was active against DNA gyrase, DHFR, deacetylase, and RhI, moxifloxacin interacted with DNA gyrase, DHFR, and deacetylase, and netilmicin was limited to DHFR and deacetylase.

These findings suggest that inhibition of MDR "superbugs" likely requires a multi-target approach, such as using complex phytochemical preparations or combining conventional antibiotics with natural extracts like blackberry.

Empirical testing confirmed the formidable challenge posed by clinical resistance. In our assays, no standard antibiotic was effective against *A. baumannii, K. pneumoniae*, or *P. aeruginosa*. For *E. cloacae*, only gentamicin, doxycycline, and netilmicin exhibited inhibitory effects, albeit with low potency. In stark contrast, the *R. fruticosus* thick fruit extract demonstrated significant inhibitory activity against all four resistant strains.

Notably, this broad-spectrum activity occurred despite the extract's relatively low anthocyanin concentration compared to clinical antibiotic doses. The extract's efficacy is likely due to phytochemical synergy. High concentrations of organic acids, approximately six times greater than total polyphenols, may complement the antimicrobial effects of anthocyanins, enhancing overall activity. This is consistent with prior studies on green tea leaf extracts, in which organic acids were identified as key contributors to antimicrobial activity.

This empirical efficacy is notable, considering the extract's relatively low anthocyanin content compared to clinical antibiotic concentrations. A key to its broad activity may be its phytochemical synergy. Phytochemical analysis revealed that the extract contains a high concentration of organic acids, present at levels six times greater than phenolic compounds. We hypothesize that these organic acids may inhibit bacterial mechanisms that anthocyanins weakly or not at all target, a premise supported by our prior research on green tea leaf extracts, in which organic acids were the primary contributors to the extracts' antimicrobial effects.

Our integrated theoretical and empirical findings support two main conclusions. First, the development of a single compound capable of broadly inhibiting MDR Gram-negative bacteria is improbable due to the multiplicity of essential and adaptive resistance mechanisms. Second, complex natural extracts, such as *R. fruticosus*, offer a promising alternative because their multi-component, synergistic actions can simultaneously disrupt several bacterial pathways.

Furthermore, plant-based formulations may offer safety advantages. Unlike broad-spectrum antibiotics, which can cause hepatotoxicity (*e.g.*, tetracyclines) or nephro- and ototoxicity (*e.g.*, aminoglycosides), natural compounds are generally associated with minimal adverse effects. Future strategies should focus on standardized, synergistic phytochemical preparations to effectively and safely counteract antimicrobial resistance.

Our integrated theoretical and practical findings lead to two major conclusions. First, the development of a single compound capable of broadly inhibiting MDR Gram-negative bacteria appears unlikely, given the multiplicity of essential and adaptive resistance mechanisms. Second, complex natural extracts like that from *R. fruticosus* offer a promising alternative due to their multi-component, synergistic action, which can simultaneously disrupt several bacterial pathways.

Furthermore, such natural formulations offer a significant safety advantage. Unlike many broad-spectrum antibiotics *(e.g.*, the hepatotoxicity of tetracyclines or the nephro- and ototoxicity of aminoglycosides), plant-based compounds are typically associated with minimal adverse effects. Therefore, future strategies should focus on developing standardized, synergistic compositions from natural sources that can effectively and safely combat antimicrobial resistance.

CONCLUSIONS

Our analysis confirms that crude blackberry extract represents a complex phytochemical matrix characterized by a high concentration of phenolic compounds, anthocyanins (notably cyanidin-3-O-glucoside), and organic acids. While *in silico* modeling revealed that no single conventional antibiotic effectively inhibits all key resistance pathways in Gram-negative bacteria, the total extract exhibited potent, broad-spectrum activity against multidrug-resistant (MDR) clinical isolates of *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae*. This disparity underscores that overcoming MDR requires a multi-target strategy, which

is inherently provided by the synergistic action of natural extracts. Consequently, standardized phytochemical preparations from *R. fruticosus* represent a promising avenue for antimicrobial development and may serve as adjuncts to potentiate the efficacy of existing drugs through rational combination therapies.

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