ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine
www.aaem.pl

Analysis of the health-promoting properties of herbal mixture B9 with the addition of whey powder

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Batjargal A, Kochan E, Rosicka-Kaczmarek J, Miśkiewicz K, Weremczuk-Jeżyna I, Grzegorczyk-Karolak I, Lichota A, Sienkiewicz M, Stańczak N, Urjin B, Koman D, Kwiatkowski P, Hymos A, Khurelbat D, Dudzińska E. Analysis of the health-promoting properties of herbal mixture B9 with the addition of whey powder. Ann Agric Environ Med. doi:10.26444/aaem/217848

Abstract

Introduction and Objective. Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal disorder characterized by recurrent disturbances in bowel habits and abdominal discomfort. Modulation of the gut microbiota and oxidative balance using plant-derived metabolites represents a promising supportive strategy for functional gastrointestinal disorders. The aim of this study is to evaluate the *in vitro* biological activity and cytocompatibility of the herbal mixture B9 supplemented with whey protein in the context of intestinal health.

Materials and Method. The phenolic profile of the B9 extract was characterized using UHPLC-ESI-MS/MS and included ellagic acid, gallic acid, chebulanin, chebulic acid, corilagin, and quercetin. Antioxidant activity was assessed using the DPPH assay; the B9 preparation exhibited strong radical scavenging activity (EC50 = 4.13 µg/mL), nearly six times higher than butylated hydroxytoluene (BHT) and comparable to Trolox. Antioxidant effects were further evaluated by measuring protein carbonyl group levels in Enterococcus faecalis ATCC 29212. Cytocompatibility was assessed by measuring the metabolic activity of normal intestinal epithelial cells (CCD 841 CoTr) using the MTT assay.

Results. The B9 preparation significantly reduced protein carbonyl levels in *E. faecalis*, indicating antioxidative activity. Co-treatment with whey increased metabolic activity of intestinal epithelial cells, with the strongest effects observed at 3.0% and 5.0% (v/v) of the B9 extract combined with 7.5% (v/v) of whey, indicating good cytocompatibility.

Conclusions. In conclusion, the B9 herbal preparation enriched with whey exhibits pronounced antioxidant activity and favourable cytocompatibility toward normal intestinal epithelial cells *in vitro*, supporting its further investigation in the context of intestinal health and functional gastrointestinal disorders such as IBS.

Key words

plant metabolites, *Terminalia chebula*, whey, antioxidant properties, intestinal epithelial cells

INTRODUCTION

Irritable Bowel Syndrome (IBS) represents a major public health issue, responsible for nearly 3% of all medical visits and accounting for about 40% of gastroenterology referrals. It is a chronic functional disorder of the gastrointestinal tract, manifested by recurrent disturbances in bowel activity, altered bowel habits, and abdominal pain or discomfort [1]. Depending on the predominant symptom, IBS is categorized into four main types: diarrhea-predominant (IBS-D), constipation-predominant (IBS-C), mixed (IBS-M), and unclassified (IBS-U) forms [2].

The pathogenesis of IBS is multifactorial, with significant evidence indicating disruptions in the intestinal microbiota as a major contributor. Both quantitative and qualitative changes in the microbial community have been linked to gastrointestinal dysfunctions and neuropsychological symptoms such as anxiety and depression. Functional bowel disorders are thus frequently associated with psychogenic

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Received: 11.06.2025; accepted: 28.01.2026; first published: 23.02.2026

components. The enteric nervous system (ENS) plays an essential role in coordinating intestinal motility and mediating the interplay between peripheral and central processes. It functions as a complex neuronal network maintaining bidirectional communication with the central nervous system (CNS), forming what is known as the brain–gut axis [1].

Recent research has shown that modulation of intestinal microbiota can influence immune responses [3]. In patients with IBS-D and IBS-C, disruption of the intestinal epithelial barrier has been documented, correlating with clinical manifestations, such as abdominal pain, bloating, and irregular bowel movements. Compared to healthy individuals, IBS patients exhibit reduced microbial diversity and compositional imbalance. Clinical studies demonstrated that rifaximin – a broad-spectrum, poorly absorbed antibiotic – can reduce abdominal pain and bloating in IBS by decreasing pathogenic bacterial populations and attenuating inflammation and intestinal permeability [4]. Therefore, microbiome-centred therapeutic strategies are of growing clinical importance [3].

Effective IBS management remains complex and requires a tailored therapeutic plan. Patient education, dietary modification, and psychological support form the cornerstone of non-pharmacological interventions. Mild forms of the disease can often be controlled through stress reduction and lifestyle adjustments, whereas pharmacological treatment is guided by the predominant symptom pattern [5].

Growing interest has focused on the therapeutic manipulation of intestinal microflora. In particular, the use of plant-derived metabolites offers a promising approach for mitigating IBS symptoms [6]. In this context, the aim of the presented study is to evaluate the biological activity and cytocompatibility of a combination of traditional Mongolian herbal extracts supplemented with whey protein, with relevance to intestinal health and functional gastrointestinal disorders.

Whey protein concentrate contains major proteins, such as β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and bovine serum albumin (BSA), along with bioactive components including lactoferrin (LF), lactoperoxidase (LP), immunoglobulins, and transforming growth factor β (TGF-β). These components are resistant to digestion and exhibit protective functions in the gut by downregulating pro-inflammatory cytokines produced by macrophages. Animal studies have demonstrated that fortifying infant formula with LF and TGF-β2 may prevent intestinal inflammation [7]. Hence, it is hypothesized that the addition of whey to herbal preparations may support their biological activity and cytocompatibility, potentially enhancing their relevance for intestinal epithelial function.

Several scientific publications have documented the significant effects of soy isoflavones and vitamin D in reducing inflammatory markers in plasma and protease activity in faeces among women with IBS. Other plant metabolites with proven IBS-related activity include resveratrol, curcumin, fennel, cumin, and pepper. *Terminalia chebula* is a particularly notable candidate which exhibits anti-anxiety, antidepressant, calming, and antioxidant properties. This raw material enhances digestion, relieves constipation, reduces intestinal spasms, alleviates visceral pain, and may contribute to the modulation of intestinal microflora [1].

MATERIALS AND METHODS

Preparation of samples. Herbal mixture B9 was obtained as dry powder (Mon-Intra Co., Ltd, Mongolia) which included: chebulic myrobalan fruit (*Terminalia chebula*) – 296 mg, bearberry fruit (*Arctostaphylos uva-ursi*) – 292 mg, pepper fruit (*Capsicum annuum*) – 120 mg, bedstraw herb (*Galium odoratum*) – 120 mg, Peruvian Maca root (*Lepidium meyenii*) – 120 mg, sage herb (*Salvia officinalis*) – 52 mg, cornflower (*Centaurea cyanus*) – 4 mg (Tab. 1). Whey powder was purchased from the District Dairy Cooperative in Krasnystaw, Poland.

Table 1. Constituents of herbal mixture B9

Constituent number	Name of plant	Family	Common name	Raw material	Content [mg]
1	*Arctostaphylos uva-ursi* (L.) Spreng.	*Ericaceae*	bearberry	fruit	292
2	*Terminalia chebula* Retz.	*Combretaceae*	chebulic myrobalan	fruit	292
3	*Capsicum annuum* L.	*Solanaceae*	pepper	fruit	120
4	*Galium odoratum* (L.) Scop.	*Rubiceae*	bedstraw	herb	120
5	*Lepidium meyenii* Walp.	*Brassicaceae*	Peruvian Maca	root	120
6	*Salvia officinalis* L.	*Lamiaceae*	sage	herb	52
7	*Centaurea cyanus* L.	*Asteraceae*	cornflower	flower	4

Preparation of B9 extracts. The dry powder of B9 and whey powder was weighed in the amount of 100 mg and was extracted in a thermoblock with orbital shaking (900 RPM) using 1 ml of deionized water. Extraction was carried out at ~95 °C for 60 min. Next, the samples were cooled down for 60 min to room temperature and centrifuged at 1,5000 × g for 10 min. The supernatant was filtered with 0.45 µm PES membrane and transferred to a new tube [8].

Qualitative and quantitative analysis of phenolic compounds by UHPLC-DAD. The quantitative determination of phenolic constituents in the herbal mixture was conducted based on the method proposed by Oracz et al. [9], with modifications introduced in our laboratory. Analyses were performed on a Dionex UltiMate 3000 HPLC + system, equipped with a UV–Vis detector and an Accucore C18 column, 2.6 µm, 150 × 2.1 mm i.d., (Thermo Scientific, PA, USA), maintained at 28 °C. Injection volume – 10 µl. Separation was carried out under gradient elution conditions using 1% formic acid in water as phase A and acetonitrile as phase B, at a flow rate of 0.35 mL min^{-1}. The gradient profile was as follows:
0 min – 1% B; 8 min – 5% B; 15 min – 8% B; 20 min – 10% B; 25 min – 15% B; 35 min – 20% B; 40 min – 25% B; 50 min – 90% B; 53 min – 90% B; 58 min – 1% B; 65 min – 1% B.

Identification of analytes was achieved through comparison with standard solutions. Detection was carried out at 280 nm for hydroxybenzoic acids, 320 nm for hydroxycinnamic acids, and 365 nm for flavonols. Data acquisition and processing were performed using Chromeleon 6.8.1 Chromatography Data System software.

Identification of phenolic compounds by UHPLC-ESI-MS/MS. Phenolic compounds in the extract were identified following the procedure of Oracz et al. [9], with minor adjustments. A Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (Thermo Scientific™) coupled to a UHPLC Transcend™ TLX-1 system was employed. Separation was achieved on an Accucore™ C18 column (2.6 µm, 150 × 2.1 mm i.d., (Thermo Scientific, PA, USA), thermostated at 28 °C with 10 µl injections.

The mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B), applied in a gradient at a flow rate of 0.35 mL min^{-1}, using the same gradient programme as in the UHPLC-DAD analysis. The mass spectrometer operated in both positive and negative ESI modes with a capillary voltage of 4500 V, capillary temperature of 325 °C, drying gas temperature of 400 °C, and nitrogen as the drying and collision gas (flow 10 L min^{-1}). The instrument was run in Parallel Reaction Monitoring (PRM) mode with collision energy of 25 eV and a resolution of 35,000. Spectra were collected within an m/z range of 50–750, and MS^2 data were acquired using high-energy collisional dissociation (HCD).

Data control and processing were performed with Q Exactive Tune 2.1, Aria 1.3.6, and Thermo Xcalibur software. Compound identification was based on retention times, UV–Vis spectra, full ESI–MS spectra, and MS/MS fragmentation patterns, verified against reference standards and literature data.

Antioxidant activity assays (DPPH, ABTS, and FRAP). The antioxidant potential of the B9 extract was evaluated using ABTS, DPPH, and FRAP assays. ABTS assay: the radical cation decolourisation test was carried out according to Re et al. [10]. The $ABTS^{+}$• solution was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate (Sigma-Aldrich) and allowing the mixture to stand for 16 h in darkness at room temperature. To perform the assay, 2 mL of extract (2–1000 µg mL^{-1}) was added to 2 mL of ABTS solution, and absorbance was read at 734 nm after 10 min. DPPH assay: free radical scavenging activity was estimated as described by Brand-Williams et al. [11]. Equal volumes (2 mL) of extract and 0.2 mM DPPH methanolic solution (Sigma-Aldrich) were mixed and incubated for 30 min at room temperature. Absorbance was measured at 517 nm. For both ABTS and DPPH tests, the results were expressed as EC_{50} values, corresponding to the extract concentration that achieved 50% radical scavenging.

FRAP assay: ferric reducing antioxidant power was determined according to Pulido et al. [12]. The FRAP reagent contained 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3$•$6H_2O$, and 0.3 M acetate buffer (pH 3.6) in a ratio of 2.5:2.5:45 (mL). The mixture was pre-incubated at 37 °C for 15 min. The reaction was initiated by combining 3 mL FRAP reagent, 0.3 mL water, and 0.1 mL extract, and absorbance was recorded at 595 nm. Antioxidant activity was expressed as µM Trolox equivalents per gram of extract.

All spectrophotometric readings were obtained using a UV-Vis Spectrophotometer (Beijing Rayleigh Analytical Instrument Corp., Beijing, China). Trolox and BHT (butylated hydroxytoluene) (Sigma–Aldrich) served as positive controls.

Quantification of protein carbonyl groups. Quantification of protein carbonyl groups is one of the indicators of oxidative damage [13, 14]. The study determined whether the tested compound affects the concentration of carbonyl groups in *Enterococcus faecalis* ATCC 29212. The bacteria were incubated with the compound with a final concentration of 190 µg/ml for 24 hours at 37 °C, under aerobic conditions. The spectrophotometric method with 2,4-dinitrophenylhydrazine (DNPH) was used to determine the concentration of carbonyl groups in proteins.

Cell cultures. Human normal colonic epithelial cells (CCD 841 CoTr) were cultured at 34 °C in a humidified atmosphere containing 5% CO_2, according to established protocols for this cell line, in a 1:1 mixture of RPMI 1640 and DMEM medium (Sigma-Aldrich). The combined medium was supplemented with 10% foetal bovine serum (FBS) and an antibiotic mixture containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma-Aldrich).

MTT assay. Cells were grown for 24 h in 96-well plates in 100 µL of culture medium per well, and supplemented with the B9 herbal extract at final concentrations of 2.5%, 3.0%, 5.0%, and 7.5% (v/v), either alone or in combination with 7.5% (v/v) whey.

The MTT assay is based on the conversion of a yellow tetrazolium salt to purple formazan crystals by metabolically-active cells, a reaction primarily catalyzed by mitochondrial succinate dehydrogenase. After removal of the MTT solution, the formazan crystals precipitated at the bottom of the wells were dissolved in 100 µL of DMSO per well. Absorbance was measured at 550 nm using an Infinite F50 Plus microplate reader (Crailsheim, Germany). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test, and differences were considered statistically significant at $p < 0.05$.

RESULTS

Phenolic compounds in B9 extract. B9 dry powder extract was subjected to UHPLC-ESI-MS/MS for the identification of phenolic compounds. Analysis showed the presence of 20 metabolites (Tab. 2) which included three groups of phenolic compounds: phenolic acids -being derivatives of hydroxybenzoic acid (metabolites 1–3, 10, 15) and hydroxycinnamic acid (metabolites 6 and 9), ellagitannins (metabolites 4–5, 7–8, 11–14, 17–18) and flavonoids (metabolites 16, 19–20)

HPLC analysis showed that phenolic acids were the richest fraction in terms of quantity in the B9 extracts, constituting 66.5% of all determined ingredients. Ellagic acid (157.57 mg/g d. w.) was the quantitatively dominant metabolite, not only among the group of phenolic acids, but also among all identified compounds (Fig. 1; Tab. 2). Corilagin (25.71 mg/g d.w.), chebulic acid (22.41 mg/g d.w.), and chebulanin (18.81 mg/g d.w.), were present in an amount approximately seven-times lower than ellagic acid. However, their contents were significantly higher than other ingredients belonging to ellagitannins, which accounted for almost 33% of the total content of identified phenolic compounds. Quercetin and its two derivatives represented flavonoids, but their level was low – only 1.6 mg/g d.w. (Fig. 2; Tab. 3).

Table 2. Characterization of phenolic compounds in studied extract by LC–-MS using ESI negative ion mode

Peak number	Retention time (RT) MS (min)	Phenolic compound	[M-H]- (m/z) a – precursor ion b – product ion	MS/MS (m/z)
1	2.02	Gallic acid	169.01[a] > 125.02[b]	81.03
2	4.98	Protocatechuic acid	153.02[a] > 109.03[b]	-
3	7.90	4-hydroxybenzoic acid	137.02[a] > 93.03[b]	-
4	9.72	Vescalagin	933.06	631.05
5	9.78	Punicalagin A	1083.05	781.05, 600.99
6	12.94	5-O-caffeoylquinic acid	353.09	191.05
7	13.66	Punicalagin B	1083.05	781.05, 600.99
8	15.72	Unidentified ellagitannin	1085.00	-
9	17.78	4-O-caffeoylqunic acid	353.09	191.05
10	18.62	Syringic acid	197.04	169.01
11	19.08	Chebulanin	651.00	169,01, 275.02, 481.06
12	20.35	Corilagin	633.07[a] >301.00[b]	169.01, 275.02, 463.05
13	26.27	Chebulagic acid	953.08[a] >301.00[b]	337.02, 615.06
14	26.53	Chebulic acid	355.00[a]>337.00[b]	301.00
15	26.48	Ellagic acid	301.00[a] > 301.00[b]	-
16	27.11	Quercetin 3-O-rutinoside	609.14[a]>300.03[b]	-
17	30.12	Casuarinin	935	-
18	30.22	Chebulinic acid	955.00>955.00	301.00, 617.07
19	34.80	Quercetin 3-rhamnoside	447.09[a]>301.03[b]	-
20	38.13	Quercetin	301.03[a]>151.00[b]	179.00

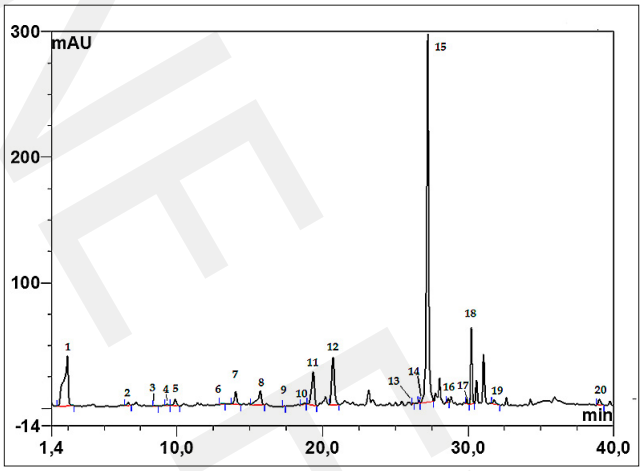

Figure 1. HPLC chromatogram demonstrated peaks of metabolites isolated from B9 extract. Peaks: 1) gallic acid (RT-2.4), 2) protocatechuic acid (RT-6.65), 3) 4-hydroxybenzoic acid (RT-8.45), 4) vescalagin (RT- 9.30), 5) punicalagin A (RT-9.89), 6) 5-O-caffeoylquinic acid (RT- 13.10), 7) punicalagin B (RT14.03), 8) unidentified ellagitannin (RT-15.72), 9) 4-O-caffeoylqunic acid (RT-17.36), 10) syringic acid (RT-18.77), 11) chebulanin (RT-19.35), 12) corilagin (RT-20.71), 13) chebulagic acid (RT-26.20), 14) chebulic acid (RT-26.61), 15) ellagic acid (RT-27.22), 16) quercetin 3-O-rutinoside (RT-28.62), 17) casuarinin (RT-29.89), 18) chebulinic acid (RT-30.22), 19) quercetin 3-rhamnoside (RT-31.79), 20) quercetin (RT- 39.01).
RT – retention time [min].

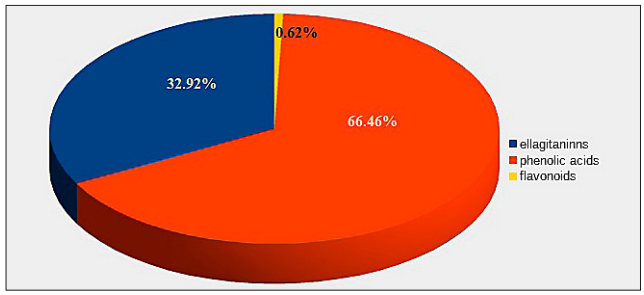

Figure 2. Percentage of total phenolic acids, ellagitannins and flavonoids in relation to all studied metabolites in B9 extracts

Table 3. Content of phenolic compounds in B9 extracts

Phenolic compound	Content [mg/100g]±SD
Phenolic acids	
4-hydroxybenzoic acid	16.23 ± 0.15
ellagic acid	**15756.81**±151.56
gallic acid	**1091.26** ±5.34
protocatechuic acid	56.99 ± 0.46
syringic acid	120.82 ± 1.15
5-O-caffeoylquinic acid	9.59 ± 0.09
4-O-caffeoylqunic acid	1.15 ± 0.04
Ellagitannins	
casuarinin	123.79 ± 1.28
chebulagic acid	27.87 ± 0.21
chebulanin	**1880.53** ± 16.67
chebulic acid	73.89 ± 0.65
chebulinic acid	**2240.56** ±2.30
corilagin	**2571.42** ± 12.78
punicalagin A	163.25 ± 1.54
punicalagin B	293.58 ± 2.17
unidentified ellagitannin	1010.10 ± 8.98
vescalagin	61.25 ± 0.56
Flavonoids	
quercetin	77.97 ± 0.79
quercetin 3-O-rutinoside	29.03 ± 0.31
quercetin 3-rhamnoside	52.87 ± 0.61

DPPH and ABTS radical scavenging assays. Results are expressed as the means of three replicates ± SD. Values marked with the same letter are not significantly different at $p < 0.05$, according to Tukey's multiple range test.

DPPH and ABTS radical scavenging assays were used to evaluate the antioxidant properties of B9 extract. In addition, the antioxidant activity of the extracts was evaluated based on their ability to reduce iron ions (FRAP). These antioxidant assays are often used to determine the antioxidant potential of plant extracts [15].

Table 4. The DPPH and ABTS radical scavenging assays

Antioxidant assay	B9	BHT	Trolox
FRAP mM Trolox/g	5.28 ± 0.02[a]	3.67 ± 0.03[b]	-
DPPH EC_{50} (µg/mL)	4.13 ± 0.06[a]	24.20 ± 1.24[b]	4.72 ± 0.0[a]
ABTS EC_{50} (µg/mL)	1.81± 0.06[a]	10.02 ± 0,51[b]	4.44 ± 0.05[c]

In the DPPH and ABTS assays, the antioxidant activity was expressed as the ability to reduce the initial absorption of DPPH or ABTS by the extracts at different concentrations. B9 extract showed great DPPH scavenging capacity (EC50 = 4.13 µg/g). The EC50 value obtained for B9 extract was almost six times higher compared to synthetic antioxidant BHT and comparable to Trolox (Tab. 4). The ABTS test revealed high free radical scavenging activity by extract B9 (EC50 = 1.81 µg/mL), this antioxidant potential was stronger with compared to BHT and Trolox. The FRAP test showed that B9 extract exhibited strong reducing power (5.28 mM Trolox/g). The activity of B9 extract was also significantly higher compared to BHT (Tab. 4).

Concentration of carbonyl groups in Enterococcus faecalis assay. Based on the obtained test results (Fig. 3), a decrease in the concentration of carbonyl groups in *E. faecalis* after administration of the tested compound was demonstrated compared to the control group (bacteria not treated with methanol extract from the herbal mixture). This result may indicate the antioxidant properties of the tested herbal mixture against *E. faecalis* ATCC 29212.

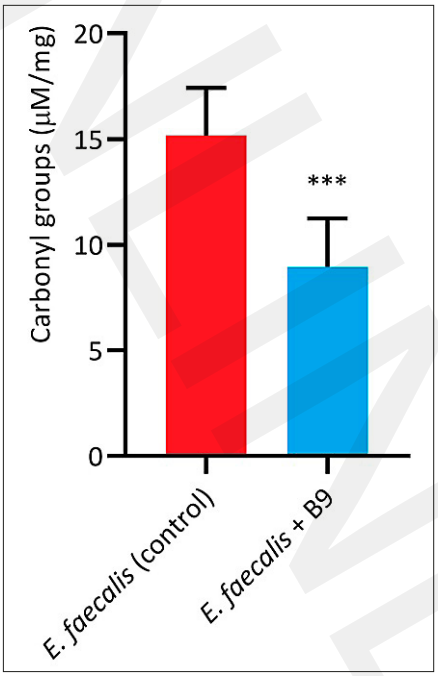

Figure 3. Concentration of carbonyl groups expressed in µM/mg of protein in *Enterococcus faecalis* without and after incubation (24 h, 37 °C, aerobic conditions) with B9 (190 µg/ml). *** $P<0.001$

MTT assay using normal intestinal epithelial cells (CCD 841 CoTr). The metabolic activity of normal intestinal epithelial cells (CCD 841 CoTr) was evaluated using the MTT colorimetric assay. The cells were exposed to the B9 herbal mixture at final concentrations of 2.5%, 3.0%, 5.0%, and 7.5% (v/v), as well as to whey at a single concentration of 7.5% (v/v).

Treatment with the B9 herbal mixture alone did not reduce cellular metabolic activity compared to control cells. In contrast, co-treatment with whey resulted in a significant increase in metabolic activity, particularly at 3.0% and 5.0% (v/v) of the B9 mixture (Fig. 4). These results indicate good cytocompatibility of the tested preparations and a concentration-dependent enhancement of metabolic activity in the presence of whey.

Effect of the B9 herbal mixture alone (red bars) and in combination with whey (7.5% v/v; blue bars) on the metabolic activity of CCD 841 CoTr cells (1×10^5 cells/mL) after 24 h of incubation. Results are expressed as a percentage of control values (set to 100%). Data represent means ± SD (n = 3). Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$).

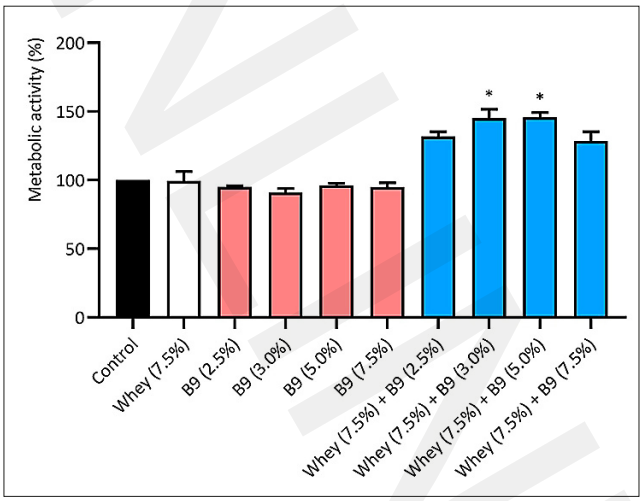

Figure 4. Measurement of cell metabolic activity using the MTT assay

Microscopic image B demonstrated that treatment with the B9 herbal mixture combined with whey resulted in increased cell density and confluence of normal intestinal epithelial cells (CCD 841 CoTr) compared to the untreated control (A), without visible signs of cytotoxicity or morphological alterations (Fig. 5).

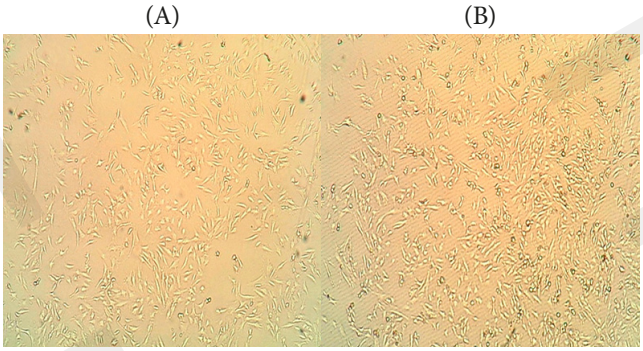

Figure 5. Microscopic images of normal intestinal epithelial cells (CCD 841 CoTr). (A) Untreated control cells. (B) Cells treated with the B9 herbal mixture (3.0 %) and whey (7.5 %) for 24 h

DISCUSSION

The dominant components of the analyzed B9 mixture were powdered *Terminalia chebula* fruit in quantity of 296 mg, and bearberry fruit (Arctostaphylos uva-ursi) in quantity of 292 mg. Terminalia chebula is a tree belonging to the native flora of southern and southeast Asia, from the *Combretaceae* family. Chebulinic acid and other ellagitannins from *T. chebula* show numerous biological activities, including

antitumour, antiatherogenic, anti-inflammatory, antioxidant, hepatoprotective, and antidiabetic effects [16]. According to the review article by Bag et al. [17], among phenolic acids, ellagic acid is responsible for antibacterial activity against intestinal bacteria such as *Clostridium perfringens* and *Escherichia coli*, and gallic acid also shows antibacterial, antiviral, and antioxidant action.

The current phytochemical analysis of the tested herbal mixture B9 showed a high content of phenolic acids – ellagic acid (15,756.81 mg/100 g) and gallic acid (1,091.26 mg/100 g). The dominant ingredients with proven health-promoting effects were also ellagitannins – corilagin (2,571.42 mg/100 g), chebulinic acid (2,240.56 mg/100 g), and chebulanin (1,880.53 mg/100 g). Corilagin, a secondary metabolite of plants from the *Combretaceae* family, is known for its antioxidant and anti-inflammatory properties [18].

The second main component of the tested B9 mixture is bearberry (*Arctostaphylos uva-ursi*), a shrub from the *Ericaceae* family, native to the circumboreal region and subarctic Northern Hemisphere, but has spread to many regions of Europe and Asia. In bearberry fruits, the dominant active compounds are also phenolic acids such as ellagic and gallic acids, hence their high content in the B9 mixture. Phenolic acids are known for their antioxidant and anti-inflammatory properties. For example, gallic acid can decrease pro-inflammatory cytokine release (IL-6, TNF-α, and IL-1β), inhibit cell proliferation and adhesion (inflammatory infiltrate), hence its usefulness in the treatment of inflammatory diseases [19, 20, 21].

Other compounds with rich medicinal potential described in recent scientific literature were also identified in the tested B9 mixture, including syringic acid (120.82 mg/100 g), casuarinin (123.79 mg/100 g), punicalagin A (163.25 mg/100 g), punicalagin B (293.58 mg/100 g), and quercetin (77.97 mg/100 g). Somade et al. [22] proved that syringic acid can significantly decrease IL-6, TNF-α, iNOS, COX-2, and NF-κB levels in induced inflammation, and also reduce oxidative stress markers, showing antioxidant effects [23] in rat models. Punicalagin, an ellagitannin polyphenol, also exhibits antioxidant and anti-inflammatory properties [24], by inhibiting TNF-α, IL-1β, and IL-6 production in RAW264.7 macrophages. Quercetin protects against oxidative stress, maintains oxidative balance by inducing GSH synthesis and increasing the expression of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) [25].

Some studies suggest that *Enterococcus faecalis*, through overgrowth or translocation within the gastrointestinal tract, may induce inflammation of the intestinal mucosa by producing pro-inflammatory cytokines and activating leukocytes. Moreover, like many other bacteria, Enterococcus faecalis employs strategies to evade immune surveillance, leading to dysbiosis and subsequent pathological changes. A strong relationship exists between bacterial dysbiosis associated with Enterococcus faecalis and the development of IBS symptoms [26]. It has been proven that *E. faecalis* produces significant amounts of extracellular superoxide (O2–) and reactive oxygen species, such as hydrogen peroxide (H2O2) and hydroxyl radicals, which can damage colonic epithelial cells and DNA both *in vitro* and *in vivo* [27].

Since oxidative stress contributes to protein modification, damaging their structure and impairing biological functions, the presented study investigated whether the herbal preparation B9 has a protective effect. Based on the obtained findings, it can be concluded that the B9 herbal mixture exhibits antioxidant activity against *E. faecalis*.

It has been established that phenolic compounds in herbal mixtures possess 3–5 times greater antioxidant capacity than vitamins C or E *in vitro* [28]. Previous studies by the current authors have also demonstrated the strong antioxidant activity of a broad spectrum of phenolic compounds in another tested herbal mixture, Gurgem-7 [8]. The research showed that the B9 herbal mixture has a robust antioxidant effect against *Enterococcus faecalis*, which may be relevant in the context of intestinal oxidative stress associated with functional gastrointestinal disorders, including IBS. Additionally, the protective effect of the herbal mixture on the intestinal mucosa is enhanced by the addition of whey powder, which contains antimicrobial whey proteins.

Whey proteins are known for their antimicrobial and immunomodulatory properties [29]. The increase in MTT signal observed in normal intestinal epithelial cells treated with whey and the B9 herbal mixture, should be interpreted primarily as an enhancement of cellular metabolic activity rather than a direct increase in cell number. Whey is known to contain bioactive peptides, amino acids, and micronutrients that may support epithelial cell metabolism and mitochondrial function. Therefore, the observed effects likely reflect the nutritive and trophic properties of whey, as well as the good cytocompatibility of the herbal formulation. Importantly, none of the tested concentrations induced a decrease in metabolic activity, indicating the absence of cytotoxic effects toward normal intestinal epithelial cells. Research indicates that cow's milk contains hundreds of naturally occurring peptides, many homologous to antimicrobial and immunomodulatory peptides such as globulin, α-lactalbumin, lactoferrin, bovine serum albumin, immunoglobulins, and lactoperoxidase [29]. Additionally, cow's milk contains B vitamins, including vitamin B5, essential for the production of coenzyme A (CoA), a crucial cofactor in numerous enzymatic reactions related to energy metabolism. Therapeutic strategies aimed at maintaining CoA balance are promising, as they improve immune cell function [30]. Although the present study did not directly assess epithelial barrier function, the preserved morphology and increased metabolic activity of normal intestinal epithelial cells suggest good cytocompatibility of the tested formulation.

CONCLUSIONS

The presented analysis indicates that the herbal mixture B9, particularly when supplemented with whey, exhibits biologically relevant *in vitro* activities, including antibacterial effects against *Enterococcus faecalis* and pronounced antioxidant properties. Moreover, the formulation showed good cytocompatibility toward normal intestinal epithelial cells and supported cellular metabolic activity without signs of cytotoxicity.

Taken together, these findings suggest that B9 supplemented with whey may represent a promising supportive formulation for further investigation in the context of intestinal health and functional gastrointestinal disorders, such as irritable bowel syndrome. However, additional studies, including *in vivo* and clinical investigations, are required to confirm its therapeutic potential.

Funding

This work was supported by the Medical University of Lublin, Grant number DS 675.

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