# HPLC-DAD and HPLC-ESI-MS-MS profiles of hydroalcoholic extracts of *Chamaemelum nobile* and *Mentha pulegium*, and study of their antihemolytic activity against AAPH-induced hemolysis.

Mustapha Tacherfiout[a*], Sarah Kherbachi[a], Meriem Kheniche[a], Marco Mattonai[b], Ilaria Degano[b], Erika Ribechini[b], Bachra Khettal[a]

[a] : Laboratoire de Biotechnologies Végétales et Ethnobotanique, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algérie.

[b] : Dipartimento di Chimica e Chimica Industriale, Università di Pisa, 56124 Pisa, Italy.

Mustapha Tacherfiout: mustapha.tacherfiout@univ-bejaia.dz

Sarah Kherbachi: sarah.kherbachi@snv.univ-bejaia.dz

Meriem Kheniche: meriem.kheniche@snv.univ-bejaia.dz

Marco Mattonai: marco.mattonai@dcci.unipi.it

Ilaria Degano: ilaria.degano@unipi.it

Erika Ribechini: erika.ribechini@unipi.it

Bachra Khettal: bachra.khettal@univ-bejaia.dz

* Corresponding author at : Laboratoire de Biotechnologies Végétales et Ethnobotanique, Faculté des Sciences de la Nature et de la Vie, Université de Bejaia, 06000 Bejaia, Algérie.

E-mail address: mustapha.tacherfiout@univ-bejaia.dz (M. Tacherfiout)

**Abstract**

*Chamaemelum nobile* (Roman chamomile) and *Mentha pulegium* (pennyroyal mint) are two herbs used in traditional medicine throughout the world, including Algeria to treat several diseases. The present research aimed to study the phenolic composition of *C. nobile* (CN) and *M. pulegium* (MP) hydroalcoholic extracts, as well as their antioxidant and antihemolytic effects on 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced hemolysis in human erythrocytes. Spectrophotometric estimation of total polyphenols, total flavonoids, flavones and flavonols and condensed tannins showed that CN was richer in total flavonoids, flavones and flavonols and condensed tannins (15.65 ± 0.77 mg QE/g, 4.29 ± 0.77 mg QE/ and 28.73 ± 1.47 mg CE/g, respectively for CN extract, against 7.41 ± 0.77 mg QE/g, 2.3 ± 0.24 mg QE/g and 23.21 ± 1.59 CE/g for that of MP), while MP was richer in total polyphenols (81.78 ± 1.19 mg GAE/g and 54.4 ± 2.69 mg GAE/g, respectively for MP and CN extracts). The CN and MP hydroalcoholic extracts were subjected to HPLC-DAD and HPLC-ESI-MS-MS analysis. A total of 46 compounds were detected, of which 39 were identified. For the CN extract, 33 compounds were detected, among which 27 were identified. In contrast, for the MP extract, only 19 compounds were detected and 18 identified. The compounds found in the two extracts are divided into five groups based on their structures: phenolic acids, quinic acid esters, flavonoids, caffeic acid oligomers and fatty acids. However, caffeic acid oligomers were only present in *M. pulegium* extract. Dicaffeoylquinic acid was the compound that gave the highest peak area in CN extract, while in MP extract rosmarinic acid was the most abundant. Both extracts showed good antioxidant activity. The $IC_{50}$ for DPPH radical scavenging activity were 19.98 ± 0.91 µg/mL for MP extract and 52.77 ± 1.53 µg/mL for CN extract. The MP extract had a reducing power of 223.74 ± 1.08 mg ascorbic acid equivalent/g extract, while CN's was 130.65 1.69 mg Asc E/g. Both extracts were also significantly effective in reducing AAPH-induced oxidative damage. Pretreatment of human erythrocytes with various dosages of the two extracts in the presence of AAPH significantly reduced hemolysis in a dose-dependent manner, with $IC_{50}$s of 127.48 ± 3.14 µg/mL for the CN extract and 129.52 ± 2.15 µg/mL for the MP extract. Lipid peroxidation induced by AAPH and estimated by MDA levels revealed that both extracts significantly reduced MDA levels at doses of 200 and 300 µg/mL, particularly MP extract, where MDA levels were practically identical to those of control erythrocytes cultured only in PBS. Our finding provide evidence that that *C. nobile* and *M. pulegium* are important sources of antioxidants that have a considerable protective effect on the erythrocyte membrane against free radicals generated by

AAPH. These antioxidant and antihemolytic properties may protect against diseases caused by free radicals.

**Keywords:**

*Chamaemelum nobile, Mentha pulegium*, Antioxidants, AAPH-hemolysis, Lipid peroxidation

## 1. Introduction

Erythrocytes (red blood cells) are the most prevalent blood cells which are responsible for carrying oxygen from the lungs to the body's various tissues via the bloodstream **(Pandey and Rizvi, 2010)**. Their biconcave disk form allows them to exchange gases more efficiently. The normal lifespan of erythrocytes is 120 days and are produced in the bone marrow by the erythropoietin regulating effect **(Hamidi and Tajerzadeh, 2003)**. Erythrocytes are under continuous stress by oxygenation and deoxygenation cycles as well as from reactive oxygen species (ROS). **(Barodka et al., 2014)**. ROS includes both oxygen radicals and non-radical species which are generated in cells during normal metabolism, especially throughout the energy production process by a variety of enzymatic and non-enzymatic processes **(Bayr, 2005)**. Moreover, in mature erythrocytes without mitochondria, the oxygenation (ferric state) and deoxygenation (ferrous state) cycles of heme iron lead to the production of superoxide and then hydrogen peroxide **(Fibach and Dana, 2019)**. When the production of ROS via exogenous or endogenous factors takes over the antioxidant systems, oxidative stress is generated **(Beaudeux et al., 2006; Fibach and Dana, 2019)**, which can be the initial etiology or the potentiator of several human pathologies such as cancer, cataract, atherosclerosis, diabetes, cardiovascular disorders, rheumatism, etc. **(Favier, 2006)**. This excess of ROS and oxidative stress on the erythrocyte membrane mainly affects proteins and lipids leading to cross-linking of membrane proteins, lipid peroxidation or a combination of both **(Edwards and Fuller, 1996)**. Due to the richness of their membrane in polyunsaturated fatty acids, as well as their high concentrations of cellular oxygen and hemoglobin, erythrocytes are particularly vulnerable to oxidative stress damages, especially lipid peroxidation **(Hseu et al., 2014)**. This lipid peroxidation in turn induces physical and chemical alterations of the membrane leading to changes in the chemical composition, lipid distribution and the packing rate of erythrocytes **(Spengler et al., 2014)**. In addition, lipid peroxidation leads to secondary oxidation products such as aldehydes like malondialdehyde (MDA) and 4-hydroxy-2- ,3-trans-nonenal (HNE), isoprostanes (IsoP) and oxysterols **(Michel et al., 2008)**, which can

damage DNA and produce mutations, impact protein production, and cause additional damage by cross-linking proteins **(Pandey and Rizvi, 2010)**.

Herbal medicine has been the primary source of primary health care around the world since ancient times. In Algeria, medicinal and aromatic plants are mainly used in rural areas, especially by the elderly who know the secrets that are passed on from one generation to another **(Reguieg, 2011)**. The therapeutic virtues of these plants are often attributed to their secondary metabolites such as alkaloids, terpenoids, phenolic acids, flavonoids, tannins, saponins...etc. **(Wink, 2015)**. According to the World Health Organization (WHO), almost 80% of the world's population still uses plant-based medications **(Uritu et al., 2018)**. *Chamaemelum nobile* (Roman chamomile) and *Mentha pulegium* (pennyroyal mint) commonly called “Babounge” and “Feliou” respectively, are two plants known in Algeria and used in traditional medicine.

Chamomile is represented by Roman chamomile (*Chamaemelum nobile*) and German chamomile (*Matricaria chamomilla*), two varieties well known worldwide. Chamomile is recognized as a universal treatment for almost all common ailments of human beings due to its healing powers **(Russo et al., 2021; Srivastava and Gupta, 2015)**. It has been used in traditional medicine as anti-inflammatory, anti-infective, sedative **(Sharifzadeh et al., 2016)**, to treat influenza, gastrointestinal disorders, anxiety, convulsions, rheumatic pain and muscle spasms, mucosal ulceration, ulcerations and hemorrhoids **(Tai et al., 2020)**, and like antiseptic, disinfectant, bactericidal, antibiotic, fungicidal and vermifuge **(Guimarães et al., 2013)**. Every day, over one million cups of chamomile tea are consumed around the world **(Bhaskaran et al., 2012)**. *Chamaemelum nobile* (L.) is a perennial herb found in Western Europe, North Africa and North America that belongs to the Asteraceae family **(Carnat et al., 2004; Guimarães et al., 2013)**. In the United States, *C. nobile* is listed as GRAS (generally recognized as safe) **(Zhao et al., 2014)**. *C. nobile* is rich in essential oils, mainly esters of angelic acid and tiglic acid. It also contains a-pinene, farnesene, and sesquiterpene lactones of the germacranolide type such as nobilin and 3-epinobilin **(Srivastava and Gupta, 2015)**. It is also characterized by the presence of other secondary metabolites as flavonoids consisting either of flavones such as apigenin and luteolin, or flavonols such as quercetin and patuletin, which are present in various forms such as aglyco-, mono-, and di-glycosides and/or acyl derivatives, terpenoids, coumarins, hydroxycoumarins, polysaccharides, steroids, and organic acids that are responsible for its beneficial effects **(Bhaskaran et al., 2012; Zhao et al., 2014)**.

*Mentha* is a genus of 61 species belonging to four sections (*Pulegium*, *Tubulosae*, *Eriodontes*, and *Mentha*) in the Lamiaceae family **(Benabdallah et al., 2018)**, which is one of the most important plant families whose best known members are a variety of aromatic spices such as thyme, oregano, mint, sage, basil, rosemary, savory, etc. **(Uritu et al., 2018)**. In Algeria, fifteen species of *Mentha* are listed including five important species, among which *Mentha pulegium* **(Brahmi et al., 2020)**, which is a species of flowering plant commonly called pennyroyal (European), squaw mint, mosquito plant or pudding grass, native to Europe, North Africa and the Middle East **(Miraj and Kiani, 2016)**. *M. pulegium* has been traditionally used to treat flatulent dyspepsia and intestinal colic with its carminative and antispasmodic characteristics **(BOULKBACHE-MAKHLOUF et al., 2014)**. It is also used as an emmenagogue, abortifacient, and to cure colds, influenza, smallpox, and tuberculosis, as well as to accelerate latent menstruation **(Miraj and Kiani, 2016)**.

Phenolic compounds are plant secondary metabolites with defense, growth, and developmental functions. They are a well-studied family that includes over 8000 distinct phenolic structures. They are often in the form of esters and glycosides and have at least one aromatic ring to which one or more hydroxyl groups are attached **(Carocho and CFR Ferreira, 2013)**. High consumption of phenolic-rich fruits and vegetables is associated with health benefits. Phenolic compounds are known for their antioxidant power and present a wide range of activities such as antimicrobial, antiviral, anti-inflammatory, anti-tumor, anti-cancer, anti-mutagenic, reduction in coronary heart disease risk, anti-allergenic, and anti-artherogenic **(Balasundram et al., 2006; Umar Lule and Xia, 2005)**.

To our knowledge, no study has been conducted on the anti-hemolytic properties of *Chamemelum nobile* and *Mentha pulegium* phenolic compounds. Therefore, the aim of the present work is to characterize the phenolic compounds of the hydroalcoholic extracts of the two plants using colorimetric methods, HPLC-DAD and HPLC-MS-MS, as well as to investigate their antioxidant and antihemolytic activities against hemolysis induced by 2,2'-Azobis(amidinopropane) dihydrochloride (AAPH) in human erythrocytes.

## 2. Material and methods

### 2.1 Reference polyphenols

Gallic acid (98%, Fluka), 3,4-dihydroxybenzoic acid (≥ 97%, Fluka), 4-hydroxybenzoic acid (99%, Sigmal-Aldrich), 3-hydroxybenzoic acid (99%, Sigma-Aldrich), vanillic acid (97%,

Sigma-Aldrich), syringic acid (98%, Sigma-Aldrich), caffeic acid (97%, Sigma-Aldrich), chlorogenic acid (≥ 95%, Sigma-Aldrich), p-coumaric acid (≥ 98%, Sigma-Aldrich), ferulic acid (99%, Sigma-Aldrich), sinapic acid (≥ 99%, Fluka), o-coumaric acid (97%, Sigma-Aldrich), trans-cinnamic acid (97%, Sigma-Aldrich), ellagic acid (97%, Lancaster), rutin (≥ 95%, Fluka), quercitrin (> 99%, Sigma-Aldrich), myricetin (> 95%, Biochemika), naringenin (≥ 95%, Fluka), quercetin (≥ 98%, Sigma-Aldrich), and genistein (97%, Alfa-Aesar) were used as reference polyphenols. Two stock solutions, M1 and M2, were prepared in methanol. Each solution contained ten polyphenols at a concentration of approximately 10 ppm, as follows: M1 – gallic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, p-coumaric acid, sinapic acid, trans-cinnamic acid, rutin, myricetin, quercetin. M2 – 3,4-dihydroxybenzoic acid, 3-hydroxybenzoic acid, syringic acid, chlorogenic acid, ferulic acid, o-coumaric acid, ellagic acid, quercitrin, naringenin, genistein.

### 2.1. Plant material

The aerial parts of *Chamaemelum nobile* and *Mentha pulegium* were purchased from an herbalist in the locality of Bejaia (north-eastern Algeria). They were authenticated by Dr. Abdelazize Franck Bougaham, from Laboratory of Applied Zoology and Animal Ecophysiology, Faculty of Nature and Life Sciences, University of Bejaia. The aerial parts of both plants were cleaned, dried and ground with an electric grinder. The particles obtained were sieved to obtain a uniform powder.

### 2.2. Preparation of hydroalcoholic extracts

The hydroalcoholic extracts were obtained with a 70/30 v/v mixture of ethanol and distilled water. Fifty grams of each powder were mixed with 500 mL of ethanol- distilled water and stirred at room temperature for 24 h. The mixtures were filtered and the supernatants were recovered. The same procedure was carried out a second time. The two recovered supernatants were mixed and the solvent was evaporated to obtain a dry extract. To eliminate the lipophilic fraction of the sample, the dry extracts were solubilized in distilled water and washed three times with hexane in a separatory funnel. To obtain the dry extracts, the distilled water was removed entirely.

### 2.3. Phytochemical analysis

#### 2.3.1. Determination of total polyphenols content

Total polyphenols content (TPC) of the two extracts were determined according to the protocol described by **(Wolfe et al., 2003)** with some modifications. 250 µL of each extract was combined with 1.25 mL of Folin-Ciocalteu (0.1 N) and 1 mL of 7.5% sodium carbonate solution was added after 5 min. The absorbance was measured with a spectrophotometer at 740 nm after 90 min of incubation. The total phenolic content was quantified as mg of gallic acid equivalents per g of extract (mg GAE/g) using a gallic acid standard curve.

### 2.3.2. Determination of total flavonoids content

Aluminium chloride was used for the determination of total flavonoids content (TFC), following the method described by **(Quettier-Deleu et al., 2000)**. A volume of 1 mL of each extract was mixed with 1 mL of aluminum chloride (2%) solution, homogenized, and the absorbance at 410 nm measured 15 min later. The total flavonoids content was quantified as mg of quercetin equivalents per g of extract (mg QE/g) using a quercetin standard curve.

### 2.3.3. Determination of flavones and flavonols content

Flavones and flavonols content was determined according to the method reported by **(Kosalec et al., 2004)**. 250 µL of each extract was mixed with 750 µL 96° ethanol, 50 µL of aluminum chloride solution (10%), 50 µL of potassium acetate (1 mol/l), and 1.4 mL of distilled water. The concentration of flavones and flavonols was estimated in the same conditions using quercetin for building the calibration curve. The results are given as mg of quercetin equivalents per g of extract (mg QE/g).

### 2.3.4. Determination of condensed tannins content

Condensed tannins react with vanillin in the presence of an acid to produce a red complex. A volume of 1.5 mL of vanillin (4%) solubilized in methanol was mixed with 250 µL of each extract. 750 µL of concentrated sulfuric acid was then added. The absorbance was read at 500 nm after 15 min incubation at room temperature. Results were represented as milligram (+)-catechin equivalents/g of extract (mg CE/g) based on a catechin standard curve **(Sun et al., 1998)**.

## 2.4. HPLC-DAD and HPLC-ESI-MS-MS analysis

Hydroalcoholic extracts of *C. nobile* and *M. pulegium* were redissolved in bidistilled water to obtain solutions containing around 10 mg/g. Before each analysis, aliquots of these solutions

were diluted to 1 mg/g. The two stock solutions M1 and M2 were used as reference samples for these analyses.

### 2.4.1 HPLC-DAD analysis

Experiments were carried out using an HPLC system comprised of a PU-2089 quaternary pump (Jasco International Co.) with a degasser, an AS 950 auto sampler (Jasco International Co.), and a GECKO 2000 column oven (Amchro GmbH). A MD-2010 diode-array detector (Jasco International Co.) was used for detection, with a resolution of 4 nm and a wavelength range of 200-650 nm. The injection volume was set at 20 µL. An Ascentis Express RP-Amide column (10 cm x 2.1 mm, particle diameter 2.7 m, Sigma-Aldrich, USA) was used in conjunction with an Ascentis Express RP-Amide guard column (5 mm x 2.1 mm, particle diameter 2.7 µm, Sigma-Aldrich, USA) to achieve separation. Water (A) and acetonitrile (B) were used as eluents, both containing 0.3% (v/v) formic acid. The flow rate of the mobile phase was 0.4 mL/min, and the temperature of the column oven was 40 °C. For all experiments, the following gradient was used: 0-3.75 min at 100% (A); 3.75-19.50 min from 100% to 89% (A); 19.50-27.75 min from 89% to 79% (A); 27.75-44.25 min from 79% to 60% (A); 44.25-50.25 min from 60% to 39% (A); 50.25-51 min from 39% to 0% (A); 51.00-52.50 at 0% (A). The re-equilibration process took 12 min. ChromNAV (version 1.12.01) was used to process HPLC-DAD chromatograms.

### 2.4.2 HPLC-ESI-MS-MS analysis

Experiments were performed using an HPLC 1200 Infinity chromatographic device connected through a Jet Stream ESI interface to a 6530 Infinity Q-ToF tandem mass spectrometer (Agilent Technologies, USA). Separation was achieved using the same column, eluents and gradient used for HPLC-DAD experiments. For the ESI interface, $N_2$ (purity > 98%) was used as a drying and sheath gas. The following conditions were used for ionization in negative ion mode: drying gas temperature 350 °C, flow 10 L/min, capillary voltage 4.5 kV; nebulizer gas pressure 35 psi; sheath gas temperature 375 °C, flow 11 L/min. MS acquisition ranges were 100-1700 m/z and MS-MS acquisition ranges were 90-1700 m/z. The collision-induced fragmentation was carried out using $N_2$ (99.999% purity) as the collision gas at a potential of 20 V. The mass axis was calibrated daily with the Agilent HP0321 tuning mix (Agilent Technologies, USA), which was prepared in acetonitrile. MassHunter Workstation (version B.04.00, Agilent Technologies, USA) was used to process HPLC-ESI-MS-MS chromatograms. The compounds were identified primarily through their MS and MS-MS

spectra, as well as comparisons to literature references. Confirmation of identified compounds in the HPLC-ESI-MS-MS chromatograms was supported, when possible, by the analysis of the corresponding peaks in the HPLC-DAD chromatograms.

### 2.5. Antioxidant activities

#### 2.5.1. DPPH radical-scavenging activity

In the presence of an antioxidant, the purple radical DPPH (2,2-Diphenyl-lpicrylhydrazyl) is reduced to yellow diphenylpicryl hydrazine. 2.45 mL of various concentrations of each extract were mixed with 50 mL of DPPH radical (5 mM in methanol). The absorbance of the mixtures was measured at 517 nm after 30 min of incubation in the dark. Quercetin was used as positive control. The formula below was used to calculate the percentage of DPPH radical scavenging activity **(Maisuthisakul et al., 2007)**.

$$\text{DPPH radical scavenging activity (\%)} = [\ A_0 - A_1) / A_0\ ] \times 100$$

$A_0$ is the absorbance of the control solution (containing only DPPH);

$A_1$ is the absorbance of the DPPH solution containing plant extract, or positive control

#### 2.5.2. Reducing power

The reducing power of the two extract was determined as described by **(Kosalec et al., 2013)**. A volume of 250 µL of each extract was mixed with 500 µL of phosphate buffer (0.2 M, pH 6.6) and 500 µL of potassium ferricyanide (1%). After 20 min of incubation at 50 °C, 500 µL of a 10% trichloroacetic acid (TCA) solution were added. 500 µL of this mixture were taken and added to 500 µL of distilled water and 100 µL of ferric chloride (0.1%), and the absorbance was read at 700 nm. Ascorbic acid was used as standard.

### 2.6. Antioxidant tests using the human erythrocyte model (antihemolytic activity)

#### 2.6.1. Preparation of erythrocytes suspension

Blood was collected from healthy donors and was provided by the CTS (Centre de Transfusion Sanguine = Blood Transfusion Center, Bejaia, Algeria). Blood was centrifuged in heparinized tubes at 3000 rpm for 10 minutes. The supernatant and buffy coat were removed and the red blood cells were then washed three times with a phosphate buffered saline solution pH 7.4 (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH

7.4). The red blood cells pellet was suspended in PBS to obtain hematocrit of 10% which is used immediately after its preparation **(Rafat et al., 2010)**.

### 2.6.2. Hemolytic activity of extracts on red blood cells

The extract were prepared in 5% DMSO dissolved in PBS pH 7.4. An aliquot of 250 µL of erythrocyte suspension (10% hematocrit) was mixed with 500 µL of different concentrations (25-1000 µg/mL) of each extract. The mixture was incubated in a water bath at 37 °C for 4 h, with gently mixing every one hour. A positive control was prepared by substituting the PBS solution with distilled water (considered as 100% hemolysis). After 4 h of incubation, all tubes were adjusted to 4.5 mL with PBS solution and centrifuged for 10 min at 2000 rpm. The released hemoglobin was measured at 540 nm and the hemolysis rate was calculated as follows **(Rafat et al., 2010)**.

$$\text{Hemolysis rate (\%)} = [(A_{\text{extract}} / A_{\text{positive control}}] \times 100$$

$A_{\text{extract}}$: Absorbance of hemoglobin in tubes treated with the extract

$A_{\text{positive control}}$: Absorbance of hemoglobin in tubes treated with the distilled water

### 2.6.3. Antihemolytic effects of the extracts on AAPH induced hemolysis

The study of the antihemolytic effect of hydroalcoholic extracts of CN and MP was tested on AAPH [2,2'-azobis(amidinopropane) dihydrochloride] induced hemolysis followed the protocol reported by **(Yang et al., 2017)** with slight modifications. A volume of 250 µL of the 10% erythrocyte suspension was pre-incubated at 37 °C for 30 min with different concentrations of the two hydroalcoholic extracts (50-300 µg/mL). 500 µL of an AAPH solution in PBS was added to reach a final concentration of 200 mM. The incubation was continued until 4 h. Then, all tubes were adjusted to 4.5 mL with PBS solution and centrifuged for 10 min at 2000 rpm. Ascorbic acid was used as standard. The hemolysis rate was estimated by measuring the released hemoglobin at 540 nm as previously mentioned. The percentage inhibition of AAPH-induced hemolysis of the two extracts was calculated as follows.

$$\text{Hemolysis inhibition (\%)} = [(A_{\text{AAPH}} - A_{\text{extract}}) / A_{\text{AAPH}}] \times 100$$

$A_{\text{AAPH}}$: Absorbance of hemoglobin in tubes treated only with 200 mM of AAPH

$A_{\text{extract}}$: Absorbance of hemoglobin in tubes treated 200 mM of AAPH and extracts

### 2.6.4. Effect of the extracts on erythrocyte lipid peroxidation

In order to evaluate the effect of the two extracts on lipid peroxidation of erythrocytes, the levels of malondialdehyde (MDA) were measured using thiobarbituric acid reactive substances (TBARS) assay **(Okoko and Ere, 2012)** with slight modifications. The extracts were used to pre-treat the erythrocyte suspension, which was subsequently treated with 200 mM AAPH, incubated, and centrifuged as previously described. 250 µL of the supernatant were collected and combined with 1 mL of the thiobarbituric acid solution (0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.2 M HCl). The mixture was vortexed before being incubated for 1 h at 100°C. After cooling, the mixture was centrifuged at 3000 rpm for 5 min, and the absorbance was measured at 532 nm. A calibration curve was performed under the same conditions using 1,1,3,3-Tetramethoxypropane (a protected form of malondialdehyde) (0-10 µM). The MDA level was determined and expressed as µM/mL of the erythrocyte suspension (ES).

### 2.7. Statistical analysis

All measurements were performed in triplicate and the results expressed as mean ± standard deviation (SD). The statistical analysis was performed by Graph Pad Prism 6.0 software. Statistical significance was assessed by Student's t-test or one-way ANOVA followed by Tukey's multiple comparison tests. The differences were considered as significant when $P < 0.05$. The IC50 values were determined using the software Origin 9.5.

## 3. Results

### 3.1. Phytochemical analysis

Total polyphenols, total flavonoids, flavones and flavonols and condensed tannins contents are shown in Table 1. Total polyphenols contents, as gallic acid equivalents, were determined by Folin-Ciocalteu colorimetric method that gives a blue coloration during phenols oxidation. TPC was higher in the hydroalcoholic extract of *M. pulegium* (81.78 ± 1.19 mg GAE/g of extract) compared to that of *C. nobile* extract (54.4 ± 2.69 mg GAE/g extract). However, total flavonoids contents, and flavones and flavonols contents, which give a yellow color in presence of aluminum chloride and expressed as milligram quercetin equivalents, were higher in *C. nobile* extract (15.65 ± 0.77 mg QE/ g extract and 4.29 ± 0.77 mg QE/ g extract, respectively), compared to those of *M. pulegium* extract (7.41 ± 0.77 mg QE/ g extract and 2.3 ± 0.24 mg QE/ g extract, respectively). Condensed tannins content expressed as milligram

catechin equivalent were 28.73 ± 1.47 mg CE/ g extract for *C. nobile* extract and 23.21 ± 1.59 mg CE/ g extract for *M. pulegium* extract.

### 3.2. Compounds identified by HPLC-DAD and HPLC-MS-MS

A list of all the identified compounds in hydroalcoholic extracts of *C. nobile* and *M. pulegium* is presented in Table 2, along with details regarding the raw formula, molecular weight and spectroscopic/spectrometric properties. 39 different species were identified in total; 27 in *C. nobile* extract and 18 compounds in *M. pulegium* extract, while 7 peaks could not be assigned to any compound. The data regarding these peaks are reported nonetheless for completeness of information. The identified compounds can be classified based on their structures, and five main categories can be distinguished:

***Phenolic acids*** (#2, 4, 8, 15) – These are the most basic polyphenols and can be found in a wide range of plant species. These compounds were identified by comparison with the analytical standards in the stock solutions M1 and M2.

***Quinic acid esters*** (#6, 11, 13, 16, 17, 18, 27, 28, 35) – These compounds are characterized by the presence of ester bonds involving the carboxylic group of one or more phenolic acid molecule and the hydroxy groups of a quinic acid molecule. The most common phenolic acid among these compounds is caffeic acid, but a small number of quinic acid esters (#16, 17, 18) was also found with p-coumaric and ferulic acids. Most quinic acid esters showed a characteristic fragment ion at *m/z* 191, corresponding to the quinate anion. Moreover, the presence of quinic acid does not significantly alter the UV-Vis absorption of the phenolic acid moieties, which retain their characteristic wavelengths. These spectroscopic and spectrometric properties proved fundamental in the identification of all members of this category.

***Flavonoids*** (#14, 21, 23, 24, 25, 26, 30, 31, 32, 33, 37, 39, 42, 44, 46) – Flavonoids are a class of well-known polyphenols with a carbon skeleton of C6-C3-C6. Flavonoids, together with phenolic acids, are present in a wide range of plant species and are responsible for the antioxidant and nutraceutical properties of many plant-based foods. Flavonoids are a very large group of molecules with several isomers, so identifying them in these samples was difficult. By comparing the flavonoids to the standard mixture M1, rutin (#25) was easily detected, but the other flavonoids were only tentatively identified based on their pseudomolecular ions and fragment ions.

***Caffeic acid oligomers*** (#22, 29, 36, 38, 41) – Only the hydroalcoholic extract of *Mentha pulegium* revealed the presence of caffeic acid oligomers. They are ester-linked oligomers made up of two, three, or four caffeic acid or 3,4-dihydroxyphenyllactic acid groups. These compounds have UV-Vis spectra that are similar to those of the corresponding monomers.

***Fatty acids*** (#3, 5, 19, 43) – Fatty acids are generally non-polar compounds, thus it is reasonable to hypothesize that the majority of them were removed during the hexane wash process. The chromatograms did, however, reveal some of the most polar fatty acids. Since they do not significantly absorb UV-Visible light, none of the compounds in this group were detected in the HPLC-DAD chromatograms.

***C. nobile*** **hydroalcoholic extract**: The chromatograms are shown in Fig. 1. The HPLC-MSMS profile of this extract was richer than that of *Mentha pulegium*, and two major peak groups could still be identified. The first group, which eluted between 11 and 21 min, featured abundant peaks attributed to caffeic acid (#8) and quinic acid esters (#13, 17), the most intense of which was chlorogenic acid (#13). The presence of flavonoids, flavonoid glucosides, and dicaffeoylquinic acids dominated the second group of peaks, which eluted between 24 and 37 min. Contrary to quinic acid monoesters, quinic acid diesters were not detected in the chromatograms of *Mentha pulegium*. The most intense peaks in this region were attributed to a methylmyricetin glucoside isomer (#24) and to two dicaffeoylquinic acid isomers (#28, 35) whose structure is presented in Fig. 3, A.

***M. pulegium*** **hydroalcoholic extract**: The chromatograms are presented in Fig. 2. Based on their retention time, the major peaks in the HPLC-MS-MS chromatogram can be divided into two groups. The first group, with retention times in the range 11-16 min, showed two main peaks belonging to a glucoside of tuberonic acid (#12) and to gallocatechin (#14). Tuberonic acid is a jasmonate, which is a class of hormones that controls plant growth and development. The other peaks in this group belong to phenolic compounds such as caffeic acid (#8) and chlorogenic acid isomers (#11, 13). The second group of peaks, eluting in the range 25-35 min, was dominated by the presence of rosmarinic acid. This compound, whose structure is presented in Fig. 3, B, is obtained by the formation of an ester bond between caffeic acid molecule and 3,4-dihydroxyphenyllactic acid. Other caffeic acid-based oligomers, such as salvianolic acids (#22, 41) and lithospermic acid (#38), were also attributed to the other main peaks in this area. Small peaks belonging to flavonoids (#23, 25, 44) were also detected.

### 3.3. *C. nobile* and *M. pulegium* hydroalcoholic extracts have a good antioxidant activity

The scavenging activities of DPPH radical by the two hydroalcoholic extracts of *C. nobile* (CN) and *M. pulegium* (MP) are presented in Fig. 4. Both extracts exhibited high antioxidant capacity with a dose dependent manner. MP hydroalcoholic extract gave the best DPPH radical scavenging activity compared to CN hydroalcoholic extract. Indeed, the MP extract gave scavenging activity of DPPH radical of 76.9 ± 0.74% at concentration of 50 µg/mL. However, the CN extract gave scavenging activity of DPPH radical of 84.2 ± 3.57% at concentration of 125 µg/mL. This is confirmed by the $IC_{50}$ values obtained (MP: IC50 DPPH = 19.98 ± 0.91 µg/mL; CN: IC50 DPPH = 52.77 ± 1.53 µg/mL). The $IC_{50}$ of quercetin used as standard was 2.85 ± 0.05 µg/mL.

The reducing power test assesses the ability of antioxidants such as phenolic compounds in plant extracts to donate an electron to ferric ions, converting them into ferrous iron ions. In this study, the reducing power of the two extracts was expressed as milligrams of ascorbic acid equivalent per gram of extract (mg Asc E/g extract). According to the results, the MP extract has almost twice the reducing power (223.74 1.08 mg Asc E/g extract) than the CN extract (130.65 1.69 mg Asc E/g extract).

### 3.4. Antihemolytic activity on human erythrocyte

#### 3.4.1. *C. nobile* and *M. pulegium* hydroalcoholic extracts have no hemolytic effect on human erythrocytes

Different concentrations of the hydroalcoholic extracts of CN and MP were tested on human erythrocytes to evaluate their hemolytic activity. In comparison to total hemolysis induced by distilled water, incubation of erythrocytes with both extracts (25-1000 g/mL) caused essentially minimal hemolysis (Fig. 5). CN extract hemolysis rates range from 2.26 ± 0.46% to 3.16 ± 1.19% for the CN extract, while the MP extract hemolysis rates range from 1.93 ± 0.06% to 3.05 ± 0.97%. The statistical analysis showed a significant difference ($P < 0.05$) between hemolysis rates generated by the two extracts and distilled water.

#### 3.4.2. *C. nobile* and *M. pulegium* hydroalcoholic extracts attenuate AAPH-induced hemolysis in human erythrocytes

Treatment of erythrocytes with 200 mM AAPH resulted in a hemolysis rate of 56.66 ± 1.30% compared to total hemolysis induced by distilled water ($P < 0.05$). The rate of AAPH-induced hemolysis is reduced in a dose dependant manner when erythrocytes are pretreated with varying doses of the two hydroalcoholic extracts of CN and MP, and with ascorbic acid used

as control (Fig. 6.). Indeed, hemolysis rates decreased slightly after 4 h of incubation in the presence of 200 mM AAPH and pretreatment with 50 and 100 µg/mlL hydroalcoholic extracts of CN and MP, compared to the control treated only with 200 mM AAPH (CN: 51.88 ± 1.12% and 42.36 ± 2.23%; MP: 55.31 ±0.59% and 49.02 ± 1.23%, at concentrations of 50 and 100 µg/mL, respectively). These rates correspond to hemolysis inhibition percentages of 8.42 ± 1.98% and 25.23 ± 3.95% for CN extract, and 2.37 ± 1.04% and 13.48 ± 2.17% for MP extract (Table 3). However, at higher doses of both extracts (150, 200 and 300 µg/mL), the hemolysis rates decreased very sharply to 7.61 ± 0.34% for the CN extract and 7.69 ± 0.53% for the MP extract at the 300 µg/mL dose, corresponding to hemolysis inhibition rates of 86.46 ± 0.53% and 86.56 ± 0.84, respectively. The $IC_{50}$ value for CN extract was 127.48 ± 3.14 µg/mL and for MP extract was 129.52 ± 2.15 µg/mL (Fig. 6. and Table 3). Pretreatment of erythrocytes with 300 µg/mL ascorbic acid used as a control gave an inhibition rate of 95.38 ± 1.86%.

### 3.4.3. *C. nobile* and *M. pulegium* hydroalcoholic extracts decrease AAPH-induced lipid peroxidation in human erythrocytes.

Our results show that when erythrocytes were treated with 200 mM AAPH for 4 h, MDA levels increased by around 400% (123.08 ± 8.35 µM/mL ES) compared to control erythrocytes grown just in PBS (31.6 ± 4.74 µM/mL ES). The efficacy of CN and MP extracts to counteract AAPH-induced membrane lipid peroxidation was assessed using different doses of the two extracts. Pre-treatment of the erythrocyte suspension for 30 min with both extracts (50-300 µg/mL) before the addition of 200 mM AAPH resulted in a decrease in the rate of lipid peroxidation (MDA levels), except for the 50 and 100 µg/L doses of the MP extract. Indeed, this decrease is very significant at 200 and 300 µg/mL concentrations of both extracts, with MDA levels close or similar to those of control erythrocytes cultured alone in PBS, especially for the MP extract. The MDA levels after pretreatment of erythrocytes with 200 and 300 µg/mL are respectively, 59.88 ± 9.98 and 42.53 ± 5.15 µM/mL ES for the CN extract, and 41.1 ± 11.8 and 28.51 ± 1.42 µM/mL ES for the MP extract. This equates to a decrease in MDA generation of 51.35% and 65.44% for the CN extract, and 66.6% and 76.83% for the MP extract, compared to the positive control treated only with 200 mM AAPH. MDA rate in erythrocytes pretreated with 300 µg/mL ascorbic acid used as standard (20.67 ± 1.42 µM/mL ES) is lower even than that of control erythrocytes cultured only in PBS (31.6 ± 4.74 µM/mL ES)

## 4. Discussion

Roman chamomile (*C. nobile*) and pennyroyal mint (*M. pulegium*) are two very well-known plants in the world. In Algeria, they are used thanks to their richness in essential oils to treat various diseases. Our work aimed to characterize the phenolic compounds of *C. nobile* and *M. pulegium* and to study their effects against AAPH-induced hemolysis in human erythrocytes. For this reason, extraction of phenolic compounds from both plants was carried out using a binary solvent mixture of ethanol and water (70-30 v/v respectively). The choice of this binary solvent was due to the fact that previous studies have reported that binary solvent systems are more efficient for the extraction of phenolic compounds compared to the mono-solvent system (pure ethanol or water) **(Thoo et al., 2010; Zhang et al., 2007)**. In addition, ethanol and water also offer the advantages of being GRAS (Generally Recognized as Safe), inexpensive, green solvents and can be used directly in food and pharmaceuticals **(Monroy et al., 2016)**.

The results of the phytochemical analysis showed that *M. pulegium* is richer in total polyphenol (81.78 ± 1.19 mg GAE/g) than *C. nobile* (54.4 ± 2.69 mg GAE/g). The Folin-Ciocalteu assay is a non-specific method and is based on the electron transfer from phenolics to phosphomolybdic/phosphotungstic acid complexes in alkaline medium **(Ainsworth and Gillespie, 2007)**. When comparing our findings to those described in the literature, we find that the *M. pulegium* analyzed in this study had higher total polyphenol content than the one grown in the same region of Bejaia (Algeria) (6.1 ± 0.5 mg GAE/g for ethanolic extract) **(Fatiha et al., 2015)**, that the one grown in Mahdia (Tunisia) (37.4 mg GAE/g for methanolic extract) **(Hajlaoui et al., 2009)**, and also the one grown in Portugal (71.7 ± 2.1 and 57.9 ± 1.6 mg PE/g for ethanolic and water extract, respectively) **(Mata et al., 2007)**. The extract of *C. nobile* (54.4 ± 2.69 mg GAE/g), on the other hand, was found to be lower in total polyphenol than the hydroalcoholic extract (methanol-water) (65.15 ± 3.62 mg GAE/g) of the Roman chamomile farmed in Portugal, but higher than the decoction and infusion extracts (16.55 ± 0.32 and 44.39 ± 1.07 mg GAE/g, respectively) **(Guimarães et al., 2012)**.

Conversely, *C. nobile* was found to be richer in total flavonoids and flavones and flavonols (15.65 ± 0.77 and 4.29 ± 0.77 mg QE/g, respectively) than *M. pulegium* (7.41 ± 0.77 and 2.3 ± 0.24 mg QE/g, respectively). These compounds form a yellow complex with aluminum. It is one of the most commonly used methods for the determination of total flavonoids and their different classes in food and medicinal plant samples. Nevertheless, the results of this

procedure depend on the structure of the individual flavonoids present **(Pękal and Pyrzynska, 2014)**. The total flavonoid content of *M. pulegium* in the present study was higher than that grown in the same region of Bejaia (Algeria) (0.85 ± 0.01 mg QE/g) **(Fatiha et al., 2015)**, but lower than that grown in Saudi Arabia (16.95 ±1.48 mg RE/g) **(Osman, 2013)**, and in Mahdia (Tunisia) (33.83 mg CE/g) **(Hajlaoui et al., 2009)**. For *C. nobile*, Carnat et al. (2004) **(Carnat et al., 2004)** found that the infusion of Roman chamomile grown in Anjou (France) contained 236 mg/L flavonoids, the majority of which were apigenin derivatives.

Finally, the condensed tannin contents of the two extracts of Roman chamomile and pennyroyal mint were very close: 28.73 ± 1.47 and 23.21 ± 1.59 mg EQ/g, respectively. These levels are higher than those reported in the bibliography for both plants. Indeed, Hajlaoui et al. (2009) **(Hajlaoui et al., 2009)** reported a rate of 3.07 mg EC/g for *M. pulegium* from Mahdia (Tunisia), while Amokrane et al. (2016) **(Amokrane et al., 2016)** found a rate of 0.81 ± 0.001 mg EC/g dry matter for *C. nobile* collected in Constantine (Algeria).

Variations in the contents of the different compounds are noted between our results and those reported in the bibliography. These variations can be explained by the lack of specificity of the Folin-Ciocalteau (for total polyphenols) and aluminium chloride (for total flavonoids) methods as explained above. They could also be due to the difference of the solvent used during the extraction (ethanol-water, ethanol, water, methanol-water, infusion, etc.) as well as the period and the place where the plants were harvested.

Previous work has been carried out to characterize the phenolic composition of different extracts of *C. nobile*. According to Carnat et al (2004) **(Carnat et al., 2004)**, the Roman chamomile infusion from Anjou (France) contains flavonoids such apigenin and its derivatives, luteolin and its derivatives, and chamaemeloside, as well as hydroxycinnamic acids like dicaffeoyl derivative, chlorogenic acid, and neochlorogenic acid. In contrast, the crude methanolic extract of Roman chamomile from Pál Bobvos (Hungary) contained eupafolin as the major flavonoid, followed by luteolin, hispidulin and apigenin **(Sándor et al., 2018)**. In addition, over 30 phenolic compounds in Roman chamomile from the Trás-os-Montes region (Portugal) were identified. Flavonoids, phenolic acids, and their derivatives made up the majority of the substances. The most abundant compounds were 5-O-caffeoylquinic acid and a derivative of apigenin **(Guimarães et al., 2013)**. In this work, we have characterized in the CN extract three phenolic acids (3,4-dihydroxybenzoic acid, caffeic acid and p-coumaric acid), nine quinic acid esters (dicaffeoylquinic acid, feruloylquinic acid,

chlorogenic acid ...etc.) and eleven flavonoids and their derivatives (apigenin glucoside, rhamnetin glucoside, luteolin...etc.). Dicaffeoylquinic acid was the most abundant in terms of relative peak area. Furthermore, two fatty acids, hydroxypimelic acid and azelaic acid, were detected even after the extract was defatted with hexane in a separating funnel.

On the other hand, we characterized two phenolic acids (3,4-dihydroxyphenyllactic acid and caffeic acid) in the MP extract, as well as three quinic acid esters (Chlorogenic acid isomer A and B and Chlorogenic acid) and four flavonoids (Gallocatechin, Hesperidin, Rutin and Jaceidin). In addition, five caffeic acid oligomers (salvianolic acid I, rosmarinic acid, salvianolic acid B and C and lithospermic acid) were also characterized, among which rosmarinic acid gave the highest signal. Finally, like the CN extract, two fatty acids (pimelic acid and trihydroxy octadecadienoic acid) were also detected in the MP extract. Our findings are generally consistent with previous research on the composition of M. *pulegium* in various phenolic compounds. Indeed, several extracts of *M. pulegium* have been shown to contain various flavonoids of different classes **(Alharbi et al., 2021; Gülçin et al., 2020; Taamalli et al., 2015)**, as well as various phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids derivatives) **(Politeo et al., 2018; Taamalli et al., 2015)**. As in this study, rosmarinic acid was the most abundant compound in extracts of *M. pulegium* from Bihać (Bosnia-Herzegovina) **(Politeo et al., 2018)**.

In the present study, we have investigated the in vitro antioxidant activities of the two hydroalcoholic extract of *C. nobile* and *M. pulegium* using two different assays: DPPH radical-scavenging activity and reducing power. DPPH radical-scavenging activity is the simplest method of free radical scavenging and has been widely used to assess the antioxidant potential of different compounds or extracts. The phenolic compounds as antioxidants reduce the odd electron of the nitrogen atom in DPPH by giving it a hydrogen atom **(Kedare and Singh, 2011)**. Phenolic compounds with reducing potential are electron donors that reduce potassium ferricyanide ($Fe^{3+}$) to potassium ferrocyanide ($Fe^{2+}$) which forms a ferrous complex that absorbs at 700 nm when reacting with ferric chloride **(Singhal et al., 2014)**. Our results indicate that the hydroalcoholic extract of MP has a very high DPPH radical scavenging activity (IC50 = 19.98 ± 0.91 µg/mL), higher than those of ethanolic extract of MP (IC50 = 32.2 ± 0.6 µg/mL) grown in the same region of Bejaia (Algeria) **(Fatiha et al., 2015)** and methanolic extracts of MP from the region of El-Tarf (Algeria) (IC50 = 25.66 ± 1.50 µg/mL) **(Benabdallah et al., 2016)**. The hydroalcoholic extract of CN also has a good DPPH radical scavenging activity (IC50 = 52.77 ± 1.53 µg/mL), but not to the same extent as MP extract.

554 However, it was more effective in scavenging the DPPH radical in comparison to the
555 infusion, methanolic, and decoction extracts of Roman chamomile grown in Portugal, which
556 had IC50s respectively of 408.46 ± 11.34, 621.64 ± 6.84, and 1477.30 ± 71.99 g/mL
557 **(Guimarães et al., 2013)**. This ability of both extracts to quench the DPPH radical is likely
558 owing to their phenolic compounds which can provide hydrogen atoms or donate electrons,
559 via a free-radical attack on the DPPH molecule, rendering it as a stable bleached diamagnetic
560 molecule, which explains the decrease in absorbance at 517 nm **(Mathew et al., 2015)**.

561 Red blood cells are a common biological model for evaluating free radical-induced oxidative
562 damage and investigating the antioxidant and anti-hemolytic activities of various compounds
563 or extracts **(Banerjee et al., 2008)**. This is owing to the polyunsaturated fatty acid content of
564 erythrocyte membranes and the redox reactions of hemoglobin during oxygen transport, both
565 of which are significant promoters of reactive oxygen species **(Bammou et al., 2016)**. The
566 anti-hemolytic properties of Roman chamomile and pennyroyal mint were investigated on
567 AAPH-induced oxidative hemolysis. AAPH is a water-soluble peroxyl radical initiating azo
568 compound, which generates free radicals when decomposed at physiological temperatures.
569 Overproduction of these highly reactive free radicals induces chain oxidation of erythrocyte
570 membrane lipids and proteins, resulting in the loss of membrane integrity and the release of
571 hemoglobin (hemolysis) or death of healthy erythrocytes **(Yang et al., 2017; Yuan et al.,**
572 **2005)**. First, the study of the hemolytic effect of both CN and MP extracts showed that they
573 have no cytotoxic effect on human red blood cells at concentrations ranging from 25 to 1000
574 µg/mL. We have also demonstrated for the first time that Roman chamomile and pennyroyal
575 mint have excellent anti-hemolytic activities against AAPH-induced hemolysis. Indeed, pre-
576 incubation of human erythrocytes with various doses of both extracts significantly reduced
577 AAPH-induced hemolysis. This reduction was substantially greater at doses between 150-300
578 µg/mL for both extracts. Hemolysis inhibition rates were greater than 85% at a dose of 300
579 µg/mL for both extracts. These potent antioxidant and anti-hemolytic properties are most
580 likely owing to the abundance of various phenolic compounds in both plants, which are
581 known for their scavenger effect against free radicals. Therefore, when added to the
582 incubation media, the various phenolic compounds included in both CN and MP extracts can
583 quench the peroxyl radicals generated before they can damage the erythrocyte membrane
584 components and cause oxidative hemolysis **(Mendes et al., 2011)**.

585 Flavonoids have been shown to interact with the lipid and protein components of the red
586 blood cell membranes, enhancing the stability and decreasing the fluidity of the erythrocyte

membranes, lowering hemolysis rates **(Chaudhuri et al., 2007)**. In addition, flavonoids may also enhance the aggravation of van der Waals interactions inside the lipid bilayer, which may be a source of membrane stabilization **(de Freitas et al., 2008)**. It has also been reported that flavonols (quercetin, rutin, kaempferol…etc.) and their glycosides, particularly those with ortho-dihydroxyl functionality, are also powerful antioxidants that protect human red blood cells from oxidative hemolysis caused by free radicals (AAPH) **(Dai et al., 2006)**.

Because their membrane lipids are high in polyunsaturated fatty acids, erythrocytes are highly susceptible to peroxidation. An excess of oxidative stress can initiate lipid peroxidation with the loss of a hydrogen atom from the fatty acyl chains, which subsequently spreads in the form of a chain reaction **(Mendes et al., 2011)**. To evaluate the effect of both CN and MP extracts on lipid peroxidation, MDA levels were determined by the thiobarbituric acid reactive substances (TBARS) assay. Despite the limitations of its analytical specificity and robustness, this approach is frequently regarded as a good indication of oxidative stress levels. It involves combining lipid peroxidation products, particularly MDA, with thiobarbituric acid (TBA) to create MDA-TBA2 adducts known as TBARS, which are measured using spectrophotometry at 532 nm **(De Leon and Borges, 2020)**. Our results showed that MDA levels were significantly decreased in red blood cells pretreated with both CN and MP extracts compared to those treated with AAPH alone. This demonstrates that they are efficient in preventing lipid peroxidation caused by AAPH-generated free radicals. MDA levels were almost similar to those of control red blood cells cultured in PBS at dosages of 200 and 300 µg/mL, notably for the MP extract. MDA is a biomarker aldehyde generated by polyunsaturated fatty acids that is commonly used to quantify lipid peroxidation in the context of oxidative stress. Many disorders have high levels of it in biological samples. Therefore, lowering its levels by various techniques (dietary changes, antioxidant supplementation, or medicines) is usually seen to be advantageous to one's health **(Tsikas, 2017)**.

## 5. Conclusion

In summary, both plant extracts were rich in phenolic compounds. The extract of *Chamaemelum nobile* showed a higher number of chromatographic peaks, and a higher content of both flavonoid glucosides and quinic acid esters. The extract of *Mentha pulegium* was characterized by the presence of salvianolic acids, which are caffeic acid oligomers. The compound giving the most intense peak was rosmarinic acid. Furthermore, we showed for the first time that Roman chamomile and pennyroyal mint are potent antioxidants and antihemolytics in human erythrocytes, and that they attenuate AAPH-induced hemolysis.

Both plants' antihemolytic action was demonstrated by a significant reduction in AAPH-induced hemolysis rates and a significant reduction in erythrocyte membrane lipid peroxidation.

## Conflicts of interest

The authors declare that there is no conflict of interest


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