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Phenolic profile and effects of the acetone fractions obtained from the inflorescences of *Calluna vulgaris* (L.) Hull in vaginal pathogenic and non-pathogenic bacteria

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Abstract

The phenolic profile and the antibacterial activity against pathogenic commensal vaginal bacteria exhibited by different fractions of the acetone extract of heather was assessed. The acetone extract of *Calluna vulgaris* (L.) Hull was fractionated by silica gel column chromatography through an eluent system of increasing polarity, obtaining 10 different fractions (**Fr 1** to **Fr 10**). The phenolic profile was analyzed by HPLC-DAD-ESI/MS. Type B (epi)catechin dimers, (-)-epicatechin and (+)-catechin were the main phenolic compounds present in the fractions. The antibacterial activity was also analyzed against pathogenic bacteria and the effect in the beneficial microflora was also accessed. Some of the obtained fractions revealed the capacity to inhibit pathogenic microorganisms without affecting the beneficial microflora, especially **Fr 7** and **Fr 8**. For instance, *Neisseria gonorrhoeae* was inhibited by both of the fractions, while **Fr 7** was more active against *Klebsiella pneumoniae* and *Morganella morganii*, and **Fr 8** against methicillin resistance *Staphylococcus aureus* (MRSA) and methicillin susceptible *Staphylococcus aureus* (MSSA), without affecting *Lactobacillus* strains. This study corroborates the therapeutic use of this matrix in traditional medicine.

Keywords: *Calluna vulgaris* (L.) Hull, acetone extract fractions, phenolic profile, antibacterial activity, vaginal microbiota.

1. Introduction

The availability of effective antibacterial agents is becoming lower as result of the capacity that pathogenic microorganisms have developed to create different forms of resistance. Thus, the search for new and effective antibacterial agents has been considered a global priority.[1,2] In the search for new antimicrobial agents, particular attention has been given to natural products as result of their richness and diversity in phytochemical compounds with high bioactive properties. Among the natural resources, medicinal plants have been the most studied based on the ancient ethnopharmacological knowledge. These studies have scientifically demonstrated the therapeutic potential for which these plants have been used over the time.[3,4]

Calluna vulgaris (L.) Hull (commonly known as Scotch heather, common heather and ling) is the only species of *Calluna* genus (*Ericaceae*). It is a small shrub native to Europe and North Africa and was introduced in other countries such United States of America, Australia and Canada. Infusions and decoctions are the most described forms of consumption of this shrub in folk medicine. This shrub exhibits a broad spectrum of biological activities being used for the treatment of rheumatic pain and arthritis [5,6] and as a tranquilizer and sedative.[7,8] Moreover, this matrix is used for the treatment of infections related to the urinary tract, that highlights the medicinal effects as result of their anti-inflammatory, depurative, diuretic and antiseptic properties.[5,6,9,10] Extracts obtained from the inflorescences have demonstrated antibacterial activity against several bacterial strains, some of which were clinical isolates responsible for urinary infections, such as *Escherichia coli*, *Klebsiella penumoniae*, *Pseudomonas aeruginosa* and *Neisseria gonoeehoeae*.[11]

Extracts obtained from the different plant parts of heather (*i.e.* flowers, leaves, roots, and seeds) have been studied as an attentive to prove their folk medicine uses through

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scientific methodologies. Different therapeutic properties such as antioxidant,[6,12] anti-inflammatory,[6,10] anti-nociceptive,[10] cytotoxic,[13] antiviral [14] and enzymatic inhibition [6] were studied and scientifically validated.

The phytochemical composition contains a large variety of molecules, resulting from both primary and secondary metabolism. In recent studies, it was possible to identify the four isoforms of tocopherol, with α-tocopherol as the major compound [9,11]; as also the presence of some organic acids such as oxalic, quinic, ascorbic and citric acids.[11] Moreover, myricetin-3-*O*-glucoside and myricetin-*O*-rhamnoside were the main phenolic compounds found in acetone, methanol and aqueous extracts of heather inflorescences.[11] These class of bioactive molecules have been described as the compounds responsible for the mentioned biological properties found in this species.[6,12] The content in phenolic compounds can be influenced by the environmental conditions, such as the stress level that plants are subjected during their development, by the altitude and by the harvest season.[15] The study of the influence of altitude in terms of bioactive compounds, have demonstrated that plants growing in lower altitudes present lower amounts of phenolic compounds and, consequently lower antioxidant capacity.[16] Extracts of heather roots, obtained by an hydromethanolic (80:20) extraction, revealed the presence of catechins and procyanidins.[15] Triterpenoids such as ursolic and oleanolic acids were identified in chloroform extracts of cuticular waxes of heather flowers and leaves and have different biological activities, particularly anti-inflammatory activity.[17]

A study on the antibacterial activity of different aqueous (infusion and decoction) and organic extracts (*n*-hexane, dichloromethane, ethyl acetate, acetone and methanol) was recently conducted by some of the authors,[11] and as result of the high demonstrated potential, in the present study the acetone extract was fractionated by gradient elution

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through column chromatography on silica gel. The resulted fractions were characterized for their composition in phenolic compounds and their antibacterial potential against pathogenic microorganisms and non-pathogenic bacteria of vaginal microflora was also evaluated.

2. Materials and methods

2.1. Standards and reagents

Silica gel 0.060-0.200 mm, 60 A was obtained from Acros Organics (Gell, Belgium). Aluminum-backed silica-gel plates Sil G/UV_{254}, with 0.20 mm thickness, were obtained from Macharey-Nagel (Düren Neumann Neander, Germany). Phenolic compound standards were purchased from Extrasynthèse (Genay, France). Tryptic Soy Broth (TSB), Man Rogosa and Sharpe agar (MRS), were obtained from Biomerieux (Marcy l0Etoile, France). Blood agar with 7% sheep blood and MacConkey agar plates were acquired from Liofilchem (Roseto degli Abruzzi, Italy). The dye *p*-iodonitrotetrazolium chloride (INT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the solvents used were of analytical grade, used as received and were commercially obtained from Fisher Scientific (Lisbon, Portugal). Water was treated with a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Plant material

The dried inflorescences of *Calluna vulgaris* (L.) Hull (heather) were purchased from the Portuguese Herbal company "*Girassol"*. The plant material was reduced to a fine powder (~20 mesh) and stored at 4 ^{o}C with protection from light until further analysis.

2.3. Fractionation of the acetone extract

The acetone extract (5 g) was obtained according to Mandim *et al*.[11] being next dissolved in the smallest possible amount of CH_2Cl_2, mixed with silica gel and evaporated to dryness (Büchi R-20, Flawil, Switzerland). The mixture was placed on the top of a silica gel column and the dry-loaded extract was fractionated by gradient elution column chromatography, applying the elution system: CH_2Cl_2; CH_2Cl_2/EtOAc - (90/10), (80/20), (70/30), (60/40), (50/50), (40/60), (30/70), (20/80), (10/90); EtOAc; EtOAc/acetone - (90/10), (80/20), (70/30), (60/40), (50/50), (40/60); acetone; acetone/MeOH - (80/20), (60/40), (10/90); MeOH; MeOH/formic acid - (99/1), (97/3), (95/5). This increasing polarity elution system, allowed to obtain twenty-nine fractions that were grouped in ten final fractions (**Fr 1-10**), according to their TLC profiles, being **Fr 1** the one with lower polarity grade and **Fr 10** with higher polarity. The obtained fractions were evaporated to dryness under reduced pressure at 40 ºC (Büchi R-20, Flawil, Switzerland).[18]

2.4. Phenolic profile of the obtained fractions

A known mass of each fraction was re-dissolved in 20% MeOH at a final concentration of 20 mg/mL and filtered through a 0.22 µm nylon syringe. The phenolic compounds analysis was performed by High Performance Liquid Chromatography (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) with a diode array detector and coupled to mass spectrometry (Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA) equipped with an ESI source, following a methodology previously described by Bessada *et al*.[19].

The tentative identification of these compounds was also performed according to their retention times, the UV and mass spectra, and with available literature information. The compound´s quantification was accessed by comparing the area of the chromatographic

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peaks using commercial calibrations curves, when possible, or calibration curves of similar compounds when a commercial standard was not available. The results were expressed in mg per g of extract.

2.5. Evaluation of the antibacterial activity

Microorganisms and growth conditions. The majority of the pathogenic bacteria were clinical isolates from the local health unit of Bragança, Northeast of Portugal. Four Gram-positive bacteria: *Enterococcus faecaliis* obtained from urine, methicillin resistant *Staphylococcus aureus* (MRSA) isolated from expectoration and methicillin susceptible *Staphylococcus aureus* (MSSA) from a wound exudate; and six Gram-negative bacteria: *Morganella morganii* and *Pseudomonas aeruginosa* both isolated from expectoration, *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* isolated from urine, and two commensal pathogenic strains: *Neisseria gonorrhoeae* ATCC 49226 and a variable Gram: *Gardnerella vaginalis* ATCC 14018 (Liofilchem, Roseto degli Abruzzi, Italy), were tested. To evaluate the effects on the vaginal microbiota, non-pathogenic commercial microorganisms of the vaginal microflora were tested, namely: *Lactobacillus plantarum* DSM 12028, *Lactobacillus delbrueckii* subs. *bulgaricus* LMG 6901 and *Lactobacillus casei* NCTC 6375, kindly provided by the Catholic University of Porto.

All the bacteria were grown in TSB/MRS culture medium according to each bacterial strain needs, and further incubated for 24 hours, at 37 ºC except in the cases of *N. gonorrhoeae*, *G. vaginalis* and the *Lactobacillus* strains that were incubated for 48 hours, at 37 ºC with 10% CO_2.

Minimal inhibitory concentrations (MICs): The obtained fractions were dissolved in TSB/MRS culture medium and 5% DMSO at a final concentration of 20 mg/mL. The

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fractions exhibiting the higher antibacterial activity against pathogenic bacteria were also tested in microorganisms belonging to the vaginal microflora: **Fr 7**, **8**, **9** and **10**.

The MICs were determined by the microdilution method and the colorimetric assay using INT (*p*-iodonitrotetrazolium chlorite, 0.2 mg/mL). Briefly, 190 µL of the stock solution (20 mg/mL) were added to the first well of the 96-well microplate. Successive dilutions were carried out over the wells containing 90 µL of culture medium. Afterwards, 10 µL of inoculum (1.5×10^8 CFU/mL) were added to all the wells containing the tested concentrations in the range of 0.156 to 20 mg/mL.

The microplates were further incubated in an oven (Jouan, Berlin, Germany) at 37ºC for 24 h for *E. coli*, *K. penumoniae*, *M. morganii*, *P. mirabilis*, *P. aeruginosa*, *E. faecalis*, MRSA and MSSA. In the case of *N. gonorrhoeae*, *G. vaginalis* and the *Lactobacillus* strains, the incubation was at 37 ºC for 48h with 10% CO_2. Three negative controls were prepared (one with medium, another one with the fractions, and a third one with medium and antibiotic); and a positive control with medium and inoculum. Ampicillin, imipenem and vancomycin were the antibiotics used.

The MICs of the samples were determined after the addition of the INT (0.2 mg/mL, 40 µL) and after incubation at 37 ºC for 30 min. The viable microorganisms reduced the yellow dye to pink.[20] The MIC was defined as the lowest concentration that prevented this change and exhibited the complete inhibition of bacterial growth.

2.6. Statistical analysis

The assays were performed in triplicate and results were expressed as mean values ± standard deviation (SD). The results were analyzed by variance analysis (ANOVA) and by a Turkey HSD test ($\alpha = 0.05$). When we have less than three samples, the results were analyzed by *t*-Student test, as a form to determine the significant differences

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between two samples, with $p = 0.05$. The statistic program used to do all this analysis was SPSS v. 23.0 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. HPLC-DAD-ESI/MS analysis of phenolic compounds identified in the obtained fractions

Peak characteristics and tentative identities of the phenolic compounds are presented in **Table 1,** being the quantification of each individual compound in each fraction, showed in **Table 2**. A total of fourty-seven compounds were identified in the acetone fractions (**Fr 1** – **Fr 10**), composed by one phenolic acid, sixteen flavan-3-ols ((epi)catechin, B-type (epi)catechin dimers and trimers), two flavonones (eriodictyol derivatives), four flavanonols (taxifolin derivatives), and twenty-four flavonols (myricetin, quercetin, isorhamnetin, and kaempferol derivatives).

Compounds 5-*O*-caffeoylquinic acid (**4**), (+)-catechin (**6**), myricetin-*O*-hexoside (**16**), myricetin-3-*O*-glucoside (**21**), quercetin-3-*O*-glucoside (**32**), quercetin-*O*-hexoside (**33** and **38**), isorhamnetin-3-*O*-glucoside (**35**), kaempferol-*O*-rhamnoside (**39**) and isorhamnetin-*O*-rhamnoside (**40**) were common compounds present in the different organic and aqueous extracts of *Calluna vulgaris* (L.) Hull inflorescences.[11,15] Nevertheless, not all of the compounds ((+)-catechin (**6**) and kaempferol-*O*-rhamnoside (**39**)) were present in the acetone extract,[11] due to the higher separation after the fractionation.

Only one phenolic acid was found in all the fractions, being identified as 5-*O*-caffeoylquinic acid (peak **4**), which was positively identified in comparison with the commercial standard.

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Sixteen compounds were assigned as flavan-3-ols (i.e., catechins and procyanidins). These compounds were assigned based on their pseudomolecular ions and MS^2 fragmentation patterns, characterised by the formation of product ions from the cleavage of the interflavan bond and retro-Diels-Alder (RDA) and heterocyclic ring fissions (HRF) of the elementary flavan-3-ol units.[21] The mass spectra evaluation does not allow establishing the position of the linkage between flavanol units (*i.e.*, C4-C8 or C4-C6) and differentiation between isomeric catechins (*e.g.*, catechin/epicatechin). Compounds (+)-catechin (**6**) and (-)-epicatechin (**11)** were positively identified according to their retention time, mass and UV-Vis characteristics by comparison with commercial standards. The remaining compounds corresponded to proanthocyanidin oligomers of the procyanidin class (*i.e.*, consisting of catechin and/or epicatechin units). Peaks **1**, **2**, **3**, **5**, **8**, **10** and **17** presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 577 and MS^2 fragmentation patterns coherent with B-type (epi)catechin dimers (*i.e.*, (epi)catechin units with C4–C8 or C4–C6 interflavan linkages). Characteristic product ions were observed at *m/z* 451 (-126 u), 425 (-152 u) and 407 (-152-18 u), attributable to the HRF, RDA and further loss of water from an (epi)catechin unit, and at *m/z* 289 and 287, that could be associated to the fragments corresponding to the lower and upper (epi)catechin unit, respectively. Similarly, compounds **7**, **12**, **14**, **15**, **18**, **19** and **22** (pseudomolecular ions $[M-H]^-$ at *m/z* 865) were assigned as B-type (epi)catechin trimers. These compounds have been reported previously in a hydroalcoholic extract of different parts of *C. vulgaris*.[9]

Peaks **20**, **24**, **26** and **30** were assigned as taxifolin derivatives based on their UV spectra and the fragment at *m/z* 303 $[taxifolin-H]^-$. Peaks **20** and **24** ($[M-H]^-$ at *m/z* 465) released a unique fragment at *m/z* 303 $[taxifolin-H]^-$ (-162 mu, loss of a hexosyl moiety). Taxifolin-3-*O*-glucoside was previously detected in flowers of *C. vulgaris*, and

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therefore compound **20** was assigned as this compound. Peak **24** was tentatively identified as taxifolin-*O*-hexoside, due to the fact that ESI/MS analysis does not allow obtaining information about the nature and position of the sugar moieties. Compound **26** ([M-H]$^-$ at *m/z* 303) was positively identified as taxifolin. Similar assumptions were performed for compounds **9** and **23** ([M-H]$^-$ at *m/z* 449) being tentatively identified as eriodictyol-*O*-hexoside. To the best of our knowledge, these compounds were not previously identified in *C. vulgaris*.

The remaining peaks corresponded to flavonol glycoside derivatives deriving from quercetin (peaks **27**, **32**, **33**, **34**, **36**, **37**, **38**, **42**, **43**, **44**, **45** and **47**), kaempferol (peak **39**), myricetin (**13**, **16**, **21**, **25**, **28**, **29** and **31**) and isorhamnetin (peaks **35**, **40**, **41** and **46**) (**Table 1**). Compound **32** (quercetin-3-*O*-glucoside) and **35** (isorhamnetin-3-*O*-glucoside) were positively identified.

Peaks **27**, **33** and **38** ([M-H]$^-$ at *m/z* 463) released a fragment at *m/z* 301 [quercetin-H]$^-$ (-162 mu, loss of a hexosyl moiety) being all identified as quercetin-*O*-hexoside derivatives. Compounds **34** ([M-H]$^-$ at *m/z* 433), **36** and **37** ([M-H]$^-$ at *m/z* 433), presented a MS^2 fragment corresponding to the loss of a pentosyl (-132 mu) and a rhamnosyl (-146 mu) moiety, respectively, being assigned as quercetin-*O*-pentoside and quercetin-*O*-rhamnoside. Similarly, compounds **13**, **16**, **21**, **25**, **28** and **31** ([M-H]$^-$ at *m/z* 479) and **29** ([M-H]$^-$ at *m/z* 463) were identified as myricetin-*O*-hexoside and myricetin-*O*-rhamnoside, respectively. Compounds **39** ([M-H]$^-$ at *m/z* 431) and **40** ([M-H]$^-$ at *m/z* 461) were assigned based on their pseudomolecular ions using a similar reasoning as for quercetin derivatives, being identified as kaempferol-*O*-rhamnoside and isorhamnetin-*O*-rhamnoside, respectively. No information about the identity of the sugar moieties and location onto the aglycone could be obtained. Peaks **42**, **43** and **44** possessed the same pseudomolecular ion ([M-H]$^-$ at *m/z* 639) and were identified as quercetin-*O*-(feruloyl)-

glucoside, due to the observation of a product ion at *m/z* 463, from the loss of feruloyl residue (176 u), as well as the late elution, since the presence of the hydroxycinnamoyl residue implies a decrease in polarity. Similarly, compounds **46** ([M-H]$^-$ at *m/z* 609) and **47** ([M-H]$^-$ at *m/z* 593) were identified as isorhamnetin-*O*-(caffeoyl)-pentoside and quercetin-*O*-(*p*-coumaroyl)-rhamnoside, respectively.

Finally, for compounds **30** ([M-H]$^-$ at *m/z* 575), **41** ([M-H]$^-$ at *m/z* 583) and **45** ([M-H]$^-$ at *m/z* 567) no precise identification of the moieties was possible, therefore they were identified as taxifolin, isorhamnetin and quercetin derivatives.

Flavonol derivatives have been extensively reported in different parts and extracts of *C. vulgaris* (L.) Hull.[9,10] To the best of our knowledge, some of the identified compounds, such B type (epi)catechin dimer and trimer, eriodyctiol-*O*-hexoside, quercetin-*O*-(feruloyl)-hexoside, isorhamnetin-*O*-(caffeoyl)-pentoside and quercetin-*O*-(*p*-coumaroyl)-rhamnoside, have not been reported previously in this species. **Fr 4** presented the highest concentration in some of the identified compounds, followed by **Fr 7** > **Fr 8** > **Fr 9** > **Fr 10** > **Fr 6** > **Fr 5** = **Fr 1** > **Fr 3** = **Fr 2**, being flavan-3-ols, such as B-type (epi)catechin dimer, (-)-epicatechin and (+)-catechin the most abundant compounds. **Figure 1** represents the phenolic profile of the acetone fraction **Fr 8**.

3.2. Antibacterial activity of the obtained fractions

Table 3 presents the MIC values (mg/mL) of the 10 fractions tested against the pathogenic and non-pathogenic bacteria.

Pathogenic commensal bacteria: All the analyzed fractions revealed activity against all the tested bacterial strains. In general, the exhibited activity is higher in the most polar fractions, suggesting that the compounds with higher polarity present in the acetone extract are the major contributors to the antibacterial potential.

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Pseudomonas aeruginosa was the most resistant microorganism (MIC values ranging from 5 to >20 mg/mL), while methicillin susceptible *Staphylococcus aureus* (MSSA) was the most susceptible one (MIC values ranging from 0.15 to 20 mg/mL). By comparing the results of fractions with the antibiotics used, the fractions **Fr 6**, **Fr 7**, **Fr 8**, **Fr 9** and **Fr 10** revealed stronger results than ampicillin against *Klebsiella pneumoniae*, *Morganella morganii* and *P. aeruginosa*.

Fr 7, **Fr 8**, **Fr 9**, and **Fr 10** were the ones that benefice more with the fractionation process, showing higher antibacterial activity presenting the lower MIC values (MIC values= 0.15 - >20 mg/mL). The opposite occurs with the fraction's **Fr 4** and **Fr 5**, that revealed higher MIC values and, consequently, lower antibacterial activity (MIC values ranging from 20 to >20 mg/mL).

When comparing the results between the antibacterial activity of the acetone extract, previously studied,[11] with the correspondent fractions, it can be seen that the fractionation process wasn't benefic against some of the analyzed bacterial strains and to all the fractions obtained. **Fr 1**, **Fr 4** and **Fr 5** revealed higher MIC values to all pathogenic bacteria (MIC values ranging from 10 to >20 mg/mL) when compared to the acetone extract (MIC values ranging from 0.6 to 20 mg/mL). The same occurs with fractions **Fr 7**, **Fr 8**, **Fr 9** and **Fr 10** against *P. aeruginosa* and *Enterococcus faecalis*, with similar or higher MIC values in comparison to the extract (MIC values ranging from 5 to >20 mg/mL for the fractions and 2.5 to 5 mg/mL for the extract). **Fr 7** and **Fr 10** demonstrated also lower potential against methicillin resistant *Staphylococcus aureus* (MRSA) (MIC values ranging from 1.25 to 2.25 mg/mL against 0.6 mg/mL of the extract). Finally, **Fr 6** demonstrated lower activity against *P. aeruginosa*, *E. faecalis*, MSSA and MRSA (MIC values of 10 mg/mL against the 0.6 to 5 mg/mL revealed by the acetone extract).

On the other hand, it can be seen that the fractionation process was benefic for some of the tested fractions. **Fr 7 – Fr 10** revealed higher potential against *Escherichia coli* (MIC value of 2.5 mg/mL, against the 5 mg/mL of the extract), *K. pneumoniae* and *M. morganii* (MIC 1.25 – 2.5 mg/mL in comparison to 5 mg/mL of the extract) and against *Proteus mirabillis* (MIC values ranging from 2.5 to 10 mg/mL for the fractions and 10 mg/mL for the extract). **Fr 6** exhibited higher potential only against *P. mirabillis* (MIC value of 5 mg/mL against the 10 mg/mL demonstrated by the extract). **Fr 8** and **Fr 9** revealed also higher antibacterial activity to MSSA (MIC value of 0.15 mg/mL in comparison to the 1.25 mg/mL demonstrated by the extract).

Both of the commercial pathogenic vaginal bacterial strains, *Neisseria gonorrhoeae* and *Gardnerella vaginallis*, seem to be strongly inhibited by the obtained fractions. **Fr 8** presented a MIC value of 0.625 mg/mL, and **Fr 7**, **Fr 9** and **Fr 10** exhibited a MIC value of 1.25 mg/mL; lower MIC values than the one exhibited by the acetone extract against these strains (MIC = 2.5 mg/mL), in the case of *N. gonorrhoeae*. For *G. vaginallis*, **Fr 7** and **Fr 10** revealed a MIC value of 2.5 mg/mL and **Fr 8** and **Fr 9** a MIC value of 1.25 mg/mL**,** when compared with the MIC of 5 mg/mL showed by the acetone extract.[11]

The antibacterial properties of heather extracts were already related but, in some of the studies, heather did not revealed a promising activity; this fact can be justified by the studied parts of this shrub, mostly roots and seems.[5] Several authors reported that there was no inhibition by this matrix in microorganisms such as *E. coli*, *K. pneumoniae*,[22] MRSA, *P. mirabilis*,[5] and *P. aeruginosa*,[5,22] contrarily to what is reported in the present work.

Pathogenic and non-pathogenic vaginal bacteria: The bacteria species belonging to the vaginal microbiota have the capacity to defend the organism of possible infections, through of limitation of the growth of potentially harmful microorganisms. Due to this fact, it is desirable that the usual antibiotics interfere the lowest possible with the benefic microflora, e.g. *Lactobacillus* sp. and, at the same time, have the capacity to inhibit or even kill the pathogenic microorganisms.

As a result of the higher antimicrobial activity exhibited by **Fr 7**, **Fr 8**, **Fr 9** and **Fr 10** against the pathogenic bacteria, their effects on the benefic bacteria belonging to the vaginal microbiota was accessed. The four fractions presented a very similar activity, with MICs in the range of 0.625 and 2.5 mg/mL. Only **Fr 7** and **Fr 8** revealed the lowest MIC values to *N. gonorrhoeae,* the vaginal pathogenic microorganism comparatively to the beneficial *Lactobacillus*: **Fr 7**, presented a MIC value of 1.25 mg/mL against *N. gonorrhoeae* and 2.5 mg/mL against the *Lactobacillus*. In its turn, **Fr 8** showed a MIC of 0.625 mg/mL against *N. gonorrhoeae* and 1.25 mg/mL against the *Lactobacillus* strains. In both cases, the MIC values are lower comparatively to the acetone extract, revealing a stronger potential provided by the fractionation process. The stronger antibacterial potential showed by the **Fr 7** and **8** is consistent the higher contents in total phenolic compounds identified in these fractions (**Table 2**).

From **Table 3** it is possible to suggest that fraction **7** was able to inhibit the pathogenic *K. pneumonia*, *M. morganii* and *N. gonorrhoeae* (MIC value of 1.25 mg/mL) without affecting the vaginal microbiota (MIC value of 2.5 mg/mL). In its turn, **Fr 8** can inhibit *N. gonorrhoeae* and almost all the Gram-positive bacteria (MIC values ranging from 0.15 to 0.625 mg/mL), also causing no changes in the vaginal microbiota (MIC value of 1.25 mg/mL).

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All the tested fractions revealed lower MIC values when compared to the ones exhibited by the acetone extract,[11] which showed MIC values of 10 mg/mL against *Lactobacillus delbrueckii* and *Lactobacillus casei*, and 20 mg/mL against *Lactobacillus plantarum*. Thus, it is possible to conclude that **Fr 7** and **Fr 8** were the most promising fractions obtained from the acetone extract of *C. vulgaris*, since the antibacterial activity was significantly improved, highlighting the capacity to inhibit pathogenic bacteria with no interference on the benefic strains. It is described in the literature that the phenolic compounds can inhibit bacteria by different mechanisms of action such as the inhibition of DNA synthesis, resulting from an effect associated with the inhibition of RNA and protein synthesis, especially in the case of Gram-positive bacteria, or through the membranes alteration in the case of the Gram-negative strains.[23]

In another report about the antimicrobial activity of naphthol derivatives, the authors described that these compounds are able to be transformed into quinone methides intermediates during the metabolic processes, and, these compounds act like alkylating agents and inhibitors of serine proteases and β-lactamases.[24]

Fractionation studies should be performed in future works, as a way to identify the compounds responsible for the demonstrated activity and the synergic and/or antagonistic effects that may be associated. Moreover, the mechanisms of action of these compounds should also be deeply investigated to better understand their interaction with the bacterial strains. To the best of our knowledge, the antibacterial effects of the fractionated acetone extract from heather constituents has never been described.

4. Conclusions

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The antibacterial potential for which *C. vulgaris* is consumed in folk medicine was studied through scientific methods. The acetone extract was previously tested revealing the highest potential, therefore, being chosen to be fractionated through a silica gel column chromatography. The 10 fractions obtained were analyzed relatively to their phenolic composition and antibacterial potential using pathogenic and non-pathogenic bacterial strains. It was possible to identified 47 phenolic compounds: 1 phenolic acid, 16 fravan-3-ol, 2 flavanones and 24 flavanols. Type B (epi)catechin dimers, (-)-epicatechin and (+)-catechin were the major compounds found.

Moreover, the fractions presenting the highest concentration of phenolic compounds (**Fr 7** and **8**) were also the ones that presented the highest antimicrobial capacity. When comparing the fractions with the acetone extract, the fractionation process significantly increased the antibacterial activity against the pathogenic microorganisms, without affecting the *Lactobacillus* strains of the vaginal microflora.

This study reports, for the first time, the improvement of the antibacterial capacity by the fractionation of the acetone extract from heather, corroborating the use of this plant in the folk medicine for antimicrobial purposes. It should also be highlighted the capacity of fractions **7** and **8,** to inhibit the pathogenic bacteria and protect human health through vaginal microbiome preservation.

Conflict of interest

There are no conflicts to declare.

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Table 1 Peak characteristics and tentative identification of phenolic compounds of the fractions of the acetone extract of *C. vulgaris* (L.) Hull.

Peak	Rt (min)	λmax (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	5.19	279	577	425(100), 289(15)	B type (epi)catechin dimer
2	5.51	279	577	425(100), 289(15)	B type (epi)catechin dimer
3	6.19	279	577	425(100), 289(15)	B type (epi)catechin dimer
4	6.26	345	353	191(100), 179(11), 173(7), 161(15), 135(6)	5-*O*-Caffeoylquinic acid
5	6.33	279	577	425(100), 289(15)	B type (epi)catechin dimer
6	6.99	279	289	245(100),203(12),137(5)	(+)-Catechin
7	7.5	280	865	711(100), 573(19), 451(24), 411(16), 289(5), 287(2)	B type (epi)catechin trimer
8	7.61	279	577	425(100), 289(15)	B type (epi)catechin dimer
9	8.12	281	449	287(100)	Eriodyctiol-*O*-hexoside
10	8.35	279	577	425(100), 289(15)	B type (epi)catechin dimer
11	9.57	279	289	245(100),203(15),137(5)	(-)-Epicatechin
12	9.85	280	865	711(100), 573(22), 451(25), 411(18), 289(6), 285(2)	B type (epi)catechin trimer
13	10.51	281	479	317(100)	Myricetin-*O*-hexoside
14	10.71	280	865	711(100), 573(19), 451(24), 411(16), 289(5), 285(2)	B type (epi)catechin trimer
15	11.23	280	865	711(100), 573(19), 451(24), 411(16), 289(5), 285(2)	B type (epi)catechin trimer
16	11.27	281	479	317(100)	Myricetin-*O*-hexoside
17	12.36	279	577	425(100), 289(12)	B type (epi)catechin dimer
18	12.47	280	865	711(100), 573(19), 451(24), 411(16), 289(5), 285(2)	B type (epi)catechin trimer
19	12.89	280	865	711(100), 573(19), 451(24), 411(16), 289(5), 285(2)	B type (epi)catechin trimer
20	14.27	291	465	303(74),285(100)	Taxifolin-3-*O*-hexoside
21	14.97	281	479	317(100)	Myricetin-3-*O*-glucoside
22	15.45	280	865	577(45),425(100), 289(12)	B type (epi)catechin trimer
23	15.47	281	449	287(100)	Eriodyctiol-*O*-hexoside

24	16.14	291	465	303(74),285(100)	Taxifolin-*O*-hexoside
25	17.14	281	479	317(100)	Myricetin-*O*-hexoside
26	17.35	285	303	285(100)	Taxifolin
27	17.49	355	463	301(100)	Quercetin-*O*-hexoside
28	17.52	281	479	317(100)	Myricetin-*O*-hexoside
29	17.68	281	463	317 (100)	Myricetin-*O*-rhamnoside
30	17.99	280	575	303(40),285(100)	Taxifolin derivate
31	18.25	281	479	317(100)	Myricetin-*O*-hexoside
32	18.5	355	463	301(100)	Quercetin-3-*O*-glucoside
33	19.09	354	463	301(100)	Quercetin-*O*-hexoside
34	21.47	353	433	301(100)	Quercetin-*O*-pentoside
35	22.52	347	477	315(100)	Isorhamnetin-3-*O*-glucoside
36	22.55	347	447	301(100)	Quercetin-*O*-rhamnoside
37	23.52	342	447	301(100)	Quercetin-*O*-rhamnoside
38	26.39	355	463	301(100)	Quercetin-*O*-hexoside
39	27.33	343	431	285(100)	Kaempferol-*O*-rhamnoside
40	28.4	346	461	315(100)	Isorhamnetin-*O*-rhamnoside
41	30.15	354	583	315(100)	Isorhamnetin derivate
42	30.93	308	639	463(100),301(32)	Quercetin-*O*-(feruloyl)-hexoside
43	31.36	306	463	301(100)	Quercetin-*O*-(feruloyl)-hexoside
44	31.36	308	639	463(100),301(32)	Quercetin-*O*-(feruloyl)-hexoside
45	33.49	352	567	301(100)	Quercetin derivate
46	34.67	336	609	315(100)	Isorhamnetin-*O*-(caffeoyl)-pentoside
47	37.59	326	593	301(100)	Quercetin-*O*-(*p*-coumaroyl)-rhamnoside

Table 2 Quantification of individual phenolic compounds present in each fraction tested (mg/g of fraction; mean ± standard deviation).

Peak	Fr 1	Fr 2	Fr 3	Fr 4	Fr 5	Fr 6	Fr 7	Fr 8	Fr 9	Fr 10
1[a]	1.444 ± 0.003[e]	n.d.	1.100 ± 0.008[e]	206 ± 2[a]	17.2 ± 0.6[c]	8.5 ± 0.2[d]	31.6 ± 0.2[b]	n.d.	n.d.	n.d.
2[a]	0.755 ± 0.005[e]	n.d.	3.04 ± 0.01[d]	101.1 ± 0.3[a]	7.73 ± 0.02[c]	3.60 ± 0.01[d]	26.4 ± 0.7[b]	n.d.	n.d.	n.d.
3[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.68 ± 0.06	n.d.	n.d.	n.d.
4[b]	1.29 ± 0.03[a]	0.204 ± 0.002[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.90 ± 0.02[b]
5[a]	n.d.	n.d.	0.31 ± 0.01[d]	20.2 ± 2.5[a]	1.526 ± 0.002[c]	n.d.	n.d.	4.50 ± 0.07[b]	n.d.	n.d.
6[a]	n.d.	n.d.	n.d.	n.d.	n.d.	12.1 ± 0.3*	48.3 ± 0.8*	n.d.	n.d.	n.d.
7[a]	0.19 ± 0.04*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.4 ± 0.3*	n.d.	n.d.
8[a]	n.d.	n.d.	0.85 ± 0.03*	n.d.	n.d.	n.d.	11.69 ± 0.08*	n.d.	n.d.	n.d.
9[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.10 ± 0.01
10[a]	n.d.	n.d.	n.d.	n.d.	n.d.	10.32 ± 0.01	10.92 ± 0.01	n.d.	n.d.	n.d.
11[a]	n.d.	n.d.	n.d.	n.d.	n.d.	7.88 ± 0.03*	62.37 ± 1.52*	n.d.	n.d.	n.d.
12[a]	n.d.	0.105 ± 0.002[c]	n.d.	n.d.	n.d.	n.d.	n.d.	32.6 ± 0.6[a]	13.0 ± 0.2[b]	n.d.
13[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.5 ± 0.1
14[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	45 ± 1[a]	27 ± 1[b]	8.0 ± 0.2[c]	n.d.
15[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10.3 ± 0.3	n.d.	n.d.	n.d.
16[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.4 ± 0.2
17[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	11.4 ± 0.4	n.d.	n.d.
18[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.5 ± 0.2	n.d.	n.d.	n.d.
19[a]	7.24 ± 0.06[b]	n.d.	n.d.	n.d.	n.d.	0.70 ± 0.03[c]	11.67 ± 0.04[a]	n.d.	n.d.	n.d.
20[d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.26 ± 0.03[b]	44 ± 1[a]	3.14 ± 0.02[c]
21[c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.8 ± 0.4
22[a]	n.d.	n.d.	n.d.	n.d.	n.d.	1.45 ± 0.01	n.d.	n.d.	n.d.	n.d.
23[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	18.56 ± 0.05	n.d.	n.d.	n.d.
24[d]	0.84 ± 0.01*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.78 ± 0.05*	n.d.

25 [c]	2.280 ± 0.005	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
26 [d]	4.1 ± 0.1[b]	n.d.	n.d.	n.d.	n.d.	3.18 ± 0.09[c]	11.2 ± 0.2[a]	n.d.	n.d.	n.d.
27 [e]	n.d.	0.4589 ± 0.0004[a]	n.d.	0.25 ± 0.01[c]	0.301 ± 0.002[b]	n.d.	n.d.	n.d.	n.d.	n.d.
28 [c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.64 ± 0.08	n.d.	n.d.
29 [c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.9 ± 0.1
30 [d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.4 ± 0.2	n.d.	n.d.	n.d.
31 [c]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.3 ± 0.2	4.6 ± 0.1	n.d.
32 [e]	n.d.	n.d.	n.d.	n.d.	0.240 ± 0.002[c]	n.d.	n.d.	n.d.	5.4 ± 0.2[b]	18.696 ± 0.008[a]
33 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.84 ± 0.03
34 [e]	n.d.	n.d.	n.d.	n.d.	0.234 ± 0.002[c]	n.d.	n.d.	n.d.	1.80 ± 0.04[a]	0.89 ± 0.03[b]
35 [e]	n.d.	n.d.	n.d.	n.d.	0.234 ± 0.002*	0.804 ± 0.001*	n.d.	n.d.	n.d.	n.d.
36 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	29.4 ± 0.3[a]	20.0 ± 0.1[b]	2.07 ± 0.08[c]
37 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.4 ± 0.1	n.d.
38 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.745 ± 0.004	3.8 ± 0.2	n.d.
39 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.529 ± 0.002*	2.13 ± 0.03*	n.d.	n.d.
40 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.37 ± 0.04*	1.26 ± 0.04*	n.d.
41 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.15 ± 0.02	n.d.
42 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.39 ± 0.04	n.d.	n.d.
43 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.77 ± 0.05*	0.833 ± 0.005*
44 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.62 ± 0.04	1.55 ± 0.01	n.d.
45 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.95 ± 0.02	1.31 ± 0.06	n.d.
46 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.84 ± 0.02	0.93 ± 0.01
47 [e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.11 ± 0.05*	0.87 ± 0.02*	n.d.
TPC	23.55 ± 0.03[h]	0.768 ± 0.005[j]	5.29 ± 0.06[i]	327 ± 4[a]	27.4 ± 0.6[g]	48.5 ± 0.3[f]	174 ± 3[b]	146 ± 1[c]	116 ± 2[d]	58 ± 7[e]

n.d. – not detected; calibration curves: [a] catechin (y = 84950x – 23200, R^2 = 1); [b] chlorogenic acid (y = 168823x – 161172, R^2 = 0.999); [c] myricetin (y = 23287x – 581708, R^2 = 0.9988); [d] taxifolin (y = 203766x – 208383, R^2 = 1); [e] quercetin 3-*O*-glucoside (y = 34843x – 160173, R^2 = 0.9988); Different letters corresponded to significate differences ($p < 0.05$).*Means statistical differences obtained by a *t*-student test.

Table 3 Antibacterial activity of fractions of the acetone extract against pathogenic and non-pathogenic bacteria.

	Fr 1	Fr 2	Fr 3	Fr 4	Fr 5	Fr 6	Fr 7	Fr 8	Fr 9	Fr 10	Ampicillin (20 mg/mL)		Imipenem (1 mg/mL)		Vancomycin (1 mg/ml)	
Pathogenic																
						MIC					MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative																
Escherichia coli	>20	>20	>20	>20	>20	2.5	2.5	2.5	2.5	2.5	<0,15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	>20	>20	>20	>20	>20	2.5	1.25	1.25	2.5	2.5	10	20	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	>20	>20	>20	20	20	2.5	1.25	1.25	2.5	2.5	20	>20	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	>20	>20	>20	>20	>20	2.5	5	2.5	2.5	5	<0,15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>20	>20	>20	>20	>20	5	5	5	>20	>20	>20	>20	0.5	1	n.t.	n.t.
Neisseria gonorrhoeae	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	1.25	0.625	1.25	1.25	<0,15	<0.15	n.t.	n.t.	n.t.	n.t.
Gram-positive																
Enterococcus faecalis	5	20	20	20	20	5	5	5	5	5	<0,15	<0.15	n.t.	n.t.	<0.0078	<0.0078
MRSA	>20	>20	20	>20	20	5	2.5	0.625	0.625	1.25	<0,15	<0.15	n.t.	n.t.	<0.0078	<0.0078
MSSA	5	20	20	20	20	5	2.5	0.15	0.15	0.39	<0,15	<0.15	n.t.	n.t.	0.25	0.5
Variable Gram																
Gardnerella vaginallis	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	2.5	1.25	1.25	2.5	<0,15	<0.15	n.t.	n.t.	n.t.	n.t.
Non-pathogenic – bacteria																
Lactobacillus delbrueckii	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	2.5	1.25	1.25	2.5	<0.15	<0,15	n.t.	n.t.	n.t.	n.t.
Lactobacillus casei	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	2.5	1.25	1.25	2.5	<0.15	<0,15	n.t.	n.t.	n.t.	n.t.
Lactobacillus plantarum	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	2.5	1.25	1.25	1.25	<0.15	<0,15	n.t.	n.t.	n.t.	n.t.

MIC correspond to the minimum inhibitory concentration; MBC values correspond to the minimum bactericidal concentration; MIC and MBC values are in mg/mL; MRSA –methicillin resistant *Staphylococcus aureus*; MSSA –methicillin susceptible *Staphylococcus aureus*; n.t. – not tested

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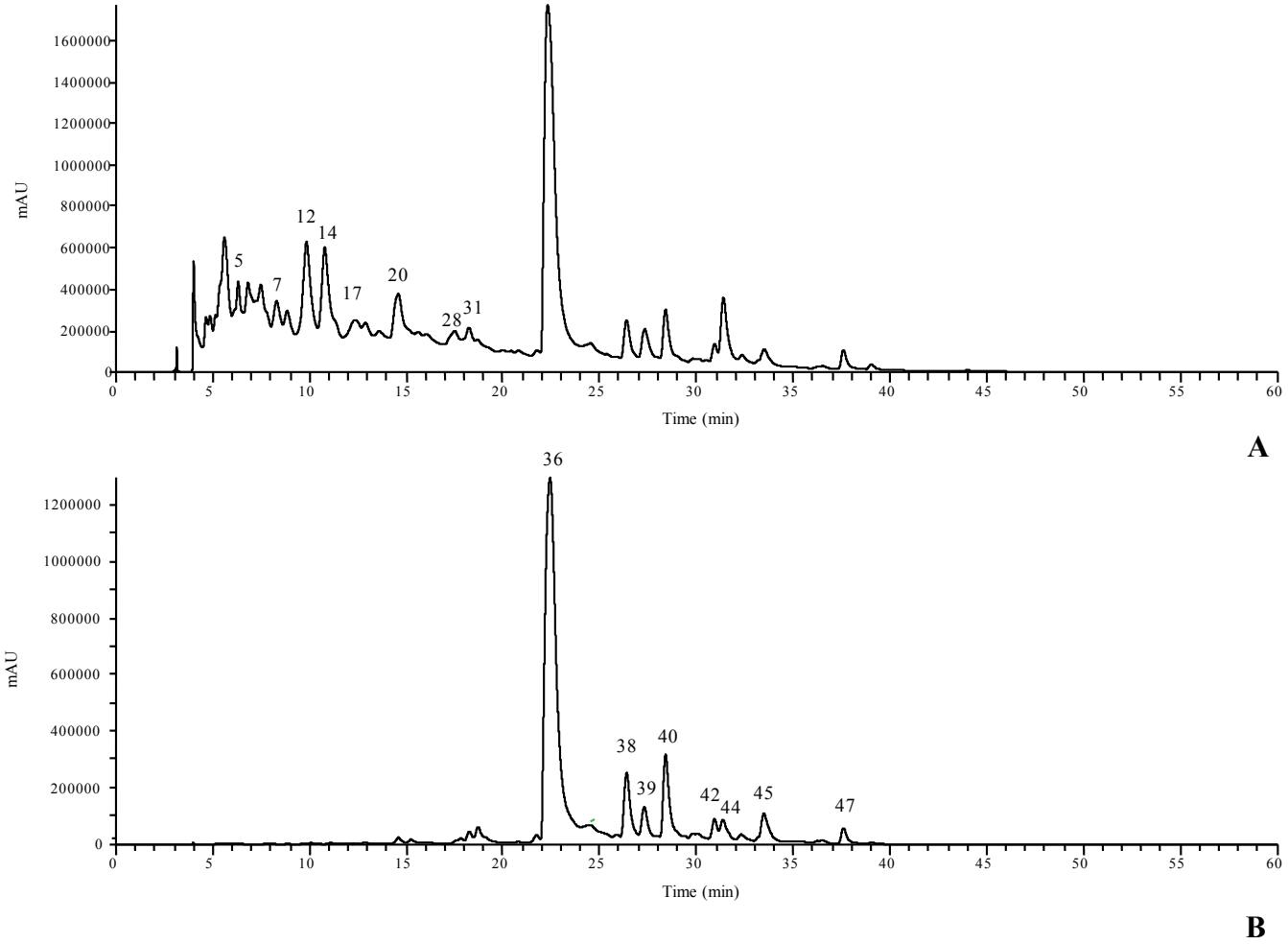

Figure 1. Phenolic profile of **Fr 8** recoded at 280 nm (**A**) and 370 nm (**B**).

Graphical abstract

Phenolic profile and effects of the acetone fractions obtained from the inflorescences of *Calluna vulgaris* (L.) Hull in vaginal pathogenic and non-pathogenic bacteria

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Calluna vulgaris (L.) Hull flower summits

Acetone extract was fractionated by silica gel column chromatography

Phenolic profile was analyzed by HPLC-DAD_ESI/MS

Antibacterial activity in vaginal pathogenic and non-pathogenic bacteria