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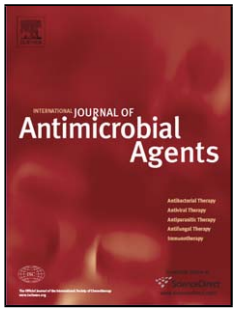

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Antimicrobial activity against *Helicobacter pylori* strains and antioxidant properties of blackberry leaves (*Rubus ulmifolius*) and isolated compounds

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Abstract

Rubus spp. (Rosaceae) provide extracts used in traditional medicine as antimicrobial, anticonvulsant, muscle relaxant and radical scavenging agents. Resistance to antibiotics used to treat *Helicobacter pylori* infection as well as their poor availability in developing countries prompted us to test the antimicrobial activity of *Rubus ulmifolius* leaves and isolated polyphenols against two *H. pylori* strains with different virulence ($CagA^+$ strain 10K and $CagA^-$ strain G21). The antioxidant activity (TEAC values) of the tested compounds ranged from 4.88 (gallic acid) to 1.60 (kaempferol), whilst the leaf extract gave a value of 0.12. All the isolated polyphenols as well as the leaf extract showed antibacterial activity against both of the *H. pylori* strains. The minimum bactericidal concentrations (MBCs) of the extract for *H. pylori* strains G21 and 10K, respectively, were 1200 μg/mL and 1500 μg/mL after 24 h of exposure and 134 μg/mL and 270 μg/mL after 48 h exposure. Ellagic acid showed very low MBC values towards both of the *H. pylori* strains after 48 h (2 μg/mL and 10 μg/mL for strains G21 and 10K, respectively) and kaempferol toward G21 strain (MBC = 6 μg/mL). A relationship between antimicrobial activity and antioxidant capacity was found only for *H. pylori* strain G21 $CagA^-$ strain.

1. Introduction

The bacterium *Helicobacter pylori* infects ca. 30% of the population in the Western world and ca. 80% of the population in many developing countries [1,2]. *Helicobacter pylori* is associated with severe pathologies, including peptic ulcer and gastric cancer [3–6]. The observation that only a subset of infected individuals develop severe gastroduodenal diseases may in part depend on the virulence of the infecting organism. Strains that possess the chromosomal insertion *cag* are endowed with an increased inflammatory potential [6–8]. *cag*-positive clones secrete a highly immunogenic protein called CagA that is linked to the development of premalignant and malignant histological lesions. Susceptibility of CagA-positive ($CagA^+$) *H. pylori* strains to antibiotics is noteworthy because the related infections significantly increase the risk for severe gastric pathologies [6].

Treatment of *H. pylori* infection consists of two antibiotics and a proton pump inhibitor. However, this is often accompanied by side effects and the selection of strains resistant to antibiotics. Thus, considerable interest has been focused on alternative/adjuvant approaches such as the use of biologically active compounds, including antioxidants from plants [9]. Several studies demonstrated the inhibitory effects on bacteria of a wide range of fruits, vegetables and their derivatives, such as berries [10], garlic [11–13], onion [14], kiwi [15], citrus [16] and wine [17], as well as plant extracts [18] and spices, in particular essential oils [19], cinnamon [20], thyme [21], propolis [22], liquorice [23], red paprika [24], tea [25] and rice [26]. In addition, further studies on the antimicrobial properties of compounds isolated from plant sources, such as resveratrol [27,28], allixin

[11], vitamin C [29], β-carotene and β-tocopherol [9,30] and garcinol [31,32], have given significant information about the existence of potential synergisms among different constituents [33].

Rubus ulmifolius is a perennial plant that grows in Italy in forest borders from sea level up to 1100 m above sea level [34]. *Rubus* spp. have been used in traditional medicine for their beneficial effects [35–39]. Blackberry leaves have been used for their anti-inflammatory, antiviral and antimicrobial properties [40] as well as their antiproliferative activity against cancer cells [41–44]. Recent studies have focused on the identification of the phenolic components of *Rubus* leaves as well as determination of their antioxidant activity [45].

In the present work, the polyphenolic components of *R. ulmifolius* leaves were identified and quantified and the radical scavenging activity of leaf extracts was then investigated. *Rubus ulmifolius* leaves and its main constituents were also tested for their antibacterial activity against *H. pylori* strains with various expression of virulence ($CagA^+$ and $CagA^-$ strains). To the best of our knowledge, the present study is the first to show a specific anti-*H. pylori* activity of *R. ulmifolius* extract.

2. Materials and methods

2.1. Plant material

Blackberry leaves (*R. ulmifolius*) were harvested during June 2004 from 'Le Capacce' farm in Fogliano (Siena, Italy) where they grow spontaneously.

Solvents and reagents were purchased from BDH (Poole, UK), except for ammonium persulphate [$(NH_4)_2S_2O_8$] obtained from Merck (Whitehouse Station, NJ) and the following compounds: [2,2'-azinobis-(3-ethilenebenzotiazolin)-6-sulfonic] acid (ABTS), quercetin 3-*O*-β-D-glucopyranoside (3,3'4',5-pentahydroxyflavone 3-beta-glucoside), kaempferol (3,4',5,7-tetrahydroxyflavone), ellagic acid (4,4',5,5',6,6'-hexahydroxydiphenic acid 2,6,2,6-dilactone), caffeic acid (3,4-dihydroxycinnamic acid), *p*-coumaric acid (trans-4-hydroxycinnamic acid), ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid) (Sigma-Aldrich, Saint Louis, MO);and gallic acid (3,4,5-trihydroxybenzoic acid), rutin (quercetin-3-rutinoside hydrate) and quercetin (3,3',4',5,7-pentahydroxyflavone dihydrate) (Fluka, Milwaukee, WI).

2.2. Matrix lyophilisation

Fresh *R. ulmifolius* leaves were homogenised in an IKA Labortechnik T25 Basic homogeniser (Staufen, Germany) and then lyophilised in an Edwards Freeze Dryer Modulo equipped with a Motors BS 5000-11 pump (Edwards, Dongen, The Netherlands) for 24 h.

2.3. Leaf phenolic extract

Powdered lyophilised leaves (300 mg) were extracted in 10 × 3 mL of $CH_3COOCH_2CH_3$ and subsequently in 10 × 3 mL of CH_3OH. After each extraction, the sample was placed in an ultrasound bath for 10 min (Bandelin Sonorex, Berlin, Germany) at a temperature >20 °C, subject to vortex for 2 min (Velp Steroglass, Perugia, Italy) and then centrifuged for 10 min at 3000 rpm (4226 Centrifuge; Prokeme, Firenze, Italy). The extract was dried with a rotating evaporator (Rotavapor® R-114; Büchi, Postfach, Switzerland) at room temperature. $CH_3COOCH_2CH_3/C_6H_{12}$ (1:0.6) was then added and the mixture was kept in the dark overnight. The brown precipitate was separated from the solution by centrifugation. Then, 10 mL of CH_3OH was added and the mixture was filtered before high-performance liquid chromatography (HPLC) injection.

2.4. HPLC analysis

Analysis of flavonoids, ellagic acid and phenolic acids was carried out on a Perkin-Elmer HPLC Series 200 system (Waltham, MA) equipped with a UV/VIS LC295 detector. Chromatograms were acquired and processed using TotalChrom software ver. 6.2.1. The column was a C18 Gemini 110 A 150 × 4 mm, 3 μm particle size (Phenomenex, Chemteck Analitica, Milan, Italy). The mobile phase was composed of solvent A (water/phosphoric acid 1%, pH 2.5), solvent B (acetonitrile) and solvent C (isopropanol). The linear gradient elution programme was as follows: 0.1 min, 95% A and 5% C; 75 min, 87% A, 3% B and 10% C; 18 min, 87% A, 3% B and 10% C; 15 min, 80% A, 10% B and 10% C; 8 min, 80% A, 10% B and 10% C; 10 min, 65% A, 25% B and 10% C; 10 min, 65% A, 25% B and 10% C; and 5 min, 50% A, 40% B and 10% C.

The flux rate was maintained at a constant value of 0.7 mL/min. The injection loop volume was 6 μL and the injection volume was 25 μL.

2.5. Identification and quantification of individual polyphenols, phenolic acids and ellagic acid

Identification of single compounds was carried out using the standard addition procedure. Quantification of flavonoids, phenolic acids and ellagic acid was performed by HPLC using six-point regression curves in the range 0.20–30 μg/mL on the basis of standards. Flavonoids and ellagic acid were determined at 254 nm; phenolic acids were monitored at 311 nm. To verify the reproducibility of the extraction method, three daily extractions for 5 days from a single sample were performed. The coefficient of variation of peak areas was always <10%.

2.5.1. Electrospray ionisation–mass spectrometry (ESI-MS) analysis

Characterisation of *Rubus* leaf extract was carried out by direct inlet flow using a LTQ ion trap mass spectrometer equipped with an ESI ion source (Thermo Finnigan, Rodano (MI), Italy), in negative ion mode.

Standard solutions of ellagic acid, kaempferol, quercetin, quercetin-3-*O*-β-D-glucopyranoside, caffeic acid, gallic acid, *p*-coumaric acid, rutin and ferulic acid were analysed in the same way.

Direct infusion was performed using a syringe pump (Unimetrics Corp., Shorewood, IL) of 500 μL volume at a flow rate of 10 μL/min. The optical system was optimised with kaempferol. The followings conditions were used: spray voltage 4.70 kV; capillary temperature 275 °C; sheath gas flow 15 arbitrary unit (a.u.); auxiliary gas flow 21 a.u.; and sweep gas flow 0 a.u.

2.6. Determination of antioxidant capacity

The antioxidant activity of isolated compounds and the extract was determined by the ABTS radical decolourisation assay [34,46]. The technique for the generation of ABTS radical ($ABTS^{\bullet+}$) involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between potassium persulphate and ABTS. The methodology evaluates the decrease in radical $ABTS^{\bullet+}$ absorbance at 751 nm in the presence of antioxidant species. The results were expressed as radical scavenging activity with respect to Trolox™ [(6-hydroxy-2,5,7,8-tetramethylchromon)-2-carboxylic acid] using the methodology described by Obòn et al. (TEAC) [47] where the TEAC values were defined as the ratios between the slopes of the straight lines, where %I (percentage of inhibition) vs. concentration of samples and Trolox™ [47] was plotted.

The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 751 nm. Increasing amounts of filtered samples were added to the hydroalcoholic solution of $ABTS^{\bullet+}$, vortexed for 30 s and the absorbance at 751 nm was read after 10 min.

2.7. Determination of minimum bactericidal concentrations (MBCs)

Two *H. pylori* strains were used to test the antibacterial activity of *R. ulmifolius* leaf extract and the isolated compounds. The $CagA^-$ G21 strain was isolated from a dyspeptic patient with non-active, non-atrophic, moderate chronic gastritis. The $CagA^+$ 10K strain was isolated from a patient with gastric carcinoma of diffuse histotype. Strain 10K was cytotoxic as it induced a vacuolating cytopathic effect on cells in culture. Strain G21 was not cytotoxic. The VacA subtype of both strains was s1/m1.

Rubus ulmifolius leaf extract and the isolated compounds were dissolved in *Brucella* broth–bovine serum with 4% dimethyl sulphoxide (DMSO) and sterilised by filtration. Samples were double diluted in *Brucella* broth–bovine foetal serum to a final volume of 100 μL using microtitre plates. Bacteria were suspended in *Brucella* broth from *Brucella* agar plates incubated for 48 h (cultures being in late log phase) and ca. 10^6 organisms (final number) were added to each dilution.

Following overnight incubation (24 h) in microaerobic conditions at 37 °C, 3 μL of each dilution was deposited onto Columbia blood agar plates, which were incubated at 37 °C in the same atmosphere for 3–5 days. Cell exposure to biocides was continued for an additional day. These 48 h samples (3 μL) were deposited on plates, which were also incubated for 3–5 days. The lowest concentration in broth whose subculture on agar showed complete absence of growth was considered the MBC.

2.8. Alignment of H^+,K^+-ATPase amino acid sequence with those of ionic pumps of Helicobacter pylori J99

The purpose of this test was to determine the target of the antimicrobial activity of *R. ulmifolius* and its extract. Certain phenolic antioxidants were shown to inhibit the gastric proton pump. Since *R. ulmifolius* extracts are mostly polyphenolic in nature, we compared the amino acid sequence of the human H^+,K^+-ATPase with that of bacterial ionic pumps. Should a homology exist, then it is likely that the bacterial targets of the substances assayed would comprise *H. pylori* ionic pump(s).

This test was performed by 'blasting' the N-acid sequences of human H^+,K^+-ATPase with the sequences of ATPases encoded by *H. pylori* strain J99, whose nucleotide sequence is available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein&cmd=search&term=).

3. Results

3.1. HPLC and ESI-MS analysis

To identify its main components, the extract of blackberry leaves was compared with a mixture of standards of gallic acid, caffeic acid, ferulic acid, ellagic acid, rutin, quercetin 3-*O*-β-D-glucopyranoside, quercetin and kaempferol by HPLC and ESI-MS analysis (Table 1). Blackberry leaf compounds were identified according to their retention times and absorbance spectra (Fig. 1).

ESI-MS analysis was utilised to confirm the presence of the identified compounds by comparison with standard solutions and by matching their molecular and fragmentation ions with literature data [48–53]. Identification of most compounds was achieved by MS^2 and MS^3 analysis. A spectrum of leaf extract obtained in negative detection is shown in Fig. 2.

Ellagic acid, quercetin and their glycosides produced negative m/z 301.0 ions, making it difficult to separate them without MS^n spectra. Further dissociation of the m/z 301.0 ion resulted in ions typical of flavonoids. Ellagic acid's ions produce higher m/z product ions in the 229.0–285.0 m/z range due to a ring structure that is more rigid than quercetin. The latter yielded products in the 100.0–200.0 m/z range.

In particular, the 301 ion dissociated to form m/z 228.9, 256.9, 178.9 and 150.9. The 256.9 ion underwent further dissociation (MS^3) to produce major ions m/z 229 and 185, characteristic of ellagic acid. The m/z 178.9 ion produced the m/z 150.9 ion, typical of quercetin.

The m/z 285.0 ion, dissociating in 150.9, 257.0, 241.0 and 249.0, was identified as free kaempferol by comparison with an authentic standard.

The m/z 463.0 compound was assigned as quercetin 3-*O*-β-D-glucopyranoside, as MS^2 yielded an ion at m/z 301.0 and the MS^3 spectrum of the m/z 301 fragment produced

two major ions at m/z 178.8 and 150.9 that matched the fragmentation pattern of quercetin.

The m/z 610.0 ion, dissociating in 301.0 and its MS^3 spectrum, produced m/z 178.9, 150.8, 273.0 and 256.9 ions and was identified as rutin.

By comparison with authentic standards, the m/z 179.0 ions (dissociating in 134.9), 169.0 (producing 124.9), 163.0 (breaking up 118.9) and 193.0 (fragmenting into 133.9, 148.9 and 177.9) were identified as caffeic acid, gallic acid, *p*-coumaric acid and ferulic acid, respectively.

The compound with an [M-H-] at m/z 433.0 was identified as a pentose conjugate of ellagic acid, as MS^2 yielded an ion at m/z 301.0 and MS^3 spectrum of the m/z 301.0 fragment produced two major ions at m/z 257.0 and 229.0, which matched the fragmentation pattern of ellagic acid.

The m/z 477.0 was tentatively identified as quercetin glucoronide (MS^2 m/z = 301.0; MS^3 m/z = 178.8 and 150.9) as the MS^3 ions showed that a quercetin aglycol, and not ellagic acid, was associated with this compound.

The compound with an [M-H-] at m/z 447.0 was assigned as a kaempferol-based compound, as MS^2 yielded the m/z 285.0 fragment. This latter fragment produced major ions at m/z 257.0, 229.0 and 241.0, matching the fragmentation pattern of kaempferol. These results agree with those conducted by other authors using mass spectrometric

analysis [49]. Table 1 reports all the identified compounds and their MS^n ion fragmentation.

Table 2 reports the polyphenol content of 100 mg of dried-freeze leaves determined by HPLC. The extract showed a high content of ellagic acid, quercetin 3-*O*-β-D-glucopyranoside and rutin, whilst polyphenolic acids were present in lower concentrations. These results are in agreement with those reported by Gudej and Tomczyl [54], who investigated the content of blackberry leaves belonging to different species of *Rubus*, not including *R. ulmifolius*. In particular, the content of quercetin, ellagic acid and kaempferol found in the present work was much higher than that found in other studies on strawberry, raspberry and blueberry leaves [54].

3.2. Antioxidant activity

The antioxidant capacity of the whole extract of blackberry leaves and of some of its components was evaluated by determining the radical scavenging capacity with respect to Trolox™ (TEAC values) (Table 3; Fig. 3). The antioxidant activity ranged from 4.9 (gallic acid) to 1.6 (kaempferol). It can be noted that the glucosylated compounds (rutin and quercetin 3-*O*-β-D-glucopyranoside) showed lower TEACs with respect to their related free form (quercetin). These results suggest that the antioxidant capacity of blackberry leaf extract was mainly due to ellagic acid that is present in high concentrations and has elevated TEAC values. Gallic acid showed higher TEACs, but was present in a much lower concentration in the sample.

Table 4 reports the inhibition percentages calculated for each compound, i.e. the percentage of inhibition towards ABTS absorbance at the concentrations determined by HPLC. These results show that the contribution of the identified compounds to the total inhibition of ABTS was ca. 58% of the total scavenging capacity.

3.3. Anti-Helicobacter pylori activity

Figure 4 shows the MBCs of polyphenolic *Rubus* leaf extract and the isolated compounds against *H. pylori* strains G21 and 10K after 24 h and 48 h exposure. The results show that all the isolated polyphenols, as well as the extract, have an antibacterial activity against both of the *H. pylori* strains. The MBC values of the extract for *H. pylori* strains G21 and 10K, respectively, were 1200 μg/mL and 1500 μg/mL after 24 h of exposure and 134 μg/mL and 270 μg/mL after 48 h of exposure.

Among the isolated compounds, ellagic acid, gallic acid and quercetin had MBC values <200 μg/mL against G21 both after 24 h and 48 h (Fig. 4A,C). In particular, after 48 h ellagic acid showed very low MBC values towards both *H. pylori* strains (2 μg/mL and 10 μg/mL for strains G21 and 10K, respectively) and kaempferol toward G21 strain (6 μg/mL). Quercetin 3-*O*-β-D-glucopyranoside caused only a 2–3 log decrease in colony-forming units (CFU) at a concentration >480 μg/mL for G21 and 10K after 24 h, whilst after 48 h it had an MBC of 480 μg/mL for G21 and 240 μg/mL for 10K. Rutin did not have any effect against G21 after 24 h, whilst it exhibited an MBC of 266 μg/mL after 48 h. An interesting and unique behaviour can be observed against 10K. After 24 h, only a

partial antimicrobial activity was observed at concentration of 130 μg/mL, whilst after 48 h of incubation at the same concentration the antimicrobial activity was complete.

3.4. Relationship between antioxidant and anti-Helicobacter pylori activities

MBC vs. TEAC values for strain G21 after 24 h and 48 h of exposure are reported in Fig. 5A,B and those for strain 10K are shown in Fig. 5C,D. The data show the existence of a relationship between MBC and TEAC values only for strain G21 after 24 h of exposure, where a decrease in MBC values with increasing antioxidant capacity of the tested samples was observed. Nevertheless, it can be noted that kaempferol showed a lower MBC than expected from its TEAC value. These results suggest that the anti-*H. pylori* activity exerted by these substances may contribute to the antioxidant capacity. It seems, in addition, that the antibacterial effects are related to different biological properties (cell targets) that are specific for each compound.

3.5. Alignments

We found strong homologies with the product of the *H. pylori* gene 889106 *copA*, a copper-transporting P-type ATPase ($E = 4 \times 10^{-10}$), and other ionic pumps (Table 5a). Homologous tracts of the gastric proton pump spanned two large segments of the entire bacterial copper-transporting ATPase. The two sections that aligned were 28% and 21% identical and 52% and 42% similar (Table 5b). Similarity refers to identical plus chemically similar amino acids.

4. Discussion

Plant extracts constitute important sources of biologically active compounds that may show significant antimicrobial properties. In this work, *R. ulmifolius*, its isolated polyphenols and the extract demonstrated antibacterial activity against two *H. pylori* strains. MBC values differ with regard to the tested samples (Fig. 6A) and the strains tested at different exposure times (Fig. 6B). The results obtained after 48 h of exposure show a general increase in antibacterial activity against both of the *H. pylori* strains. In particular, the extract appears to be much less effective after 24 h of exposure than the isolated compounds. This behaviour, which is more evident after 24 h of incubation, may be explained considering the low concentration of the single compounds in the extract and the presence of other constituents that may lack antibacterial activity.

The isolated rutin (quercetin-3-rutinoside), which is known to possess antimicrobial activity against some Gram-positive and Gram-negative bacteria [55,56], was the only polyphenol tested that showed an anti-*Helicobacter* activity against the more virulent $CagA^+$ 10K strain which was stronger than that exhibited against the $CagA^-$ G21 strain after 48h of exposure. This time-dependent behaviour suggests that such a compound may need longer times to saturate the target sites of the $CagA^+$ strains, i.e. a low affinity for the cell targets.

Ellagic acid, which kills *H. pylori* by inhibiting arylamine *N*-acetyltransferase activity [57], showed the lowest MBC values for both the *H. pylori* strains after 48 h of exposure. This result suggests that ellagic acid is an effective anti-*H. pylori* agent that could be used,

together with antibiotics, for the treatment of infections caused both by $CagA^+$ and $CagA^-$ clones.

Kaempferol was found to induce a significant decrease in the number of colonies of *H. pylori* in gerbils' stomachs after oral treatment [58]. Our data indicate that kaempferol, being active only against G21 strain after 48 h, shows a time-dependent antibacterial action, specific for $CagA^-$ strains, which are mostly associated with chronic gastritis only.

A time-dependent antibacterial behaviour has been observed also for blackberry leaf extract (Fig. 6), since MBC values for both the strains after 48 h are approximately one-fifth of those observed after 24 h of exposure.

The target of the antibacterial activity of these substances has yet to be discovered. A recent study has shown that the phenolic antioxidants of *Curcuma amada* (popularly known as mango ginger), which possesses antioxidative and antimicrobial properties against *H. pylori*, were capable of inhibiting the human gastric H^+,K^+-ATPase activity that is responsible for acid secretion [59].

We hypothesise that the target of the antibacterial action of *R. ulmifolius* and its extracts, which are polyphenols, could be one or more bacterial ion pumps. To support such a conjecture, we compared the structure of the gastric proton pump with those of *H. pylori* ion pumps and found significant linear homologies, suggesting that blackberry polyphenols kill *H. pylori* because they inactivate its ion pumps, i.e. enzymes that regulate the flux of copper and metal cations through membranes.

The different susceptibility to the blackberry leaf components of strains G21 and 10K deserves some comments. It is known that *H. pylori* infection is associated with increased production of reactive oxygen species (ROS) in the gastric mucosa. ROS may partially account for the ability of *H. pylori* to induce mucosal damage, which, in the long run, may lead to gastric cancer through the intermediate steps of atrophic gastritis and intestinal metaplasia. The capacity to promote oxidative stress appears to be especially related to the $CagA^+$ status, which is consistent with the notion that $CagA^+$ *H. pylori* strains are responsible for more severe gastric inflammation and higher gastric cancer risk [60,61]. Another explanation that could account for the different susceptibility of the two strains could be the different growth rates of the organisms tested, which may influence the MBC results. We have observed that strain G21 reached a stationary growth phase earlier than strain 10K. However, starting from the same inoculum, the number of CFU of both strains was the same after 3 days of incubation. We therefore believe that the growth characteristics of both strains had little, if any, effect on the susceptibility test results.

The observation that the strain harbouring *cagA*, i.e. 10K, was less susceptible to the substances tested cannot be transferred simply to all $cagA^+$ organisms because the various clinical isolates are very heterogeneous from a genomic point of view. However, *cagA* is also a marker for the presence of pathogenicity island (PAI) in the bacterial chromosome, an insertion that encompasses ca. 30 genes involved in virulence. The *cag* PAI genes encode substances involved in the inflammatory response and the trafficking of bacterial proteins within eukaryotic cells [6]. We assume that strains

possessing *cagA* may show a different behaviour towards chemotherapeutics, as the structures encoded by *cag* PAI might constitute a target for their antibacterial activity or, on the contrary, they may enhance access to the bacterial periplasm. In any case, should the observed low susceptibility to the polyphenols tested for strain 10K be extended to all CagA$^+$ *H. pylori* strains, such a phenomenon (i.e. decreased susceptibility to polyphenols) may help to explain why infection by CagA$^+$ *H. pylori* strains is more intimately associated with the development of gastric carcinoma.

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Ethical approval: Not required.

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Fig. 1. High-performance liquid chromatography (HPLC) chromatogram (monitored at 254 nm) of *Rubus ulmifolius* leafs extract: 1, gallic acid (t_R = 5.1 min); 2, caffeic acid (t_R = 27.6 min); 3, ferulic acid (t_R = 45.1 min); 5, ellagic acid (t_R = 85.1 min); 6, rutin (t_R = 89.3 min); 7, quercetin-3-*O*-β-D-glucopyranoside (t_R = 91.2 min); 8, quercetin (t_R = 130.7 min); and 9, kaempferol (t_R = 137.9 min). t_R, retention time.

Fig. 2. Electrospray ionisation–mass spectrometry (ESI-MS) spectrum of *Rubus ulmifolius* leaf extract.

Fig. 3. Antioxidant activity (TEAC values) of isolated compounds and leaf extract.

Fig. 4. Minimum bactericidal concentrations (MBCs) of polyphenolic *Rubus ulmifolius* leaf extract and isolated compounds: (A,B) after 24 h of exposure against *Helicobacter pylori* strain G21 (A) and strain 10K (B); and (C,D) after 48 h of exposure against *H. pylori* strain G21 (C) and strain 10K (D). Striped columns showed partial antibacterial activity, causing a decrease in colony-forming units of 2–3 logarithmic units. * Extract did not show any activity at the tested concentrations.

Fig. 5. Relationship between minimum bactericidal concentration (MBC) and antioxidant activity (TEAC values) of leafs extract and isolated compounds for (A,B) *Helicobacter pylori* strain G21 after 24 h of exposure (A) and 48 h of exposure (B) and (C,D) *H. pylori* strain 10K after 24 h of exposure (C) and 48 h of exposure (D).

Fig. 6. Minimum bactericidal concentrations (MBCs) of *Rubus ulmifolius* leafs extract and the isolated compounds (A) grouped by samples and (B) grouped by *Helicobacter pylori* strains.

Table 1

High-performance liquid chromatography (HPLC) and electrospray ionisation–mass spectrometry (ESI-MS) negative ion analysis of phenolic compounds in *Rubus ulmifolius* leaves

Peak [a]	t_R	Compound	MW	MS (m/z)	MS^2 (m/z)	MS^3
1	5.1	Gallic acid	170	169.0	124.9	
2	27.6	Caffeic acid	180	179.0	134.9	
3	45.1	Ferulic acid	194	193.0	133.9, 148.9, 177.9	
4		Coumaric acid	164	163.0	118.9	
5	85.1	Ellagic acid	302	301.0	256.9	229.0, 185.0
6	89.3	Rutin	610	610.0	301.0	178.9, 150.8, 273.0, 256.9
7	91.2	Quercetin 3-*O*-β-D-glucopyranoside	464	463.0	301.0	178.9, 150.8
8	130.7	Quercetin	302	301.0	178.8	150.9
9	137.9	Kaempferol	286	285.0	150.9, 257.0, 241.0, 249.0	
–	–	Quercetin glucoronide	478	477.0	301.0	178.8, 150.9
–	–	Kaempferol derivative	448	447.0	285.0	257.0, 229.0, 241.0
–	–	Ellagic acid pentose	434	433.0	301.0	257.0, 229.0

t_R, retention time.

[a] Numbers in the first column refer to the peaks in Fig. 1.

Table 2

Polyphenol content as determined by high-performance liquid chromatography (HPLC)

Compound	Content in 100 mg of dried-freeze *Rubus ulmifolius* leaves (μg)	% w/w
Ellagic acid	166.76 ± 8.33	0.167
Quercetin	2.17 ± 0.11	0.002
Kaempferol	1.03 ± 0.05	0.001
Rutin	95.43 ± 4.77	0.095
Ferulic acid	5.03 ± 0.15	0.005
Gallic acid	6.20 ± 0.30	0.006
Caffeic acid	4.73± 0.23	0.005
Quercetin 3-*O*-β-D-glucopyranoside	135.57 ± 6.64	0.135

Table 3

Antioxidant activity (TEAC values) of single compounds and leaf extract

Sample	TEAC
Ellagic acid	4.05 ± 0.11
p-Coumaric acid	2.08 ± 0.37
Ferulic acid	2.90 ± 0.16
Caffeic acid	1.95 ± 0.05
Gallic acid	4.88 ± 0.12
Quercetin	3.50 ± 0.03
Quercetin 3-*O*-β-D-glucopyranoside	2.27 ± 0.05
Rutin	2.16 ± 0.09
Kaempferol	1.60 ± 0.08
Leaf extract	0.122 ± 0.007
Trolox™	1.00 ± 0.05

Table 4

Percentage inhibition towards ABTS absorbance of the identified compounds in an extract sample showing a total inhibition of 55%

Compound	Concentration (mg/L)	Inhibition (%)
Gallic acid	7.70×10^{-3}	3.26
Ellagic acid	208.00×10^{-3}	10.68
Ferulic acid	4.16×10^{-3}	6.03
Caffeic acid	5.90×10^{-3}	0.00
Rutin	119.00×10^{-3}	7.15
Quercetin 3-*O*-β-D-glucopyranoside	166.00×10^{-3}	4.00
Quercetin	2.70×10^{-3}	0.00
Kaempferol	1.30×10^{-3}	0.60
Total		32

ABTS, [2,2'-azinobis-(3-ethilenebenzotiazolin)-6-sulfonic] acid.

Table 5

(a) Alignments between potassium-transporting ATPase α chain 1 (proton pump) (gastric H^+,K^+-ATPase subunit α) and proteins expressed by *Helicobacter pylori* J99

Ref.	Sequences producing significant alignments	Score (bits)	E value
NP_223072.1	Copper-transporting P-type ATPase	59.7	4×10^{-10}
NP_223445.1	Putative heavy-metal cation-transporting P-type ATPase	52.0	9×10^{-8}
NP_224114.1	Putative component of cation transport for cbb3-type oxidase	34.7	0.011
NP_222732.1	DNA primase	29.3	0.56
NP_224141.1	Type I restriction enzyme modification subunit	28.5	1.00
NP_224092.1	Hypothetical protein jhp1374	25.8	6.2
NP_223294.1	Hydrogenase, cytochrome subunit	25.4	8.0
NP_222890.1	Hypothetical protein jhp0169	25.0	10.0

(b) Alignments of human gastric H^+,K^+-ATPase with copper-transporting P-type ATPase (gene id: 889106 *copA*) from *H. pylori* J99

Ref. NP_223072.1 Copper-transporting P-type ATPase [*Helicobacter pylori* J99]

Score = 59.7 bits (143), Expect = 4e-10, Method: Compositional matrix adjust.

Identities = 32/114 (28%), Positives = 59/114 (52%), Gaps = 14/114 (12%)

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Query  702  ARTSPQQKLVIVESCQRLGAIVAVTGDGVNDSPALKKADIGVAMGIAGSDAAKNAADMIL  761

             +   PQ KL  ++  +  G IV + GDG+ND+P+L  +D+ V M   GSD +  AAD++
```

```
Sbjct  605  SNAKPQDKLNKIKELKEKGRIVMMVGDGLNDAPSLAMSDVAVVMA-KGSDVSVQAADIVS  663

Query  762  LDDNFASIVTGVEQGRLIFDNLKKSIAYTLTKNIPELTPYLIYITVSVPLPLGC  815
             +++  S+ + ++  +    N+K++  +    N           SV +PL C
Sbjct  664  FNNDIKSVYSAIKLSQATIKNIKENLFWAFCYN----------SVFIPLAC  704
```

Score = 48.5 bits (114), Expect = 9e-07, Method: Compositional matrix adjust.

Identities = 50/235 (21%), Positives = 99/235 (42%), Gaps = 23/235 (9%)

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Sbjct  230  PKTALKMHNNQQIEVLVDSIVVGDILKVLPGSAIAVDGEIIEGEG-ELDESMLSGEALPV  288

Query  237  TRSPECTHESPLETRNIAFF-------STMCLEGTVQGLVVNTGDRTIIGRIASLASGVE  289
             +      S     N +F             L   ++ +      +  I R+A   S V
Sbjct  289  YKKVGDKVFSGTLNSNTSFLMKATQDNKNSTLSQIIEMIHNAQSSKAEISRLADKVSSV-  347

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Sbjct  348  ------------FVPSVIAIAVLAFVVWLIIAPKPDFWWNFRTALEVFVSVLVISCPCAL  395
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Query  348  LATVTVCLSLTAKRLASKNCVVKNLEAVETLGSTSVICSDKTGTLTQNRMTVSHL  402
                 + + +   ++ +S     K+ +++E     + I  DKTGTLT  +  V  +
Sbjct  396  GLATPMSILVANQKASSLGLFFKDAKSLEKARLVNTIVFDKTGTLTNGKPVVKSI  450
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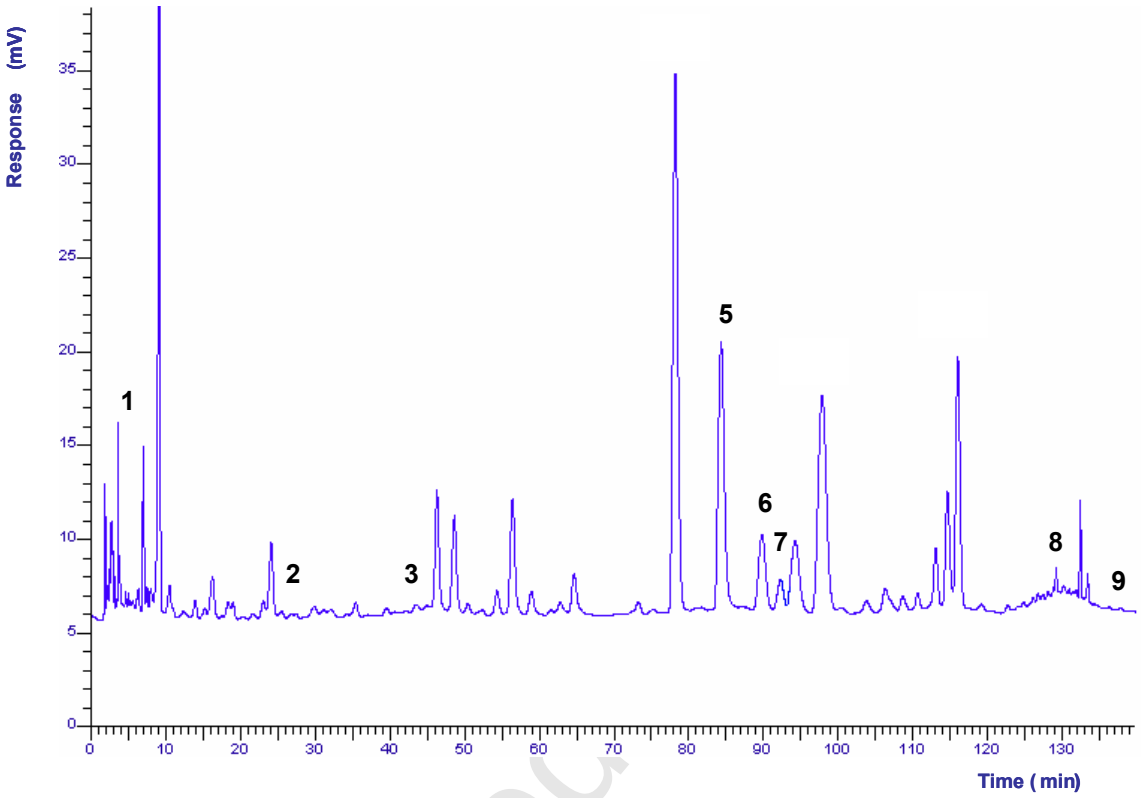

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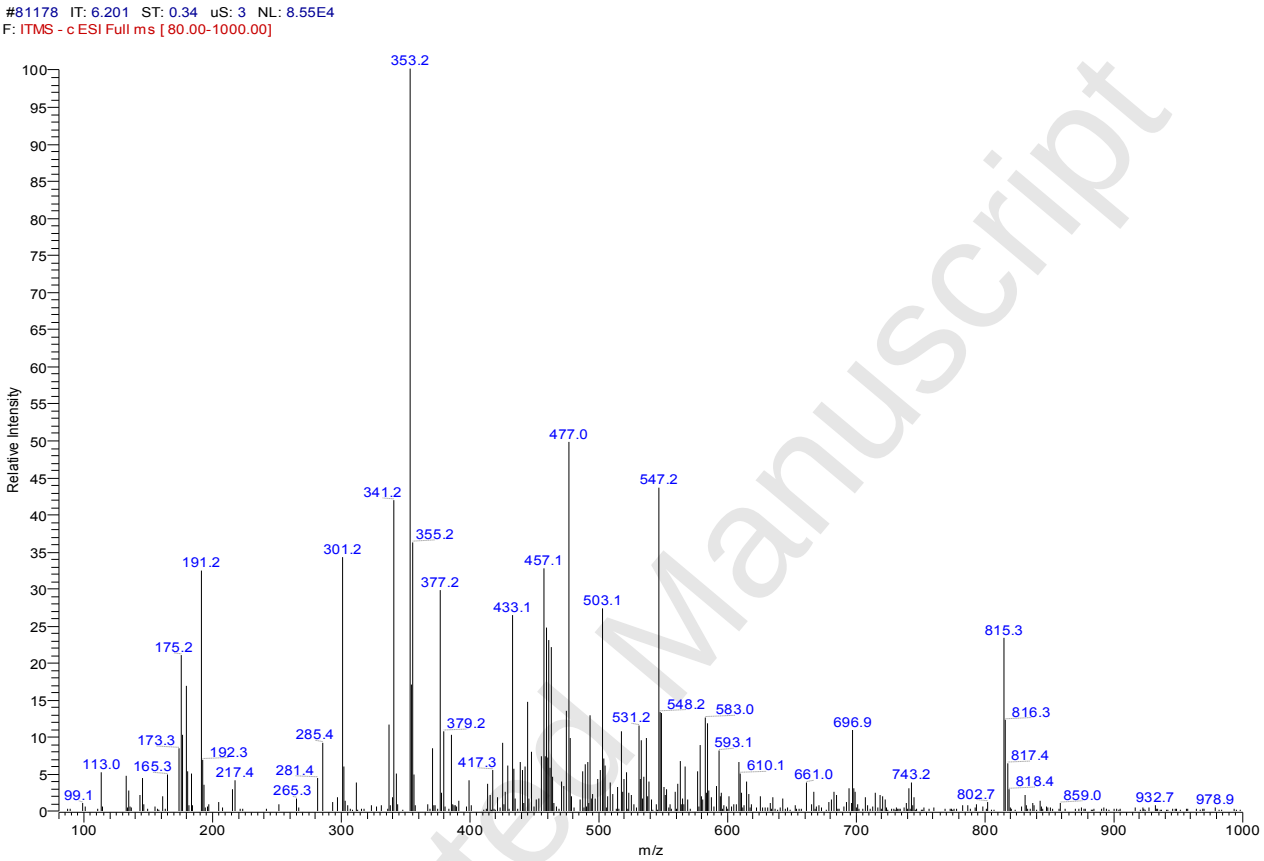

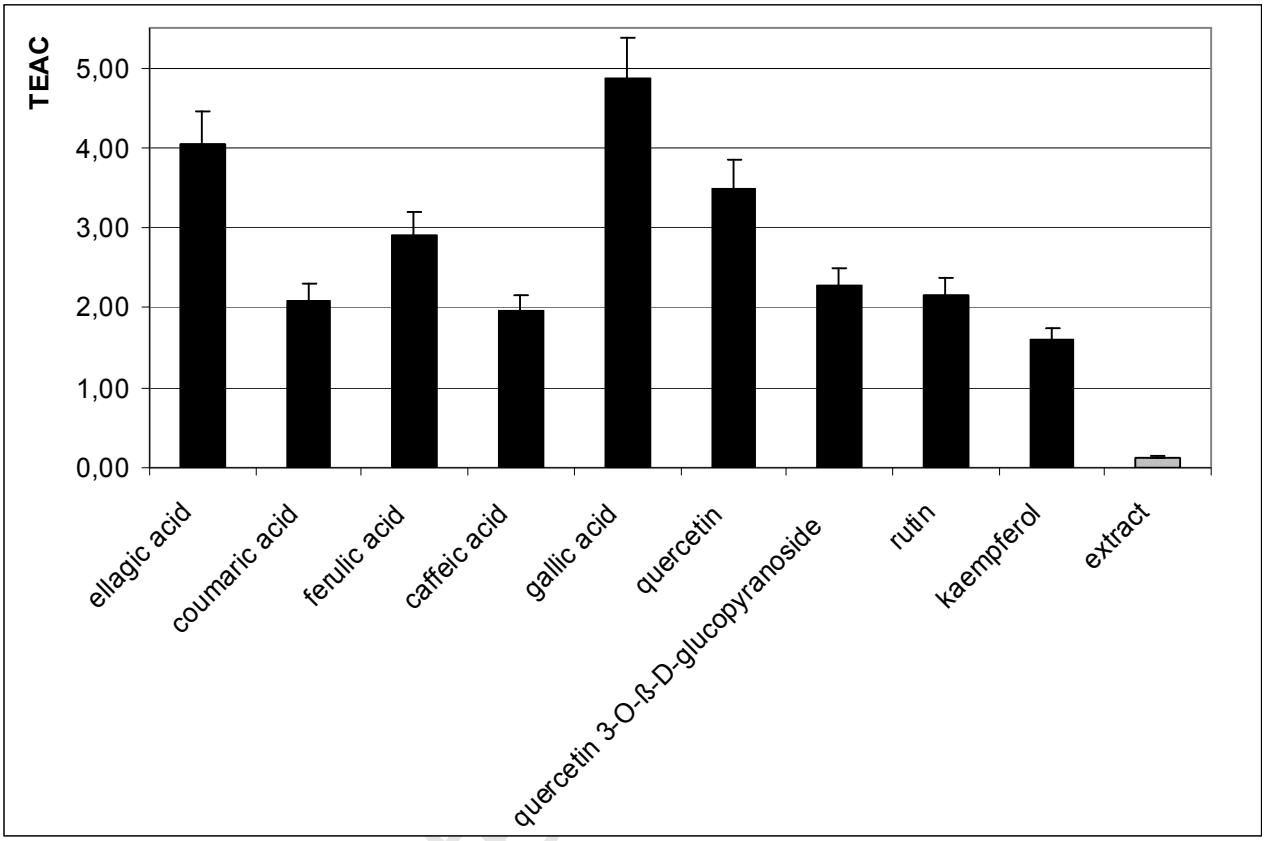

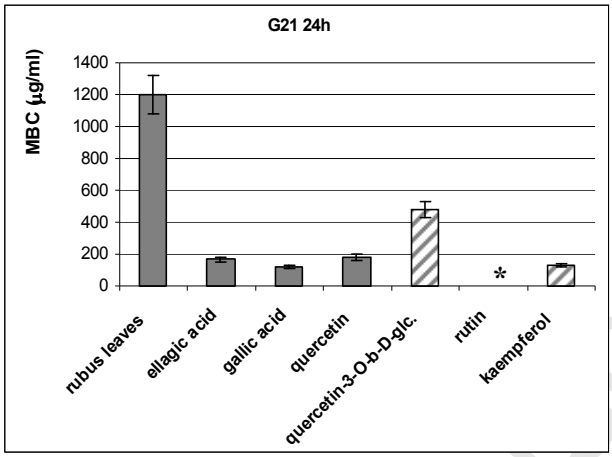

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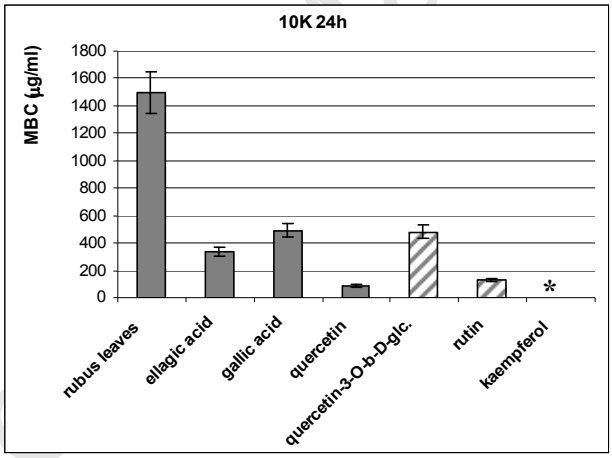

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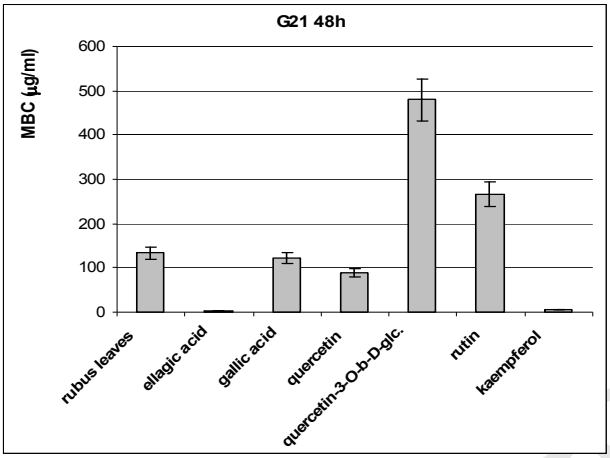

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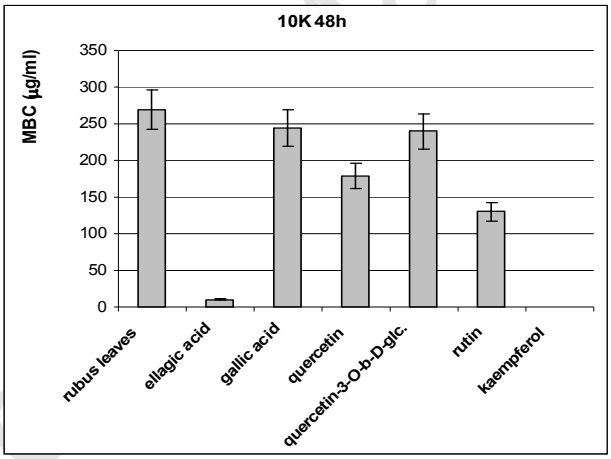

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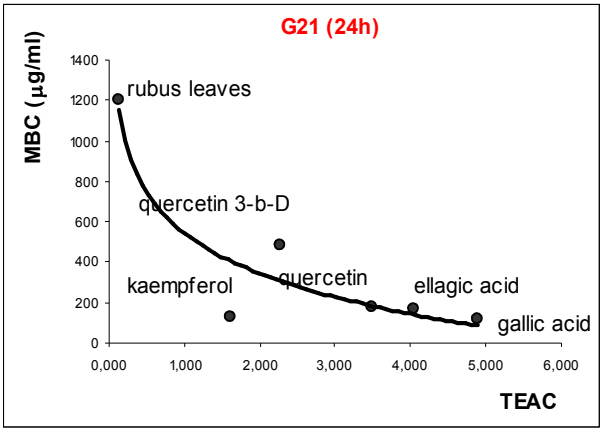

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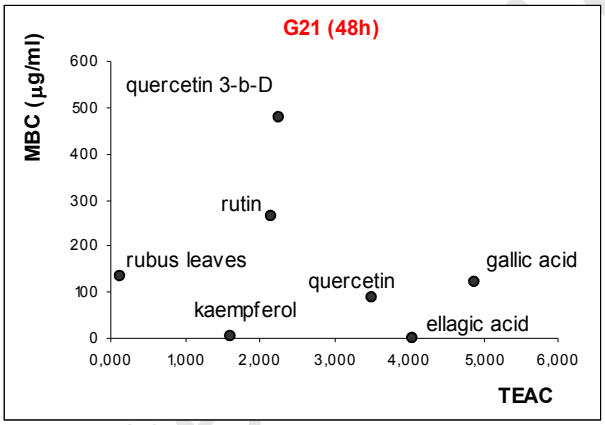

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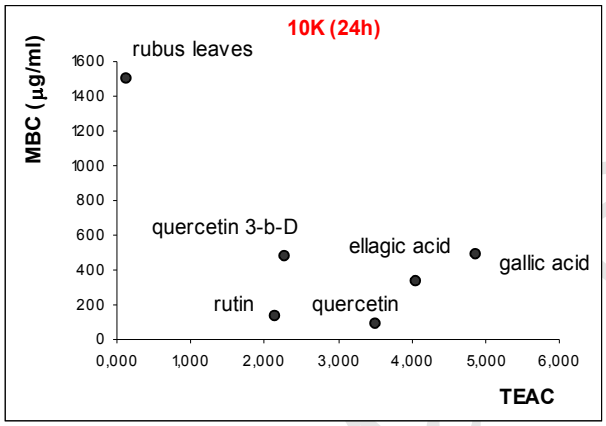

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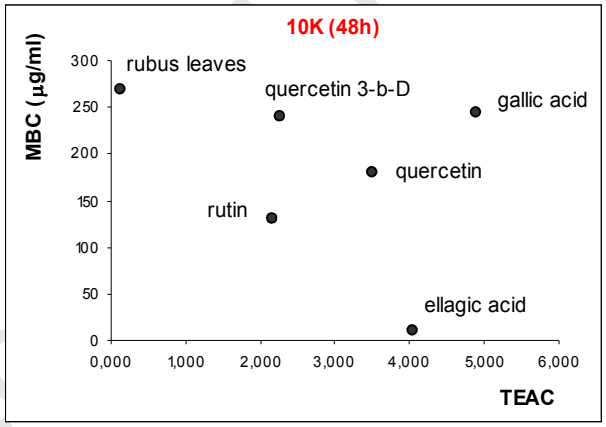

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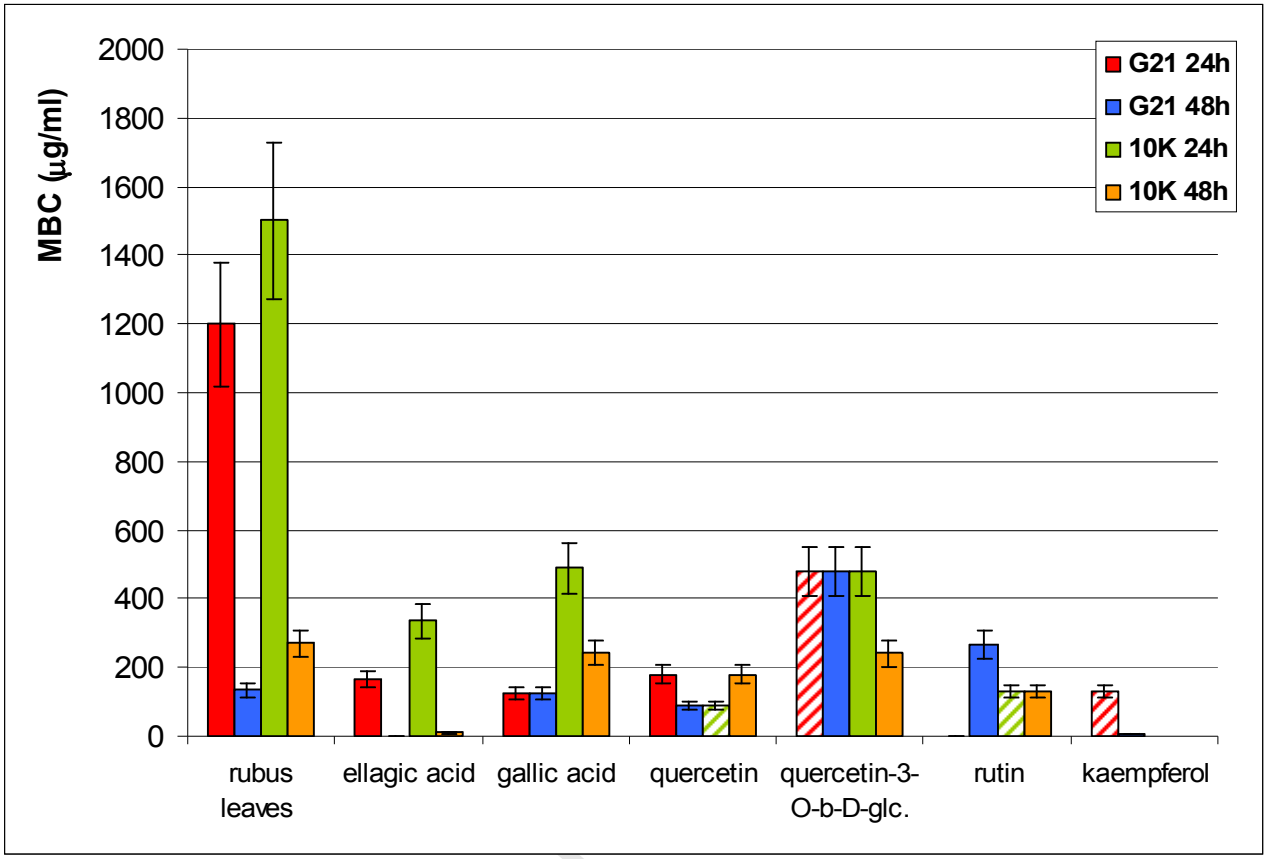

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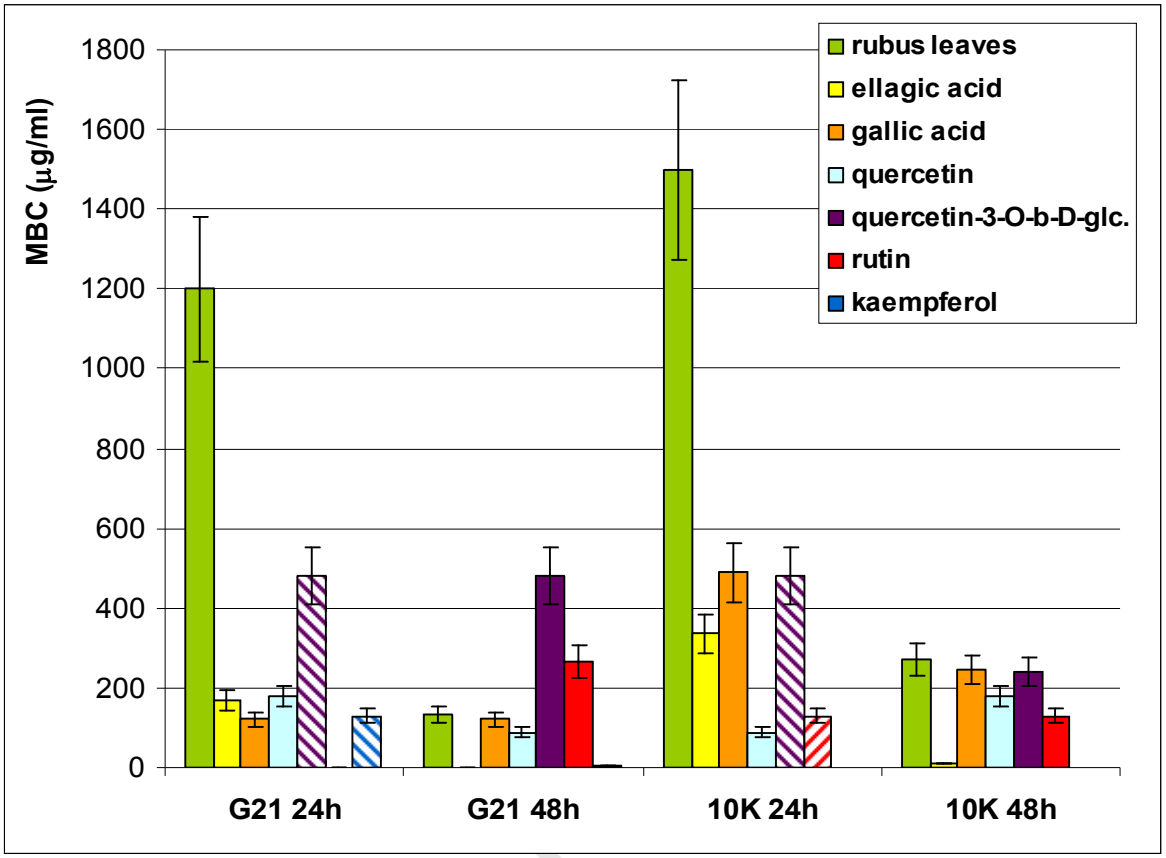

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