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Antiadhesive activity of hydroethanolic extract from bean pods of *Phaseolus vulgaris* (common bean) against uropathogenic *E. coli* and permeability of its constituents through Caco-2 cells monolayer

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ABSTRACT

Ethnopharmacological relevance: Phaseaoli pericarpium (bean pods) is a pharmacopeial plant material traditionally used as a diuretic and antidiabetic agents. Diuretic activity of pod extracts was reported first in 1608. Since then *Phaseoli pericarpium* tea figures in many textbooks as medicinal plant material used by patients.

Aim of the study: Despite the traditional use of extracts from *Phaseolium vulgaris* pericarp, limited information is available on bioactivity, chemical composition, and bioavailability of such preparations. The following study aimed to investigate the phytochemical composition, the *in vitro* permeability of selected extract's constituents over the Caco-2 permeation system, and potential antivirulence activity against uropathogenic *Escherichia coli* of a hydroalcoholic *Phaseoli pericarpium* extract (PPX) *in vitro* to support its traditional use as a remedy used in urinary tract infections.

Material and methods: The chemical composition of the extract PPX [ethanol:water 7:3 (*v/v*)] investigated by using UHPLC-DAD-MS^n and subsequent dereplication. The permeability of compounds present in PPX was evaluated using the Caco-2 monolayer permeation system. The influence of PPX on uropathogenic *E. coli* (UPEC) strain NU14 proliferation and against the bacterial adhesion to T24 epithelial cells was determined by turbidimetric assay and flow cytometry, respectively. The influence of the extract on the mitochondrial activity of T24 host cells was monitored by MTT assay.

Results: LC-MS^n investigation and dereplication, indicated PPX extract to be dominated by a variety of flavonoids, with rutin as a major compound, and soyasaponin derivatives. Rutin, selected soyasaponins and fatty acids were shown to permeate the Caco-2 monolayer system, indicating potential bioavailability following oral intake. The extract did not influence the viability of T24 cells after 1.5h incubation at 2 mg/mL and UPEC. PPX significantly reduced the bacterial adhesion of UPEC to human bladder cells in a concentration-dependent manner (0.5–2 mg/mL). Detailed investigations by different incubation protocols indicated that PPX seems to interact with T24 cells, which subsequently leads to reduced recognition and adhesion of UPEC to the host cell membrane.

Conclusions: PPX is characterised by the presence of flavonoids (e.g. rutin) and saponins, from which selected compounds might be bioavailable after oral application, as indicated by the Caco-2 permeation experiments. Rutin and some saponins can be considered as potentially bioavailable after the oral intake. The concentration-dependent inhibition of bacterial adhesion of UPEC to T24 cells justifies the traditional use of *Phaseoli pericarpium* in the prevention and treatment of urinary tract infections.

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1. Introduction

List of abbreviation

anti-UTI	against urinary tract infections
Caco-2	colonic adenocarcinoma Caco-2
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HBSS	Hanks' balanced salt solution
HMPC	The Committee on Herbal Medicinal Products of European Medical Agency EMA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NU14	uropathogenic *E. coli* strain
$OD_{640\ nm}$	optical density, determined at $\lambda = 640$ nm
PET	polyethylene terephthalate
PPX	*Phaseoli pericarpium* ethanol – water extract 7:3 (*v/v*)
T24	human epithelial cell line from bladder carcinoma (ATTC HTB-4)
TEER	transepithelial electrical resistance
TLC	thin layer chromatography
UHPLC-MS	ultra-high-performance liquid chromatography - hyphened with mass spectrometry
UPEC	uropathogenic *E. coli*
UTI	urinary tract infection

Common bean (*Phaseolus vulgaris* L.) is an annual plant, cultivated all over the world for its edible seeds. Besides the high nutritional value of the seeds and the respective processed products (Messina, 2014), the ripe seed extract containing alpha-amylase inhibiting glycoproteins is used in the weight loss supplements (Udani et al., 2018). However, the common bean pericarp, freed of the seeds, is widely used in European traditional medicine. The bean pod (pericarp) is referred to as *Phaseoli pericarpium* or *Fructus Phaseoli* sine *semine* and is monographed in the 11th edition of Polish Pharmacopoeia (PPXI) ("Polish Pharmacopoeia XI," 2017a). The quality of the herbal material is related to the content of phenolic acids, calculated as caffeic acid (minimum 0.01% *w/w*, related to the dried product). The herbal material is also part of a widely used mixture of medicinal herbal materials used for its diuretic effects (*Species diureticae*) (Länger, 2017; "Polish Pharmacopoeia XI," 2017b).

Phaseoli vulgaris L. *fructus* sine *semine* has also been monographed by the Committee on Herbal Medicinal Products (HMPC) of the European Medicine Agency (European Medicines Agency, 2013). The respective monography describes the traditional use of the herbal substance and respective herbal preparations as an adjuvant in uncomplicated urinary tract infections (UTI) by flushing of the urinary tract due to increased urine. Additionally, the HMPC monograph described the mild antidiabetic activity of the herbal material and the respective extract preparations.

Traditionally the herbal material has also been used to treat diabetes, but a detailed investigation of a potential antidiabetic effect of an aqueous pericarp extract indicated only significant glucose-lowering activity at relatively high concentrations, and thus, further investigations have been discontinued (Helmstädter, 2010). Diuretic activity of *Phaseoli pericarpium* water infusion has been reported in historical monographies (Dodoens, 1608). Since then bean pod infusion has been reported in many relevant textbooks as a diuretic - traditionally used for UTI and also as an adjuvant for treatment of arthritis and gout (Van Hellemont, 1985; Wichtl, 1994).

UTIs are one of the most common bacterial infections as they are affecting 150 million people each year with health care costs approximately US $3.5 billion in 2015 in the United States alone (Flores-Mireles et al., 2015). According to the European Association of Urology and European Section of Infection in Urology UTIs can be divided into two groups: uncomplicated (asymptomatic bacteriuria, acute uncomplicated cystitis and uncomplicated pyelonephritis and recurrent infections) and complicated (connected with acute infections of the kidney, fever, abdominal pain infections, obstructions within the urinary tract and risk factors such as male sex, neurogenic disturbances, nephropathic diseases) (European Association of Urology, 2020). UTIs are mainly caused by uropathogenic *Escherichia coli* (UPEC), but also *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, and *Proteus mirabilis* can contribute. In most cases, different UPEC strains with differing phenotypes and biochemical properties are responsible for both uncomplicated and complicated UTIs (Foxman, 2010). The significant phylogenetic group distribution difference between UTI isolates and faecal *E. coli* has been observed, as UPEC is characterised by distinct and specific virulence factors (Bahadori et al., 2019; Mojaz-Dalfardi et al., 2020).

Phytotherapeutic strategies against UTI were superseded with antibiotics in the 20th century (Nickel, 2005). However, due to increasing antibiotic resistance, alternative therapy strategies, including phytotherapeutic approaches, should be reconsidered or developed (Mazzariol et al., 2017; WHO, 2014). Moreover, it is necessary to counter recurrent UTIs, which frequently occur after antimicrobial therapy. Incidences of recurrent UTI after 3–4 months subsequent standard antibiotic treatment have been observed in up to 30% of adult women (Foxman et al., 2000). This might be associated with the immune escape strategy of the bacteria by creating biofilm-like intracellular bacterial communities in the bladder cells (Foxman and Buxton, 2013).

The present study aimed to investigate the potential influence of bean pod extract against UPEC, especially UPEC proliferation and UPEC-specific adhesion virulence. Additionally, phytochemical studies were to be performed to get a more detailed insight into the extract composition, also related to a potential investigation on the permeability of defined extract components over an *in vitro* Caco-2 model.

2. Materials and methods

2.1. General experimentation procedures

Purification of water used for HPLC was performed with the use of the Simplicity System (Merck KDaD, Darmstadt, Germany). Solvents used for chromatography (HPLC grade), methanol used to dissolve samples before UHPLC-DAD-MS^n analysis (gradient grade) and ethanol used for the extraction of plant material (analytical grade) were obtained from POCh (Gliwice, Poland). DMSO used for the MTT test was of analytical grade and was purchased from Sigma Aldrich (Saint Louis, MO, USA). Triton X and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Carl Roth (Karlsruhe, Germany).

2.2. Plant material

Common bean pericarp, *Phaseoli pericarpium*, was purchased from Kawon, Poland (batch number: 598.2019). A voucher specimen is deposited in the Herbarium of the Department of Pharmacognosy and Molecular Basis of Phytotherapy under the number [PP598.2019]. Plant material identity was confirmed by Prof. Sebastian Granica, using microscopic and TLC identification methods described in detail in the monograph of the 11th edition of Polish Pharmacopoeia ("Polish Pharmacopoeia XI," 2017a).

2.3. Preparation of hydroethanolic extract

Ethanolic extract was acquired by three-step extraction of 50 g of plant material with EtOH:water (7:3 *v/v*). The process was performed in an ultrasonic bath at 40 °C for 30 min, using 500 mL of solvent each step. Subsequently, the extract portions were filtered using paper filters (Whatman qualitative paper grade 1 (GE Healthcare, Buckinghamshire, UK)), combined and concentrated under reduced pressure followed by lyophilization to yield 3.86 g of *Phaseoli pericarpium* extract (PPX), corresponding to 7.72%, related to the starting material.

2.4. Chromatographic analysis

The UHPLC-DAD-MS^n analysis of PPX (10 mg of extract, dissolved in 1 mL MeOH:H_2O 1:1 (*v/v*)) and the samples from permeability experiments were performed on a UHPLC-3000 RS system (Dionex, Leipzig, Germany), equipped with a DAD detector and splitless connection to an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik GmbH, Bremen, Germany). UV spectra were recorded from λ = 200–450 nm. The parameters of the MS unit were as follows: nebulizer pressure: 40 psi, drying gas flow rate: 9 L/min, nitrogen gas temperature 300 °C, and capillary voltage: 4.5 kV. The mass spectra were registered by scanning from *m/z* 70 to 2200. Kinetex XB-C_{18} chromatography column was used (Phenomenex, Torrance, CA, 150 mm; 2.1 mm; 1.7 μm). The mobile phase (A): was H_2O:formic acid (99.9:0.1, *v/v*), and the mobile phase (B) was acetonitrile:formic acid (99.0:0.1, *v/v*). The gradient program was 0–60 min 5–26% B, 60–80 min 26–90% B, and the flow rate was 0.3 mL/min. The injection volume was 3 μL for the PPX solution, 2 μL for donor side samples, and 10 μL for acceptor side samples. The column oven temperature was set to 25 °C. The PPX sample was filtered through a 0.45 μm PET syringe filter (Kinesis, Cambridge, UK) prior to chromatographic analysis.

2.5. Methods of microbiology and cell biology

2.5.1. Cell lines

The T24 cell line (ATCC HTB-4) is a human epithelial cell line from the bladder carcinoma of an 82-year-old Swedish female. The cultivation of the cells was performed in Dulbecco's Modified Eagle Medium (DMEM) with high glucose and L-glutamine, supplemented with 10% (*v/v*) Fetal Bovine Serum (FBS), and 0.5% (*v/v*) penicillin/streptomycin at 37 °C and 5% CO_2. All chemicals used for cell culture were obtained from Biochrom (Berlin, Germany).

The Caco-2 cell line used in experiments was obtained from the German Collection of Microorganisms and Cell Cultures DSMZ, Leibnitz Institute, Braunschweig, Germany. The cells were cultured in 75 mL cell culture bottles with seeding density 1×10^5 cells/mL in DMEM supplemented with 20% FBS (*v/v*), 100 IU/mL penicillin, and 100 μg/mL streptomycin, and in 12.5 mL of medium. Three times a week, the cells were double-washed with 5 mL of Dulbecco's Phosphate Buffer Saline (DPBS) and the medium was changed. Passaging was performed at 80% confluency. All cultures and test plates were maintained at 37 °C in an atmosphere of 5% CO_2. Hank's Balanced Salt Solution (HBSS) was used as a transport medium in the transport experiments. All chemicals for cultivation were purchased from Biowest (Nuaillé, France).

2.5.2. Bacterial strains

Uropathogenic *E. coli* NU14 strain, cystitis isolate from a bladder-infected patient (Johnson et al., 2001) was provided by Prof. Dobrindt (University of Münster, Germany). Defrosted stocks of bacterial cultures were incubated at 37 °C for 24 h or 48 h on agar plates. Growth medium was prepared from agar 15 g (Merck, Darmstadt, Germany), Bacto-Tryptone 10 g (BD Biosciences, Franklin Lakes, USA), NaCl 8 g (AppliChem, Darmstadt, Germany), glucose 1 g (Merck, Darmstadt, Germany), granulated yeast extract 1 g (Merck, Darmstadt, Germany), $CaCl_2$ 2 g (Merck, Darmstadt, Germany), deionised water 1 L. All constituents except for glucose were dissolved in water and autoclaved. The glucose solution in deionised water was filtered through a 0.2 μm cellulose acetate membrane and added to the autoclaved medium, which was subsequently used for the preparation of agar plates.

2.5.3. Bacterial proliferation assay

Assays monitoring the influence of PPX on the bacterial growth were performed on agar grown bacteria, which were harvested after 24 h of incubation and suspended in a 1 mL liquid medium. The liquid medium was prepared analogically to the growth medium (see *2.5.2*), except for the addition of the agar. The optical density (OD) of the bacterial suspension was determined at λ = 640 nm and adjusted to 0.5/mL in the liquid medium. The PPX was dissolved in a liquid medium and sterile filtered. Syringe filters with 0.2 μm cellulose acetate membranes were used. Liquid medium served as untreated control (UC), while gentamycin 100 μg/mL 0.2 μM (Sigma-Aldrich, St. Louis, USA) was used as the positive control (PC). The extract was tested in the range of in-well concentrations from 1 mg/mL to 31.25 μg/mL. The wells with the extract tested contained 100 μL of bacterial suspension and 100 μL of extract solutions. Possible interference of the extract with the measured parameter was excluded by incorporating the control with the extract in the appropriate concentration dissolved in 200 μL liquid medium but without the addition of bacterial suspension. The plate was incubated at 37 °C, and the bacterial proliferation was monitored by measuring the OD_{640} every 60 min for 6 h and after 24 h.

2.5.4. MTT assay

Passages No 20–22 of T24 cell line were used for vitality test by determination of mitochondrial dehydrogenase activity by MTT assay (Mosmann, 1983). The test was performed in 48 well plates on cells with 90% of confluency, approximately 48 h after seeding of 2.4×10^4 cells/well in the cultivation medium. Subsequently, cells were washed with DPBS and incubated for 1.5 h and 24 h with PPX in the concentration range of 250 μg/mL to 2 mg/mL, as well as positive and negative controls, 0.1% of TritonX in DPBS and cultivation medium, respectively. After incubation of T24 cells with PPX, and before proceeding with MTT assay, cells were observed in light microscope Nikon Eclipse TS-100 (Nikon, Tokyo, Japan) in 20 × magnification to evaluate their morphology.

Passage No. 31 was used for the MTT tests on the Caco-2 cell line. The test was performed in a 24 well plate, and the cells were seeded at a density of 1×10^5 cells/well in a 20% FBS medium. The medium/test solution/reagent solution/DMSO and washing volumes were 1 mL per well. After 24 h the medium was changed to one with 10% FBS and cells cultivated for another 48 h. Subsequently, cells were washed with DPBS and incubated for 24 h with PPX in following concentrations 5.0, 2.5, 1.0, and 0.5 mg/mL, as well as positive and negative controls, 0.1% of TritonX in DPBS and cultivation medium, respectively.

After incubation with PPX, both types of cells were washed with DPBS, and 0.5 mg/mL MTT reagent in the medium was added. After 1 h of incubation, the mixture was discarded, whereas the residue dissolved in DMSO. The absorbance was measured at 560 nm (test) and 620 nm (reference). All assays were performed as three independent experiments with n = 6 replicates per experiment.

2.5.5. Flow cytometric adhesion assay

Assays monitoring the influence of PPX on the UPEC adhesion to T24 cells were based on FITC-labelling and conducted accordingly to the previous literature (Rafsanjany et al., 2013). Firstly, agar grown bacteria were harvested after 48 h of incubation and suspended in 1 mL sterile saline solution (NaCl 150 mM, Na_2CO_3 100 mM, pH 8.0). The optical density of bacterial suspension was measured in λ = 640 nm and adjusted to 8.0/mL in the sterile saline solution. All procedures conducted with the FITC solution and FITC-labelled bacteria were performed under light protection. FITC was dissolved in DMSO to obtain 10 μg/mL solution, added to the bacterial suspension in the proportion of

1:9, and incubated for 1 h at 37 °C with shaking of the suspension. After incubation, the suspension was centrifuged (10,000×*g*, 5 min) and washed 3 times with PBS to remove excess FITC. The pellet was resuspended in DMEM and OD_{640} adjusted to 4.0/mL to obtain the final suspension of bacteria.

Passages No 74–76 were used for adhesion assays on the T24 cell line. The test was performed in 6 well plates on cells with 90% of confluency, approximately 48 h after seeding of 1.25×10^5 cells/well in the cultivation medium. Subsequently, cells were washed twice with DPBS and once with DMEM without antibiotics. All assays were performed with PPX in the range of concentrations from 2 mg/mL to 250 μg/mL, as well as untreated control (cultivation medium).

Flow cytometry adhesion assays were conducted in three variations. Firstly, the 1.5 h co-incubation of T24 cells, FITC-labelled UPEC, and extracts was performed. The other two variations were based on separate incubation of either T24 cells or FITC-labelled UPEC with PPX for 1.5 h. Subsequently, the extract was washed off with DPBS (using the same conditions for bacterial suspension as above). The 1.5 h incubation of FITC-labelled UPEC suspended in DMEM with T24 cells followed, pairing PPX-treated UPEC with untreated T24 cells or PPX-treated T24 cells with untreated UPEC. The bacteria to cell ratio was 100:1 in all experiments. All incubations of UPEC with T24 cells were terminated by removing the bacterial suspension from the well and gently washing with DPBS thrice to remove UPEC unattached to T24 cells. Subsequently, T24 cells were detached from the well surface with trypsin/EDTA for 3 min, removed from wells, and centrifuged (450×*g*, 5 min). Cells were resuspended in 700 μL DMEM for fluorescence measurements using flow cytometry. For data evaluation, 10,000 counts per sample were used. Samples were measured with two technical repetitions. Three independent experiments were conducted for obtaining the data. The data was calculated per middle relative fluorescence intensity (median, M) of each sample population. Thus, the increased value of the median represented a higher number of FITC-labelled bacteria. The relative adhesion was calculated from the acquired median as a per cent of treated samples compared to the untreated control. The results are given as mean ± SD.

2.5.6. Caco-2 permeability assay

For transport studies, passage 33 was used. For the Caco-2 permeability assay, the 0.4 μm pore diameter translucent cell culture inserts and 6 well plates (effective cell monolayer surface: 4.254 cm^2; ThinCert, Greiner Bio One, Kremsmünster, Austria) were used. The cells were seeded on top of cell culture inserts at a density of 1.5×10^5 cells/cm^2 and transferred inside the cellZscopeE device (nanoAnalytics, Münster, Germany) at the first medium change. Medium and test solution volumes were 2.7 mL on the apical and 4.4 mL on the basolateral side. Since the first medium change, 10% FBS medium had been used. The cells were cultured for 21 days. Transepithelial electrical resistance (TEER) measurements were performed automatically every hour. The TEER value of all used cell monolayers was above 250 Ω/cm^2 before the experiment. 1 mg/mL PPX solution dissolved in HBSS was used as a test solution. 100 μM of propranolol in HBSS was used as a transcellular transportation marker. Apical to basolateral side transport was examined in two repetitions, and the opposite direction was examined in a single experiment. The pre-heated warmed (37 °C) test and marker solutions were added to the donor and HBSS to the acceptor sides. 25 μL samples of the donor and the acceptor side were taken after 1, 2, and 3 h of incubation (37 °C) and analysed using UHPLC-DAD-MS^n. The data were processed, and the compounds were automatically assigned to the signals after comparison with the compound library created based on the analysis of the PPX sample using Compass DataAnalysis 5.3 software (Bruker Daltonik GmbH, Bremen, Germany).

2.6. Statistical analysis

The results were presented as mean values ± SEM of the indicated number of experiments. One-way ANOVA was used to determine the statistical significance of differences between means, and Dunnett's *post hoc* test was used to compare results with the control group.

3. Results

3.1. Phytochemical composition of extract using UHPLC-DAD-MS^n

Based on the chromatographic analysis of the hydroethanolic *Phaseolus pericarpium* extract PPX (Table 1), the main constituents of the extract can be divided into three groups of compounds: phenolic acids derivatives, flavonoid glycosides, and saponins. The first eluted phenolic acid derivative was protocatechuic acid *O*-hexoside (**1**), which absorbed at 295 nm and gave pseudo-molecular ion $[M\text{-}H]^-$ at *m/z* 315 and fragmented into an aglycon $[M–C_6H_{10}O_5–H]^-$, which gave a signal at *m/z* 153. It was followed by a *p*-coumaroyl derivative of tetrahydroxyhexanedioic acid (**2**). It had maxima characteristic for *p*-coumaroyl derivatives at 312 nm, pseudo-molecular ion $[M\text{-}H]^-$ at *m/z* 355, which after the loss of *p*-coumaroyl moiety $[M–C_9H_6O_2–H]^-$ gave the signal of tetrahydroxyhexanedioic acid fragment at *m/z* 209. The phenolic fraction was also composed of an unidentified caffeic acid derivative (**3**, $[M\text{-}H]^-$ at *m/z* 525, $[M–C_9H_8O_4–H]^-$ at *m/z* 345), which had a typical caffeoyl moiety UV/vis maxima at 290 and 320 nm. The flavonoid fraction was composed mostly of various quercetin and kaempferol derivatives. Rutin (**15**), quercetin *O*-hexoside (**16**), and *O*-glucuronide (**18**, $[M\text{-}H]^-$ at *m/z* 477, $[M\text{-}GlcA\text{-}H]^-$ at *m/z* 301) were recognised as the most abundant. Lower content of quercetin *O*-pentopyranosyldeoxyhexososylhexoside (**10**, $[M\text{-}H]^-$ at *m/z* 741, $[M\text{-}Pentose\text{-}H]^-$ at *m/z* 609, $[M\text{-}Pentose\text{-}deoxyHex\text{-}H]^-$ at *m/z* 463, $[M\text{-}Pentose\text{-}deoxyHex\text{-}Hex\text{-}H]^-$ at *m/z* 301) and kaempferol *O*-pentopyranosylhexosyldeoxyhexoside (**14**, $[M\text{-}H]^-$ at m/z 725, $[M\text{-}Pentose\text{-}H]^-$ at m/z 593, $[M\text{-}Pentose\text{-}Hex\text{-}H]^-$ at *m/z* 431, $[M\text{-}Pentose\text{-}Hex\text{-}deoxyHex\text{-}H]^-$ at m/z 301), quercetin *O*-pentopyranosylhexoside (**11**, $[M\text{-}H]^-$ at *m/z* 595, $[M\text{-}Pentose\text{-}H]^-$ at *m/z* 463, $[M\text{-}Pentose\text{-}Hex\text{-}H]^-$ at *m/z* 301), kaempferol *O*-rutinoside (**19**) and *O*-glucuronide (**21**, $[M\text{-}H]^-$ at *m/z* 461, $[M\text{-}GlcA\text{-}H]^-$ at *m/z* 285) was also observed in the extract. However, the most abundant and numerous group of natural products present in the extract were saponins. Among them, the most significant quantities were detected for group B soyasaponins (Kamo et al., 2014). The highest peak was assigned to soyasaponin I (Bb) (**38**) with pseudo-molecular ion $[M\text{-}H]^-$ at *m/z* 941 and identified based on the fragmentary ions: $[M–H_2O–H]^-$ at *m/z* 923, $[M–H_2O–CO_2–H]^-$ at *m/z* 879, $[M\text{-}Rha\text{-}H]^-$ at m/z 795, $[M–H_2O–CO_2\text{-}Rha\text{-}H]^-$ at *m/z* 733, $[M–H_2O–Rha\text{-}Gal\text{-}H]^-$ at *m/z* 615 and the aglycon fragment $[Soyasapogenol\ B–H]^-$ at *m/z* 457. The lower content of soyasaponin Ba (**37**) was observed, as this compound eluted closely before **38** and was identified by the respective pseudo-molecular ion $[M\text{-}H]^-$ at *m/z* 957, $[M\text{-}Glc\text{-}H]^-$ at *m/z* 795, $[M\text{-}Glc\text{-}Gal\text{-}H]^-$ at *m/z* 633, and similarly to **38**, the aglycon fragment $[Soyasapogenol\ B–H]^-$ at *m/z* 457. The results were in accordance with **36** and **37** MS spectra provided by (Jin et al., 2007). Other soyasapogenol B based compounds present in the extract are DDMP derivatives: soyasaponin αg (**47**) and βg (**49**). They were identified based on the presence of the aglycon signal $[Soyasapogenol\ B + DDMP\text{-}H]^-$ at *m/z* 583. Soyasaponins group E was represented by soyasaponin Be (**45**), which gave pseudo-molecular ion signal at *m/z* 939 and was identified based on the fragmentary ions: $[M–H_2O–H]^-$ at *m/z* 921, $[M–H_2O–CO_2–H]^-$ at *m/z* 877, $[M–H_2O–CO_2\text{-}Rha\text{-}H]^-$ at *m/z* 731, $[M–H_2O–Rha\text{-}Gal\text{-}H]^-$ at *m/z* 613 and the aglycon fragment $[Soyasapogenol\ E\text{-}H]^-$ at *m/z* 455. The chromatogram in MS^- is depicted with annotated peaks (Fig. 1).

3.2. Influence of PPX on the proliferation of UPEC

Proliferation assay was performed in order to evaluate the potential influence of PPX on the growth of UPEC NU14. Results obtained (Fig. 2) indicated that the extract (31–1000 μg/mL) did not influence the bacteria proliferation after 24 h of incubation time. After 24 h, a statistically

Table 1
UHPLC-MS^n analysis of hydroalcoholic *Phaseoli pericarpium* extract PPX. MS data acquired in negative ionization mode – pseudo-molecular ion $[M-H]^-$ and fragmentary ions (MS^2 and MS^3), references to tentatively identified compounds. b – base signal; bolded font – parent ion for MS^3.pseudo-molecular.

Peak no.	R_t [min]	Compound	Spectrum λ max [nm]	$[M-H]^-$	MS^2 [m/z]	MS^3 [m/z]	Ref.
1	4.6	protocatechuic acid hexoside	199, 250, 295, 320	315	297, 225, 163, **153**	153	
2	7.4	*p*-coumaroyl derivative of tetrahydroxyhexane-dioic acid	204, 312	355	337, **209**, 191b	209b	Francioso et al. (2019)
3	10.5	caffeic acid derivative	286, 320	525	481, 345b, 257, 161		
4	10.7	caffeic acid derivative	211, 268, 315sh	443	425, 384, 335, 281, 237, 143		
5	12.6	unidentified dicarboxylic acid	210, 286	363	319, 275, 257		
6	14.0	caffeic acid derivative	191, 220, 277, 314	239	145		
7	17.6	unidentified compound	216, 260	563	501, 461, 419b		
8	21.0	unidentified compound	219, 309	563	545b, 517, 503, 445, 387, 321, 175		
9	27.9	unidentified compound	219, 278	245	203		
10	29.8	quercetin-3-*O*-xylosylrhamno-glucoside	218, 265, 350	741	723, **609**, 591, 475, 343, 301b, 271	463, 343b, 301, 255, 179	Price et al. (1998)
11	31.4	quercetin 3-*O*-xylosylglucoside	220, 265, 350	595	**463**, 445, 299b, 271, 179	343, 301b, 179	Lin et al. (2008)
12	33.7	unindentified compound	216, 271, 325	467	365, 323b, 305		
13	34.0	unidentified compound	270, 335	575	529, 367, 179		
14	34.6	kaempferol 3-*O*-xylosylrhamnosyl-glucoside	265, 338	725	**593**, 459, 285b	327b, 285, 229	Price et al. (1998)
15	35.0	rutin	216, 256, 352	609	301		Price et al. (1998)
16	35.8	quercetin 3-*O*-glucoside	220, 265, 350	463	301	271, 255, 211, 179b, 151, 107	Price et al. (1998)
17	36.6	unidentified compound	288	573	527, 365		
18	36.9	quercetin 3-*O*-glucuronide	216, 254, 300, 352	477	301	273,179b, 151, 107	Price et al. (1998)
19	40.2	kaempferol 3-*O*-rutinoside	221, 265, 341	593	285		Price et al. (1998)
20	40.5	quercetin mallonylhexoside	220, 264, 345	549	505	463, 301, 179	
21	42.0	kaempferol 3-*O*-glucuronide	221, 266, 332	461	285	157b, 197, 163	Price et al. (1998)
22	45.3	unidentified flavonoid	221, 269, 341	475	**299b**, 175	284b	
23	48.0	unidentified compound	266 (sh), 306	673	355		
24	48.9	undefined flavonoid	288, 335	590	567		
25	51.4	undefined flavonoid	221, 285, 339	509	465, 403, 329b, 267, 241, 223		
26	59.1	unidentified saponin	221	1265			
27	61.2	unidentified saponin	221	1249			
28	62.8	phaseoside I	221	1251	1233, 1189, 1089, 1029, 909, 891b, 867, 819, 733, 657, 569, 473, 383		Kinjo et al. (1998)
29	63.8	unidentified saponin	221, 343	1235	1217b, 1173, 1027, 865		
30	66.7	unidentified saponin	221, 277	1259			
31	67.1	unidentified free fatty acid	221	327	309, 291b, 251, 211, **195**, 183	195b	
32	68.3	unidentified saponin	221	957	941,895,793,749,689,607,541,473,357		
33	69.4	unidentified saponin	222	973	953, 909b, 825, 763, 645, 601, 555, 469, 403		
34	71.1	unidentified saponin	222	955	937, 893, 747, 629, 539, 471		
35	76.1	unidentified saponin	223	953	935, 892, 807, 745, 627, 609, 565, 537b, 469		
36	76.5	unidentified saponin	223	1021	947, 795, 695, 633, 537, 519, 479, 405, 301		
37	76.4	soyasaponin V (Ba)	223	957	941, **795**, 597, 525b, 455	633, 437	(Jin et al., 2007; Kinjo et al., 1998)
38	76.7	soyasaponin I (Bb)	223	941	923b, 879,795, 733, 615, 525, 457, 359		(Jin et al., 2007; Kinjo et al., 1998)
39	77.5	unidentified saponin	223	1027	984		
40	77.9	unidentified saponin	223, 275	875	919, 875, 729, 593, 521b, 451, 367		
41	78.1	unidentified saponin	223	925	907, 863, 779, 717, 581, 509b, 439		
42	78.3	soyasaponin III (Bb')	223	795	778, 615b, 525, 457, 409, 356, 301		Shiraiwa et al. (1991)
43	79.1	unidentified saponin	223	1011	995, 967, 951, 867, 803, 685b, 595, 525, 421		
44	79.2	unidentified free fatty acid	223	311	293, 275, 223, 171		
45	79.5	soyasaponin Be	223	939	921, 877, 731, 613b, 523, 455, 391, 307		Bianco et al. (2018)
46	80.2	unidentified saponin	223	779	599, 527, 509b, 439, 337		
47	80.3	soyasaponin αg	223	1083	1065, 983, 921, 896, 723, 651b, 564		Bianco et al. (2018)
48	81.0	unidentified saponin	223	923	905, 861, 715, 579, 507b, 437		
49	81.2	soyasaponin βg	223	1067	1049, 1005, 969, 921, 879, 741, 679, 651b, 583, 437, 377		Bianco et al. (2018)
50	87.7	unidentified compound	280, 339	301	283, 219b, 205		
51	89.6	unidentified compound	223, 285, 343	625	558, 301b		

significant increase of UPEC proliferation rate in all PPX-treated groups occurred, which might be explained by the presence of constituents of nutritional value in the extract. Similar, but less prominent, such effects have been reported previously for aqueous extracts of restharrow root (Deipenbrock et al., 2020).

3.3. Influence of PPX on the viability of T24 viability

The influence of PPX on the viability of T24 bladder cells after 1.5 h and 24 h was evaluated by MTT assay (Mosmann, 1983). For data acquired after 1.5 h in the MTT assays, no cytotoxic effect of the extract

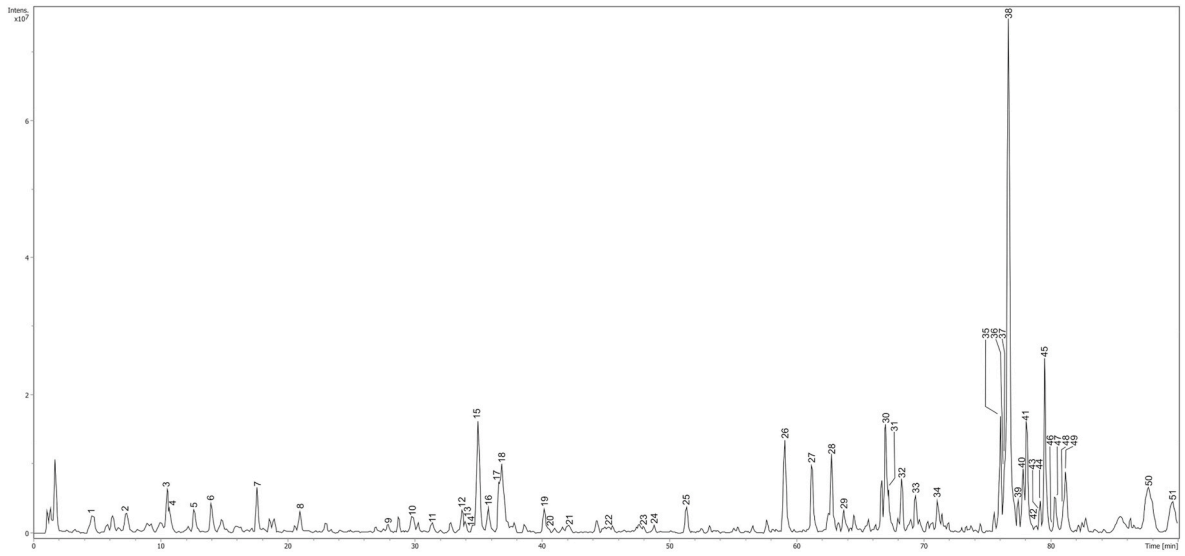

Fig. 1. UHPLC-MS^n analysis of hydroalcoholic *Phaseoli pericarpium* extract PPX. The base peak chromatogram has been recorded in the negative ionization mode. Peaks are labelled with the respective compound numbers according to the data displayed in Table 1.

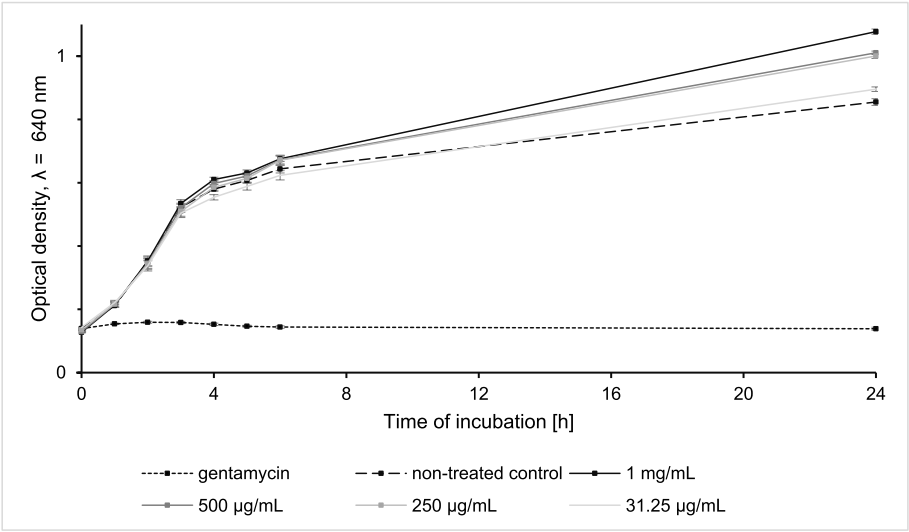

Fig. 2. Influence of PPX on the proliferation of uropathogenic *E. coli* strain NU14 over 24 h, represented by the optical density (OD $\lambda = 640$ nm) of the bacterial suspensions. Gentamycin (0.2 µM) was used as a positive control. Data are based on three independent experiments with $n = 6$ technical replicates. Results are expressed as mean ± SEM.

was observed (Fig. 3). Additionally, the effect of the extract after 24 h was also evaluated. The MTT experiments showed that PPX after 24 h caused a slight decrease in the viability of T24 cells. However, no significant concentration-dependent correlation was observed (Supplementary materials, Fig. S1), and thus, the decrease may be connected with deficiencies of accuracy and precision of the chosen method. Additionally, the observation of cell morphology after the 1.5 h and 24 h incubation of PPX with cells did not show any changes in the morphology of the cells.

3.4. Influence of PPX of the bacterial adhesion of UPEC NU14 to T24 bladder cells

The influence of PPX on the adhesion of FITC-labelled UPEC to human T24 bladder cells was monitored after 1.5 h of incubation in three different incubation protocols as described in 2.5.5. A significant decrease of bacterial adhesion to the host cells was observed during the co-incubation of UPEC with T24 host cells (Fig. 4) as well as during the pre-incubation of T24 with PPX, followed by subsequent addition of UPEC (Fig. 5). The observed antiadhesive effects turned out to be concentration-dependent. Assays with pre-incubation of UPEC with PPX did not show any influence on the bacterial adhesion (data not shown). Thus, it can be concluded that the antiadhesive effect of PPX is due to a specific impact of the extract on the host cells. Results obtained in this assay indicated that PPX has lower antiadhesive activity against UPEC adhesion to T24 bladder cells compared to other extracts described recently in the same test system and which have been recognised to have much higher antiadhesive activity as e.g. hydroalcoholic extracts from *Ononis spinosa* roots (Deipenbrock et al., 2020), *Agropyron repens* L. roots (Beydokthi et al., 2017), leaves from *Orthosiphon stamineus*, *Betula* spp., and *Urtica* spp. (Rafsanjany et al., 2013).

3.5. Assessment of permeability of the constituents of PPX using Caco-2 monolayers

The presented data show results of an experiment of raw PPX in HBSS

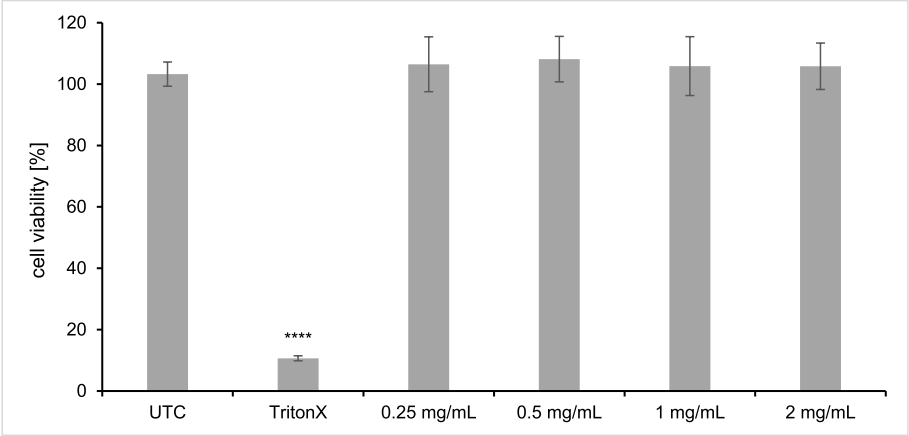

Fig. 3. Viability of T24 cells in MTT assay after 1.5 h incubation with PPX (0.25–2 mg/mL). UTC - untreated control, Triton - positive control, DMSO 5% - positive control. **** - $p < 0.001$, ** - $p < 0.01$ versus UTC. The data has been acquired in three independent experiments with $n = 6$ replicates. The results are expressed as mean ± SEM.

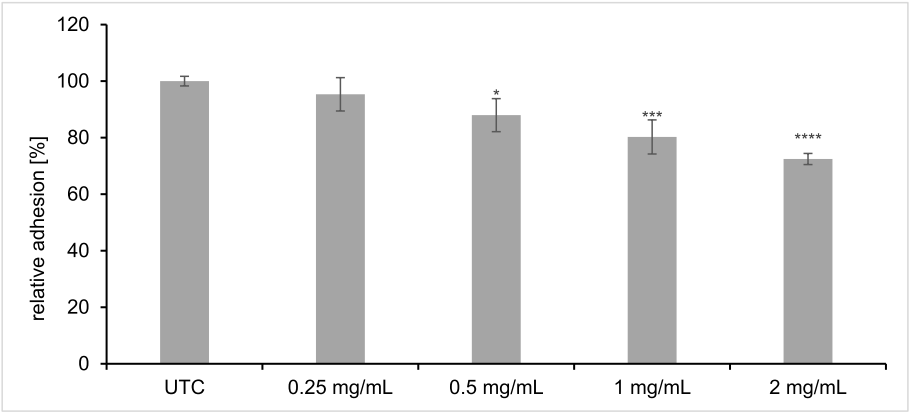

Fig. 4. Adhesion of UPEC to T24 cells (co-incubation protocol) after 1.5 h incubation with PPX (0.25–2 mg/mL). UTC - untreated control, **** - $p < 0.0001$, *** - $p < 0.001$, * - $p < 0.05$ versus UTC. The data has been acquired in three independent experiments with two technical repetitions. The results are expressed as mean ± SEM.

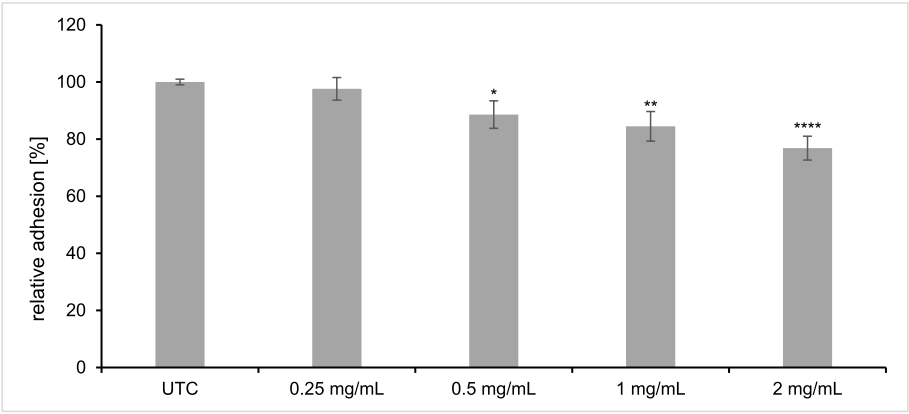

Fig. 5. Adhesion of UPEC to T24 cells (T24 pre-incubation protocol) after 1.5 h incubation with PPX (0.25–2 mg/mL). UTC - untreated control, **** - $p < 0.0001$, ** - $p < 0.01$, * - $p < 0.05$ versus UTC. The data has been acquired in three independent experiments with two technical repetitions. The results are expressed as mean ± SEM.

applicated in the donor sides, without any fractionation or isolation of pure compounds. The test solution was 1 mg/mL based on the MTT test (Fig. 6). The TEER value was monitored online using cellZscopeE device to ensure the integrity of the monolayer used in experiments. The TEER value was ca. 300 Ω/cm^2 in all experiments. In order to make raw data obtained from UHPLC-MS analysis more readable, the chromatograms and the MS signals were subtracted with the result of the blank sample (HBSS) analysis. Afterwards, the finder tool was used to assign the MS data to the chromatographic peaks, and the results were automatically compared with the library containing the compounds tentatively

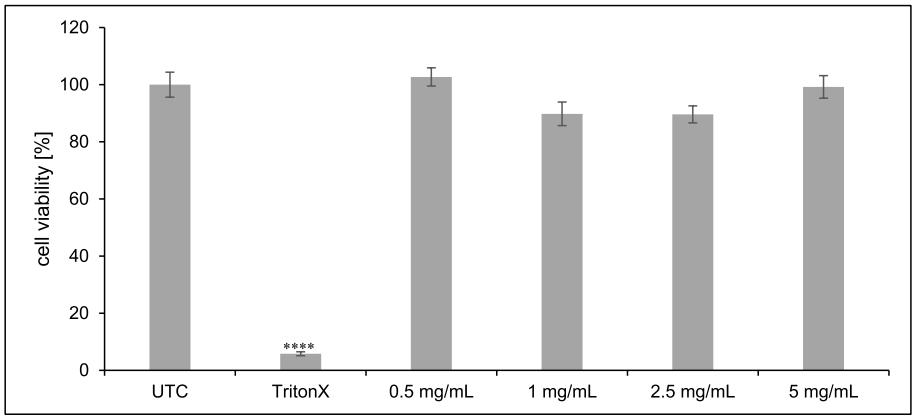

Fig. 6. Viability of Caco-2 cells with MTT assay after 24 h of incubation with PPX (0.5 to 5 mg/mL). UTC - untreated control; TritonX - positive control; **** - $p <$ 0.001 versus UTC. The data has been acquired in three independent experiments with $n = 6$ replicates. The results are expressed as mean ± SEM.

identified after raw extract analysis. Considering apical to basolateral (A to B) side transport, only rutin (**15**) was identified in the acceptor side after 1 h of incubation. In the samples taken after 2 and 3 h of incubation compounds that were the most abundant in the raw PPX - saponins (**26**, **35**, **38**, **45**) and fatty acid (**31**) were also present in the basolateral side solution (Fig. 7). In the case of the transport of natural products present in PPX in the opposite direction (B to A), the experiment showed that none of the compounds was present in the medium at the apical side after 1 h. After 2 h of incubation, a minor signal of **35** was detected, and after another hour its concentration in the acceptor side rose. Additionally, in the sample taken after 3 h the presence of flavonoids: rutin (**15**), kaempferol 3-*O*-rutinoside (**19**), and **24** was confirmed, as well as unidentified free fatty acid (**31**) and lesser signal of saponin I (Bb, **38**) (Fig. 8).

4. Discussion

The evaluation of the chemical composition of prepared extract using UHPLC-DAD-MS^n analysis revealed the presence of 51 major compounds. Several natural products, mainly flavonoids (**10**, **11**, **14**–**16**, **18**, **19** and **21**), were previously reported (Lin et al., 2008; Price et al., 1998). During the analysis, few other flavonoids were detected (**20**, **22**, **24**–**27**). Compound **20** was preliminarily identified as quercetin malonylhexoside. This is the first report on this particular compound in common bean pods. Apart from flavonoids, phenolic acids derivatives were identified in the analysed sample (**1**–**6** and **12**). The presence of phenolic acids in *Phaseoli pericarpium* was previously reported by (Łabuda et al., 2017) but no in-depth analysis leading to the characterization of occurring compounds was performed. The third large group of compounds detected in the present study are triterpene saponins. Compounds **26**–**30**, **32**–**43** and **45**–**49** were included in this group of phytochemicals based on obtained data. The presence of compounds **28**,

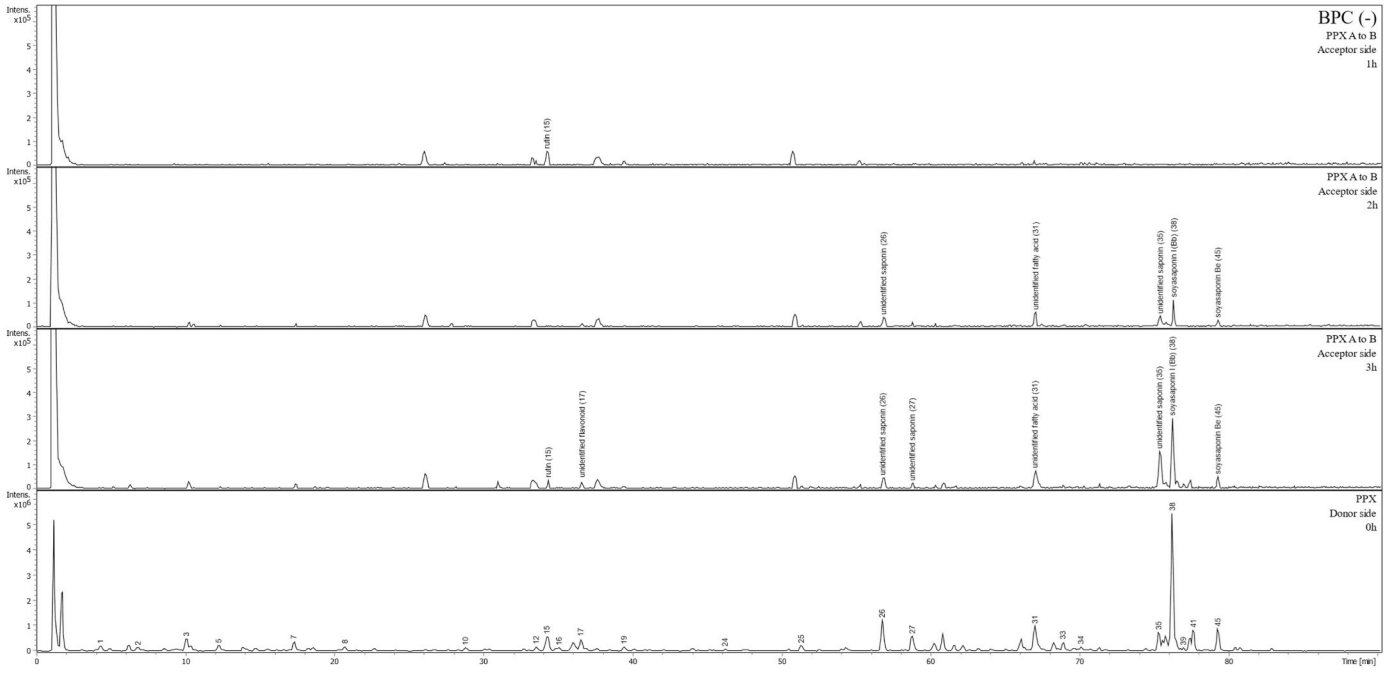

Fig. 7. Transport experiment of PPX in the direction from the apical (donor side) to the basolateral side (acceptor side) (A to B). The results expressed as base peak chromatograms in negative mode (BPC (−)) of samples of donor side mixture taken before the experiment (0 h) and of the acceptor side after 1, 2, and 3 h of incubation. All results were subtracted with the analysis of the blank sample. Captions over peaks show the compound number and tentative identification as in Table 1.

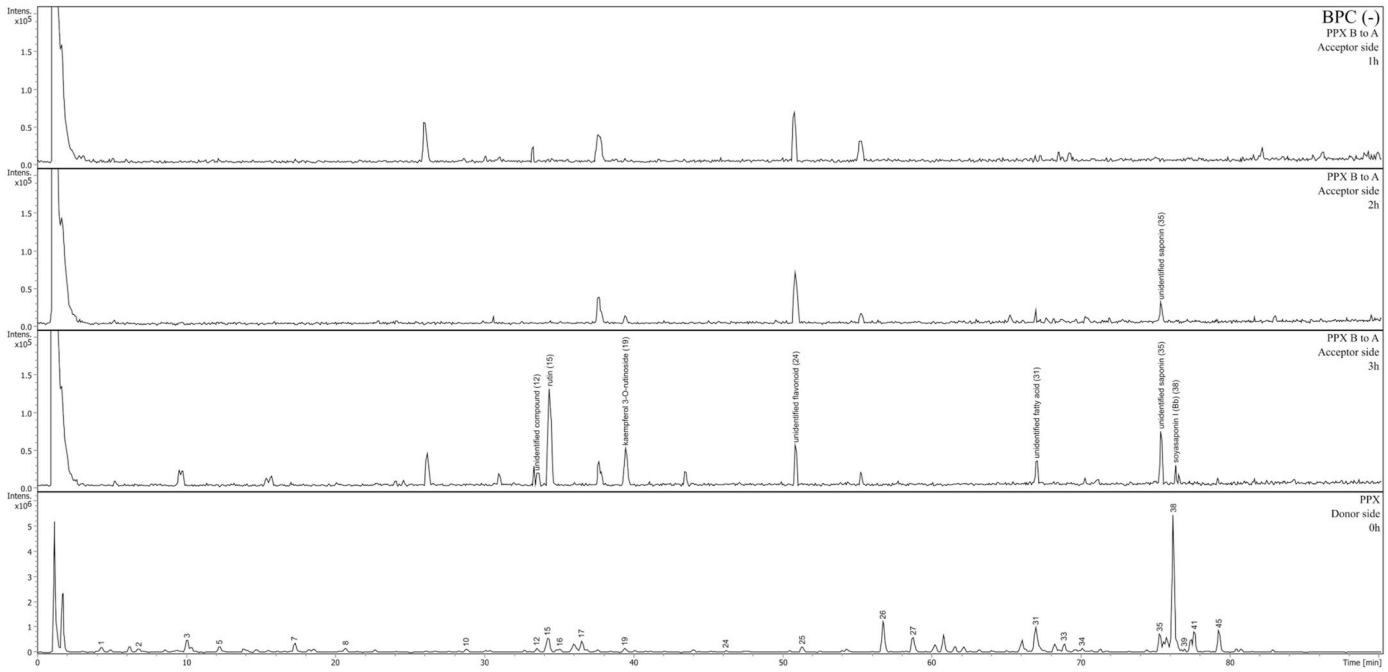

Fig. 8. Transport experiment of PPX in the direction from the basolateral (donor side) to the apical side (acceptor side) (B to A). The results expressed as base peak chromatograms in negative mode (BPC (−)) of samples of donor side mixture taken before the experiment (0 h) and of the acceptor side after 1, 2, and 3 h of incubation. All results were subtracted with the analysis of the blank sample. Captions over peaks show the compound number and tentative identification as in Table 1.

37, **38**, **45**, **47** and **49** was previously confirmed in different parts of common beans (Bianco et al., 2018; Jin et al., 2007; Kinjo et al., 1998). Compound **42** was previously detected in soybean but not in common bean (Shiraiwa et al., 1991). The current research did not confirm the presence of phaseoloside D, which was previously isolated from *Phaseolus vulgaris* (Chirva et al., 1970). The UHPLC-DAD-MS analysis did not allow the full identification of many detected compounds. However, the developed qualitative method provides a powerful tool for future phytochemical analysis of common beans and can be considered an introduction for the in-depth standardization of this medicinal plant drug.

The permeability assays using the Caco-2 model revealed that several compounds, including flavonoids and saponins, were able to cross cell monolayers and could be detected with UHPLC-DAS-MS method. Considering the transport of rutin (**15**), the results were in accordance with investigations of pure compound permeability reported before (Rastogi and Jana, 2016).

To the best of our knowledge, no studies were performed focusing on the intestinal transport of investigated saponins using cell models. The results showed possible differences in the efflux ratios within the saponins' fraction. However, that should be confirmed in the experiments on the permeability of isolated compounds. Based on the performed experiments, it can be suggested that flavonoids and soyasaponins contained in *Phaseoli pericarpium* extract can be absorbed from gastrointestinal tracts after oral intake. These two groups of compounds can be considered as potential bioavailable and bioactive constituents. However, as soyasaponins are known to be metabolised by gut microbiota to their aglycon forms – soyasapogenols (Kamo et al., 2014), the metabolised form must be taken into account, which could be tested using a mixture of the metabolites or isolated metabolites after incubation of PPX with gut microbiota *ex vivo* in further experiments.

Natural products have undeniable potential to offer new and innovative possibilities for addressing new molecular targets during the search for new antiinfectives, not only against the infecting bacteria but also by influencing the pathogen-host interplay by interacting with the cell membrane structure of the host cells. Thus, detailed investigations, as well as reinvestigations of sidelined or traditionally used herbal materials with until now not sufficiently rationalised science, can be a promising tool for the identification of new molecular targets and promising natural products. However, to assess the origin of these beneficial effects, and to justify and support the respective traditional of selected herbal materials it is crucial to accurately characterise the chemical composition of specific plant extracts, to investigate the potential bioavailability of typical and relevant marker compounds from these extracts and to investigate *in vitro* bioactivity. In investigations of natural products with anti-UTI potential, diuretic, anti-inflammatory, antimicrobial, antiadhesive and Tamm-Horsfall stimulating from the kidney activities have been pinpointed as potential underlying effects to explain the clinical activity. Diuretic drugs increase the urine volume, which might be beneficial as the infected surfaces are flushed to eliminate a part of the infectious pathogens from the urinary tract system (Shih-Bin et al., 2006). Anti-inflammatory action reduces immunological caused tissue damage to the uroepithelium. Antimicrobial and antiadhesive agents reduce bacterial proliferation and colonization of urinary tract epithelial surfaces. The antiadhesive activity of phytochemicals might be connected with interaction with bacterial outer membrane proteins (as in the case of *Agropyron repens* L. and *Zea mays* L. extracts; IC_{25} 630 μg/mL, resp. IC_{50} 1040 μg/mL), as well as with influencing the bladder epithelial (as observed for extracts from *Betula* spp., *Orthosiphon stamineus* BENTH. and *Urtica* spp.; IC_{50} 415, 1330 μg/mL, resp IC_{25} 580 μg/mL) (Rafsanjany et al, 2013, 2015a). The antiadhesive activity of the extract from *O. stamineus* was assessed to be due to a downregulation of the chaperone-usher pathway and fimbrial assembly (Beydokhti et al., 2019). Human urine samples collected after 7 days of intake of hydroethanolic *Vaccinium macrocarpon* extract caused a 49% loss of UPEC adhesion to T24 cells in comparison to the control sample (Rafsanjany et al., 2015b). It has been reported that the main targets of cranberry extract are on the one hand the induction of Tamm-Horsfall protein in the kidney (Scharf et al., 2019) and on the other side the interaction of extract compounds (mainly flavonoids) with the mannose-binding domain of type 1 fimbrial adhesion FimH of UPEC (Scharf et al., 2020). Extracts of *Apium graveloens* had significant

inhibitory activity against UPEC strains NU14 and UTI89 adhesion to T24 cells at 500 μg/mL and the pretreatment intake of this extract reduced the bacterial load in the bladder after transurethral inoculation of UPEC suspension in mice *in vivo* (Sarshar et al., 2018).

The obtained data show the anti-adhesive potential of evaluated plant material. The bioactivity was associated with the inhibition of the adhesion of UPEC to human bladder cells rather than with direct influence on UPEC proliferation of T24 viability. The molecular mechanism of the reported activity is yet to be elucidated. However, the observed bioactivity was rather weak compared to previously reported data (see above). The extract showed moderate activity with $IC_{50} > 2$ mg/mL, which should be considered a high value. However, it is not out of the question that the regular ingestion of bean pod extract can lead to the cumulation of natural products in the excreted urine at the levels that could display bioactivity against *E. coli* infections. This aspect must be verified in further *in vivo* studies.

5. Conclusion

The results of the chromatographical analysis showed that the main constituents of *Phaseoli pericarpium* hydroethanolic extract are triterpene saponins, flavonoids, and phenolic acid derivatives. The extract showed statistically significant, concentration-dependent antiadhesive activity against UPEC. Based on the comparison of the results of pre- and co-incubation experiments, it can be concluded that the antiadhesive activity of the extract is connected with influence on the epithelial cells. Some of the soyasaponins, identified in the extract, as well as rutin and free fatty acids, are able to cross the Caco-2 cells monolayer. The counter-direction experiment indicated possible differences in the efflux transport rates among the groups of compounds present in the extract. The results show that flavonoids and saponins are both the most abundant extract constituents and those able to cross the intestinal barrier, and ultimately, they may exhibit anti-UPEC activity in the bladder while being excreted in urine (El-Hawiet et al., 2010; Jaiswal et al., 2019). However, due to the oral intake of the extract, the possibility of the biotransformation of natural products in the gastrointestinal tract and the antiadhesive activity of metabolites produced this way should be evaluated in further investigations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114053.

Author contributions

Dominik Popowski Investiagtion, Visualization, Writing - Review & Editing **Karolina A. Pawłowska:** Investigation, Visualization, Writing - Review & Editing **Aleksandra Kruk** Investigation, Visualization **Melanie Deipenbrock** Investigation **Jakub P. Piwowarski** Conceptualization, Methodology, Resources, Writing - Review & Editing **Andreas Hensel** Methodology, Corrections of the Manuscript, Resources, Conceptualization **Matthias F. Melzig** Methodology, Resources **Sebastian Granica:** Conceptualization, Methodology, Resources, Writing - Review & Editing, Project administration

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