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Low concentrations of Ambrosia maritima L. phenolic extract protect endothelial cells from oxidative cell death induced by H_2O_2 and sera from Crohn's disease patients

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ABSTRACT

Ethnopharmacological relevance: A rising resort to herbal therapies in Crohn's disease (CD) alternative treatments has been recently observed due to their remarkable natural efficiency. In this context, the weed plant *Ambrosia maritima* L., traditionally known as Hachich el Aouinet in Algeria and as Damsissa in Egypt and Sudan, is widely used in North African folk medicine to treat infections, inflammatory diseases, gastrointestinal and urinary tract disturbances, rheumatic pain, respiratory problems, diabetes, hypertension and cancer.

Aim of the study: To assess an *Ambrosia maritima* L. phenolic extract for its phenolic profile composition, its potential antioxidant activity *in vitro*, and its cytoprotective effect on cultured primary human endothelial cells (ECs) stressed with H_2O_2 and sera from CD patients.

Materials and Methods: Phenolic compound extraction was performed with a low-temperature method. Extract chemical profile was attained by HPLC-DAD/ESI-MS. The extract *in vitro* antioxidant activity was assessed using several methods including cupric ion reducing power, DPPH radical scavenging assay, O-Phenanthroline free radical reducing activity, ABTS cation radical decolourisation assay, Galvinoxyl free radicals scavenging assay. Intracellular reactive oxygen species levels were evaluated in human endothelial cells by H_2DCFDA, while cell viability was assessed by MTT.

Results: The phenolic compounds extraction showed a yield of 17.66% with three di-caffeoylquinic acid isomers detected for the first time in *Ambrosia maritima* L. Using different analytical methods, a significant *in vitro* antioxidant activity was reported for the *Ambrosia maritima* L. extract, with an IC_{50} value of 14.33 ± 3.86 μg/mL for the Galvinoxyl antioxidant activity method. Challenged with ECs the *Ambrosia maritima* L. extract showed a biphasic dose-dependent effect on H_2O_2-treated cells, cytoprotective and antioxidant at low doses, and cytotoxic and prooxidant at high doses, respectively. Viability and ROS levels data also demonstrated a prooxidant and

Abbreviation: IBD, inflammatory bowel disease; CD, Crohn's disease; ROS, reactive oxygen species; AM, *Ambrosia maritima* L.; HPLC, HPLC, high performance liquid chromatography; DAD, diode array detector; ESI, electrospray ionization; MS, mass spectrometry; TPC, total phenolic content; MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, DPPH; 2,2-diphenyl-1-picryl-hydrazyl-hydrate, CUPRAC; Cupric ion reducing power, BHA; Butylated hydroxyanisole, BHT; butylated hydroxytoluene, Ascorbic acid and Trolox; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; GOR, Galvinoxyl free radicals; HUVECs, Human umbilical vein endothelial cells; H2DCFDA, 2′,7′, dichlorodihydrofluorescein diacetate.

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cytotoxic effect of CD sera on cultured ECs. Interestingly, 10 μg/mL of *Ambrosia maritima* L. extract was able to counteract both CD sera-induced oxidative stress and ECs death.
Conclusion: Our data indicated *Ambrosia maritima* L. as a source of bioactive phenolics potentially employable as a natural alternative for CD treatment.

1. Introduction

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by chronic progressive inflammation with a multifactorial etiology affecting the gastrointestinal tract, eventually leading to an uncontrolled immune response with impaired gut mucosal homeostasis (Cosnes et al., 2011). CD possesses a complex and non-specific symptomatology translated into a long-lasting subclinical disorder activity requiring complicated medication and difficult monitoring (Cosnes et al., 2011). It is generally accepted that oxidative stress, the imbalance between the generation and elimination of reactive oxygen species (ROS), is pivotal in the onset and progression of many diseases, including CD (Alemany-Cosme et al., 2021; Alzoghaibi, 2013; Fois et al., 2020; Giordo et al., 2021; Shaito et al., 2022; Thuan et al., 2018). Nonetheless, the redox-associated mechanisms underpinning IBD pathogenesis are not yet completely understood. While the specific causative agents remain ambiguous, a growing body of evidence suggests that oxidative stress represents a common thread among the multiple mechanisms involved in CD pathogenesis, such as environmental factors, impaired immune system, microbiota dysbiosis and host genetics. (Alemany-Cosme et al., 2021; Alzoghaibi, 2013; Fernanda Silva Basílio and Santos Branco, 2021; Luceri et al., 2019). In this regard, cellular and molecular damage is caused by the continuous release of ROS into the inflamed mucosal lesions microenvironment, maintaining the chronic excessive immune response (Bourgonje et al., 2019). This phenomenon is typically associated with infiltrating effector lymphocytes and increased production of proinflammatory cytokines into the intestinal mucosa, ultimately creating a loop in the continuous production of ROS (Bourgonje et al., 2019). At a molecular scale, ROS overproduction leads to cellular proteins' oxidation, thereby affecting gene regulation, DNA damage, ion transport, intermediary metabolism, and mitochondrial function accompanied by an endogenous antioxidant system reduced activity (Bourgonje et al., 2020; Luceri et al., 2019). Human and animal studies also implicated oxidative stress-associated mitochondrial dysfunction in IBD pathogenesis since early steps mechanisms of redox signaling, and redox regulation depends on mitochondrial dynamics (Ardizzone et al., 2008; Pietta, 2000; Wang et al., 2021; Xu et al., 2017). However, the role of mitochondrial dysfunction in CD-associated inflammation is not yet well defined. At the tissue level, the inflamed intestinal mucosa redox impairment propagates into the intestinal tract's deep layers, ultimately flowing into the systemic circulation (Bourgonje et al., 2020). Indeed, several studies reported high levels of oxidative stress in IBD patients' serum/plasma and revealed expanded intestinal microvasculature typical of CD patients (Ardizzone et al., 2008; Bourgonje et al., 2019, 2020; Luceri et al., 2019). Since intestinal microvessels are lined with vascular endothelial cells, the latter could be an essential component of the intestinal circulation implicated in IBD development (Wang et al., 2021).

When the endogenous antioxidant systems cannot efficiently balance the impaired cellular redox state, supplementation with exogenous antioxidants may be necessary (Pietta, 2000). Exogenous antioxidants are frequently found in food and medicinal herbs (Alsamri et al., 2021; Giordo et al., 2022; Phu et al., 2020; Quispe et al., 2021; Shaito et al., 2020b). They are mainly phenols, polyphenols (phenolic acids, flavonoids, anthocyanins, lignans, and stilbenes), carotenoids (xanthophylls and carotenes), and vitamins (vitamin E and C) (Xu et al., 2017). Due to the accumulating evidence concerning their long-term intake's protective impact against drugs and chronic diseases, plants and herbs' secondary metabolites are increasingly capturing a great interest as potent beneficial components of the human diet (Cossu et al., 2012; Crozier et al., 2006; Giordo et al., 2020). Medicinal herbs have an extensive broad of traditional applications encompassing antibacterial, antifungal, antiviral, antimalarial, antitumor properties, which depends on their phytochemical composition (Dirar et al., 2017; Phu et al., 2020; Shaito et al., 2020b).

Ambrosia maritima L. (the plant name has been checked with http://www.theplantlist.org on July 8, 2022) is a weed plant distributed mainly in Mediterranean and African countries and has been used in folk medicine to treat several conditions, including infections, gastrointestinal and urinary tract disturbances, rheumatic pain, respiratory problems, diabetes, hypertension and cancer (Nazik et al., 2020). Due to its various curative properties, *Ambrosia maritima* L. has been the focal point of different studies (Abdel Salam et al., 1984; Abdelgaleil et al., 2011; Ali Abdeldayem et al., 2021; Dirar et al., 2017; Iskander et al., 1988; Mohamed et al., 2022; Nazik et al., 2020; Picman et al., 1986). This work aims to investigate the phenolic profile of an *Ambrosia maritima* L. phenolic extract and its antioxidant and cytoprotective effect against the redox state impairment elicited by H_2O_2 and sera from CD patients on primary human endothelial cells (ECs).

2. Materials & methods

2.1. Chemical reagents

Unless stated in the text, all the reagents used were from Sigma (Sigma, St Louis, MO).

2.2. Plant material, collection and phenolics extraction

The aerial parts of *Ambrosia maritima* L. were collected in October 2015 at Ighzer Amokrane, Ouzellaguen- Bejaia (36°33′4.51″N - 4°36′29.19″E) located in the North-East of Algeria. A voucher specimen (Code: N°KR0031) was identified by Pr. K. Rebbas from the Natural and Life Department at the University of M'sila, Algeria, and deposited in his personal herbarium.

The phenolics extraction was realized as previously described with few modifications (Barberis et al., 2019; Floris et al., 2020). A low-temperature extraction (LTE) method, below 42 °C, was applied to preserve the structure of the phenols. The aerial part (leaves, flowers and stems) of *Ambrosia maritima* L. was air dried at room temperature (27 ± 3 °C), and then ground up to fine powder with a planetary ball-mill (RETSH PM100, Haan, Germany), employing a 500 mL porcelain container with 3 balls (3 cm ø) (rotation speed 0,48g/20 min). Liquid nitrogen was added to avoid possible sample oxidation and heating. The resulting powder was split into falcon vials containing approximately 5 g each and 25 mL of an hydro-ethanolic solution EtOH:H_2O (40:60, v/v). The extraction took place overnight, at 25 °C, on a planetary agitator. The vials were centrifuged for 15 min at 4627 g at 4 °C, in order to separate the pellet and recover the supernatant. The supernatant was filtered with Whatman 1 filter papers, and immediately stored at −80 °C. Once frozen the filtrate was put under a nitrogen flow to evaporate the ethanol. Then the defrosted samples were moved to the rotavapor (IKA-Werke GmbH, Staufen, Germany), operating at 38–40 °C for 10 min under vacuum, to completely remove ethanol residual. Finally, the samples were frozen again and lyophilized at −60 °C to remove water, thus obtaining a fine powder. The hydrophilic powder was put into a capped vial, and kept in a desiccator at 25 °C, until use. The yield of extraction was calculated using the following equation:

$$\text{Yield of extraction } (\%) = \frac{(\text{The weight of freeze dried recover}) \times 100}{\text{Initial weight of plant powder used}}$$

2.3. Total phenolic content (TPC)

The total phenols content was assessed using the Folin-Ciocalteu method with slight modifications (Fadda et al., 2016; Singleton and Rossi, 1965). A mixture of 1 mg of lyophilized extract and 9 mL of cold ethanol (80%) was prepared (1:10 w/v), vortexed (Stuart, U.K. model SA8.) at 1600 rpm for 2 min and centrifuged (ALC-Centrifuge 4227R, Milan, Italy) at 16,000×*g* for 15 min at 4 °C 200 μL of the extract solution were mixed with a 10% solution of Folin-Ciocalteu reagent (1 mL) and left to react for 8 min. Then 800 μL of a 0.075% sodium carbonate solution were added before incubating the sample in the dark for 1 h at room temperature (20 ± 3 °C) followed by an additional hour at 0 °C. The absorbance was read at 760 nm with a spectrophotometer (8453 Agilent Technologies, Santa Clara, CA, USA) and results were expressed as milligrams of gallic acid equivalent/g of dry weight on the basis of a gallic acid calibration curve (40–80 mg/L).

2.4. Chemical characterization of Ambrosia maritima L. Extracts

Ambrosia maritima L. extracts characterization was performed by HPLC-DAD/ESI-MS analysis as previously described (Barberis et al., 2019). Briefly, chromatographic separation was performed on an Agilent 1100 LC system that included a binary pump, Diode-Array Detector, column thermostat, degaser and a HTS-PAL autosampler (Agilent Technologies, Palo Alto CA USA). The HPLC column was a Gemini C18 (100 × 2.1 mm, 5 μm) from Phenomenex (Torrance, CA, USA) equipped with a security guard cartridge (4 × 2 mm). Briefly, two mobile phases were employed for the elution, Eluent A (Water mixed with 0.2% acetic acid – 0.01% trifluoracetic acid) and Eluent B (Acetonitrile). The flow rate was set at 0.2 mL/min with an injection volume of 5 μL, and a column temperature of 35 °C. Total run time was 50 min. The gradient profile was 0–20 min, 95%–85% A; 20–40 min, 85%–70% A; 40–50 min, and 70%–40% A. The flow rate was 0.2 mL/min from the time 0–50 min. The Diode Array Detector was set at 270 and 520 nm Bw = 4 Reference 800.

The mass spectrometer was an Agilent G1946 (MSD 1100) single stage quadrupole. The instrument was interfaced with an Electrospray Atmospheric Pressure Ionization ESI used in the positive ion mode $[M+H]^+$. The mass spectrometer was programmed to admit protonated molecules at mass range 150–850 m/z. The positive ion spray voltage was 3200 mV and the Fragmentor was 85 V. After optimization, heated nebulizer parameter was set as follows: Temperature 35 °C, Nebulizer pressure 42 psi and flow rate of drying gas 9.8 L/min. Peaks were identified on the basis of their retention time relative to external standards (t_r), UV-VIS spectra, mass spectra, phytochemical data base and their corresponding molecular masse range m/z.

2.5. Antioxidant activity

Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Ascorbic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used as reference standards in all the following *in vitro* antioxidant activities:

2.5.1. Cupric ion reducing power (CUPRAC)

The cupric acid reducing activity was evaluated following a previously described method with slight modifications (Apak et al., 2004). In each well of a 96 microplate, 50 μL of 10 mM of Cu (II), 50 μL of 7.5 mM neocuprine, and 60 μL of NH_4Ac buffer (1 M, pH 7.0) were added. Finally, 40 μL of plant extract with different concentrations were added and the plate was incubated for 60 min. The absorbance was then read at 450 nm and results were expressed as 0.50 absorbance ($A_{0.5}$).

2.5.2. DPPH radical scavenging assay

The free radical scavenging potential was assessed using the DPPH method according to Blois et al. (Blois, 1958). Briefly, 160 μL of DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) solution (60 μM) were added to 40 μL of different extract concentrations then left to react for 30 min. The reducing power was measured using a 96-microplate spectrophotometer and the absorbance was recorded at 517 nm. The results were expressed as the inhibitory concentration of 50% of free radicals (μg/mL).

2.5.3. O-phenanthroline free radical reducing activity

This assay was performed according to Szydłowska-Czerniak et al. (2008). A mixture of 30 μL of O-phenanthroline (0.5%), 50 μL $FeCl_3$ (0.2%), 110 μL methanol was added to 10 μL of different concentrations of plant extract and incubated for 20 min at 30 °C. Then the absorbance was measured at 510 nm. The results were expressed as the percentage of inhibition.

2.5.4. ABTS cation radical decolourisation assay

ABTS radical scavenging activity was determined following a method by Re et al. (1999). Briefly, 7 mM of the ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation and 2.45 mM of a potassium of persulfate solutions were mixed and incubated for 16 h at room temperature in obscurity. Later, the activated solution of $ABTS^{\cdot+}$ was gradually diluted until having a solution with an absorbance of 0.70 ± 0.2 at 734 nm. Then, 160 μL of $ABTS^{\cdot+}$ were added to 40 μL of the prepared concentrations of *Ambrosia maritima* L. extract and left to react for 10 min. The absorbance measurements were realized using a 96-microplate reader with wavelength set at 734 nm. The results were presented as IC_{50} expressed in (μg/mL).

2.5.5. Galvinoxyl free radicals (GOR) scavenging assay

Free radicals scavenging activity was assessed according to Shi et al. (2001). Briefly 160 μL of a methanolic solution of Galvinoxyl (0.1 mM) were added to 40 μL of each concentration of *Ambrosia maritima* L. extract, then, incubated for 120 min in obscurity at room temperature. The absorbance was read at 428 nm and Galvinoxyl solution was used as control.

2.6. HUVECs cell culture and media

Human umbilical vein endothelial cells (HUVECs) were acquired from Cell Application (San Diego, CA, USA) and grown as previously described (Vono et al., 2016). Briefly, HUVECs were grown in endothelial cell basal medium (EBM) containing cell growth supplement (EGM-V2 # 213K-500) as per company instructions. When confluent, cells were sub-culture at a split ratio of 1:3 and used within three passages. Unless differently specified in the text, cells were plated at 8 × 10^4 cell per well, in 96 well plates (Corning, Lowell, MA, USA), cultured for 24 or 48 h in EBM complete medium and then processed for the experiments as reported in the figure legends.

For cell culture treatments, different concentrations of *Ambrosia maritima* L. extract (1, 10, 50 and 100 μg/ml) were prepared in EBM complete medium. This concentrations range was selected according to the literature where different ranges used in previous studies from the same plant extracts or leads were applied at concentrations range of (0.001 μM–1000 μM), in case of pure compounds, or the equivalence for extracts or leads of (0,1–1000 μg) (Kuete and Efferth, 2013; Saeed et al., 2015; Said et al., 2018). In this regard, the concentration of compounds applied in the cell-based screening assays are defined according to their practicability and not by their efficiency or maximal toxic concentrations (Gerő, 2018). To assess the extract ability to counteracts oxidative stress-induced cell damage, in selected experiments HUVECs were treated with H_2O_2, or sera from CD patients and then processed as indicated in the figure legends.

2.7. Sera from patients

14 sera, 7 from CD patients and 7 from healthy controls were provided from a previous study compilation (Di Sabatino et al., 2011). The CD diagnosis was based on conventional standardized clinical, endoscopic, and histological criteria, while disease clinical phenotypes were classified as fistulizing, stricturing and luminal according to standardized parameters (Gasche et al., 2000). The sera from CD patients were collected from 4 males and 3 females with a mean age of 32.6 years in a range 21–55. Healthy controls (HC) sera were collected from 4 males and 3 females with a mean age 25.3 years in a range 21–29. (Table 1). Sera were aliquoted and stored at −80 °C before their use.

2.8. Cell metabolic activity

Cell metabolic activity (CMA) was assessed as previously described in 96-well plates (BD Falcon) by using the colorimetric test MTT (Promega, Madison, WI) (Zinellu et al., 2009). The MTT assay measures the reduction of the yellow compound 3-(4,5-dimethythiazol2-yl)- 2, 5-diphenyl tetrazolium bromide by mitochondrial succinate dehydrogenase of living cells to an insoluble purple formazan crystal (Supino, 1995) Therefore, MTT reduction is considered as an indicator of cell viability and mitochondrial metabolic activity (Rai et al., 2018). Briefly, cells were cultured as described above and then treated as described in the figure legends, then 20 μl of MTT solution (5 mg/ml) was added in each well 3 h before the end of the experiment. Next, the medium was removed, and the crystals of formazan were solubilized with acidic isopropanol (0.04 N HCl in absolute isopropanol) then absorbance was read at 570 nm wavelength using a Tecan GENios Plus microplate reader (Tecan, Switzerland) with background subtraction at 650 nm.

2.9. Intracellular measurement of ROS levels

Intracellular ROS levels were investigated by using the redox sensing probe 2′,7′-dichlorodihydrofluorescein diacetate H_2DCFDA (Boin et al., 2014). Within the cell, esterases are able to cleave the acetate groups on H_2DCF-DA, thus trapping the reduced form of the probe (H_2DCF). Intracellular ROS oxidize H_2DCF, yielding the fluorescent product, DCF. Briefly, to perform the probe loading, cells were incubated for 15 min with 5 μM of H_2DCFDA (Invitrogen) and then washed 3 times with PBS. Then, the fluorescence variation induced by the treatments was kinetically measured by reading the plate with a Tecan GENios Plus microplate reader (Tecan, Switzerland) every 20 min for 14 h (Total of 900 min) using respectively an excitation and emission wavelengths of 485 and 535 nm. All fluorescence measurements were corrected for background fluorescence and protein concentration. Data of intracellular ROS levels were expressed as means ± SD of the relative fluorescence unit (RFU) values obtained from three different experiments (Posadino et al., 2018a).

Table 1
Clinical features of selected patients with Crohn's disease (n = 7).

Variables	Patients (n = 7)
Age years (mean)	*32,6*
Age range years	*21–55*
Male	*4*
Female	*3*
Clinical phenotype	
Fistulizing	*1*
Stricturing	*3*
Luminal	*3*
Duration of disease years(mean)	*6.46*
Range of disease duration years	*0–16*

2.10. Statistical analysis

Data for all tests were expressed as mean ± SD of measurements repeated in triplicate of three different experiments. One-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons test were performed in all experiments to determine means of differences between treatments with a P value significance at ($P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$). Statistical analysis was performed using GraphPad Prism version 8.00 (GraphPad Software, San Diego California USA).

3. Results

In this study, *Ambrosia maritima* L. was investigated for its phenolic content, antioxidant activity and cytoprotective properties against oxidative-induced cell death. The employed extraction method (freeze drying cycle under low temperature conditions) allowed to obtain a 17.66% extraction yield of a desolvented hydroethanolic extract suitable for cell culture applications (Table 2).

Among the natural antioxidants present in *Ambrosia maritima* L., terpenoids have been so far the primary object of scientific investigations (Abdel Salam et al., 1984; Abdelgaleil et al., 2011; Ali Abdeldayem et al., 2021; Dirar et al., 2017; Iskander et al., 1988; Mohamed et al., 2022; Nazik et al., 2020; Picman et al., 1986); therefore, we decided to focus our attention on *Ambrosia maritima* L. phenolic components. Phenolic compounds and their chromatographic fingerprints in the *Ambrosia maritima* L. hydroethanolic extract were investigated by Folin Ciocalteu and HPLC-DAD/ESI-MS methods respectively. As depicted in (Table 2), the *Ambrosia maritima* L. extract showed a TPC value of 110.74g ± 1.06 mg of gallic acid equivalent per grams of dry weight (mg GAE/gr DW). Out of 27 detected compounds, 7 polyphenols from different classes were identified in the *Ambrosia maritima* L. extract (Table 3).

Three known phenolic acids were detected for the first time in *Ambrosia maritima* L. (3,4 Dicaffeoylquinic acid, 3,5 Dicaffeoylquinic acid and 4,5 Dicaffeoylquinic acid), some flavonoids were also detected including an acylated-*C*-glycosyl flavone (Apigenin 8C-(-6-O-Feruloyl)-glucopyranoside), an *O*-glycosyl flavone (Kaempferol -7- *O*-glucoronide) and a dihydroflavonol. Finally, a sesquiterpene lactone was also identified in the extract profile (4,5 Dihydrolychnopholide) (Fig. 1) and (Table 3).

Although widely accepted as a health benefits provider, natural antioxidants, including phenolic compounds, have been reported to act as prooxidants. (Carocho and Ferreira, 2013; Giordo et al., 2013; Pasciu et al., 2009; Posadino et al., 2013, 2015, 2019; Shaito et al., 2020a).

For these reasons, to gain more realistic information about the *Ambrosia maritima* L. phenolic extract antioxidant capacity, five *in vitro* antioxidant assays were employed (GOR, ABTS, DPPH, CUPRAC and Phenanthroline assays with vitamin C, BHA, BHT and Trolox) since they are considered as some of the standard and strongest non enzymatic antioxidant tests (Carocho and Ferreira, 2013). Results were depicted as IC_{50} for DPPH, GOR, ABTS assays and as A0.5 for CUPRAC and Phenanthroline tests. The scavenging efficacy in all performed tests was inversely proportional to the recorded IC_{50} (Table 4), and all the obtained values fluctuated in a tight interval [14.33 ± 3.86–34.26 ± 4.03]. The lowest IC_{50} was recorded in the GOR method and the highest in DPPH, while the following order was observed when considering all the

Table 2
Extraction yield and total phenolic content (TPC).

SAMPLE	*Extraction yield (%±SD)*	*Total phenols dosage (mg GAE/gr DW ±SD)*
AM	17.66 ± 3,67	110.74 ± 1.06

Values are expressed as means ± SD of three different measurements. AM: *Ambrosia maritima* L. hydroethanolic extract. GAE: gallic acid equivalent; DW: Dry weight; SD: standard deviation.

Table 3
Phenols identification using HPLC-DAD/ESI-MS from *Ambrosia maritima* L. extract.

peak	Compound	t_R	(m/z)	Species
1	Dihydroflavonol	44.236	271	$[M+H]^+$
2	3,4 Dicaffeoylquinic acid	45.5	517	$[M+H]^+$
3	Kaempferol -7- *O*-glucoronide	47.119	461	$[M+H]^+$
4	3,5 Dicaffeoylquinic acid	47.533	517	$[M+H]^+$
5	4,5 Dicaffeoylquinic acid	47.84	517	$[M+H]^+$
6	Apigenin 8C-(-6-O-Feruloyl)-glucopyranoside	51.343	609	$[M+H]^+$
7	4,5 Dihydrolychnopholide	52.387	361	$[M+H]^+$

t_R: Retention time, (m/z): molecular masse range, Species: Operating ion mode.

employed methods: GOR (14.33 ± 3.86) Phe (25.79 ± 1.63) < CUPRAC (26.2 ± 2.56) < ABTS (29.88 ± 2.69) < DPPH (34.26 ± 4.03) μg/mL. Overall, the *Ambrosia maritima* L. extract displayed an efficient antioxidant activity compared to the different standards despite the significant differences ($p < 0.05$). Among the standards, BHA was the most efficient in quenching the free radicals (0.93 ± 0.07) in Phe assay, while BHT was the less efficient (12.99 ± 0.4) in the DPPH method.

To investigate *Ambrosia maritima* L. extract biological activities, we assessed its cytotoxicity and cytoprotection, by analyzing the cell metabolic activity (CMA) under different experimental conditions (*Ambrosia maritima* L. extract alone, in the presence and absence of H_2O_2, and in the presence and absence of CD sera) using the MTT test, which indirectly expresses the cell viability (Rai et al., 2018; Supino, 1995). Potential extract toxicity was investigated by exposing the cells to increasing extract concentrations. Data reported in Fig. 2A show that the extract had no significant cytotoxic effect at the tested concentrations of 1 and 10 μg/mL, while a significant CMA reduction was observed at the two highest doses (50 and 100 μg/ml) when data were compared to untreated cells. We next detected the H_2O_2 concentration capable of eliciting 50% of cell death, which was then employed in successive experiments to investigate extract's protection against oxidative-cell damage. As depicted in Fig. 2B, cells treated with increasing H_2O_2 concentrations showed a dose-dependent CMA decline indicating 25 mM as the dosage capable to induce 50% of cell mortality.

We then investigated whether the extract would possess antioxidant properties by testing its ability to protect cells from H_2O_2-induced oxidative stress and cell death. To this end, cells were pre-treated for 24 h in both absence and presence of increasing extract concentrations and then exposed to 25 μM H_2O_2 for 2 h before the end of the experimental time. As depicted in Fig. 2C, low extract concentrations (1–10 μg/ml) were able to counteract H_2O_2-induced cell death keeping the CMA similar to the untreated cells. On the contrary, a reverse extract action was observed at high concentrations (50–100 μg/mL), which were able to significantly reduce the cell CMA to levels similar to H_2O_2-treated cells (Fig. 2C). The current findings indicate a dose-dependent extract behavior, respectively protective at low concentrations and cytotoxic at high concentrations. We next tried to better understand *Ambrosia maritima* L. pro/antioxidant behavior towards the cellular redox state. For this end, we investigated the extract capability to influence the intracellular redox state in untreated and H_2O_2-treated (25 mM) cell using the same experimental conditions employed in CMA experiments.

Table 4
Antioxidant potential of *Ambrosia maritima* L. extract compared to antioxidant reference molecules.

SAMPLES	IC_{50} GOR (μg/mL ±SD)	IC_{50} ABTS (μg/mL ±SD)	IC_{50} DPPH (μg/mL ±SD)	A0.5 CUPRAC (μg/mL±SD)	A0.5 Phen (μg/mL ±SD)
AM	14.33 ± 3.86 ^{a}	29.88 ± 2.69 ^{a}	34.26 ± 4.03 ^{a}	26.2 ± 2.56 ^{a}	25.79 ± 1.63 ^{a}
BHA	5.38 ± 0,06^{b}	1.81 ± 0.10^{b}	6.14 ± 0.41^{b}	3.64 ± 0.19^{b}	0.93 ± 0,07^{b}
BHT	3.32 ± 0,18^{c}	1.29 ± 0.30^{b}	12.99 ± 0.4^{c}	9.62 ± 0.87^{c}	2.24 ± 0,17^{c}
Ascorbic acid	5.02 ± 0.0^{b}	3.04 ± 0.05^{c}	4.39 ± 0.01^{d}	8.31 ± 0.15^{d}	3.08 ± 0.02^{d}
Trolox	4.31 ± 0.05^{d}	3.21 ± 0.06^{c}	5.12 ± 0.21^{e}	8.69 ± 0.14^{d}	5.21 ± 0.27^{e}

SD = standard deviation; IC_{50} = sample concentration at which 50% of the free radicals activity was inhibited. AM = *Ambrosia maritima* L. hydroethanolic extract, BHA = butylated hydroxyanisole, BHT = butylated hydroxytoluene. Trolox = 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid. GOR = Galvinoxyl free radicals scavenging assay. ABTS = ABTS cation radical decolourisation assay. DPPH = DPPH radical scavenging assay. CUPRAC = Cupric ion reducing power. Phen = O-Phenanthroline free radical reducing activity. Values are expressed as means ± SD of three different measurements. Means followed by a common letter are not significantly different ($p < 0.05$ for one-way Anova and Newman-Keuls multiple comparison tests).

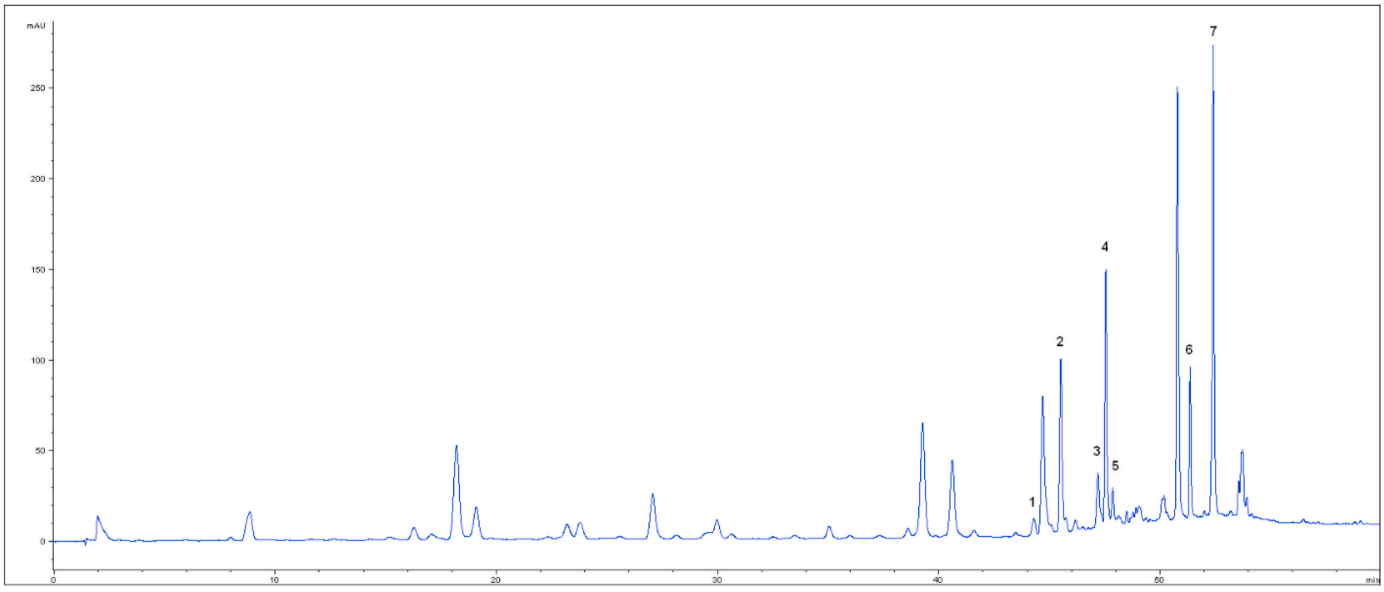

Fig. 1. HPLC-DAD profile of *Ambrosia maritima* L. hydroethanolic extract. (1) Dihydroflavonol, (2) 3,4 Dicaffeoylquinic acid, (3) Kaempferol -7- *O*-glucoronide, (4) 3,5 Dicaffeoylquinic acid, (5) 4,5 Dicaffeoylquinic acid, (6) Apigenin 8C-(-6-O-Feruloyl)-glucopyranoside, (7) 4,5 Dihydrolychnopholide.

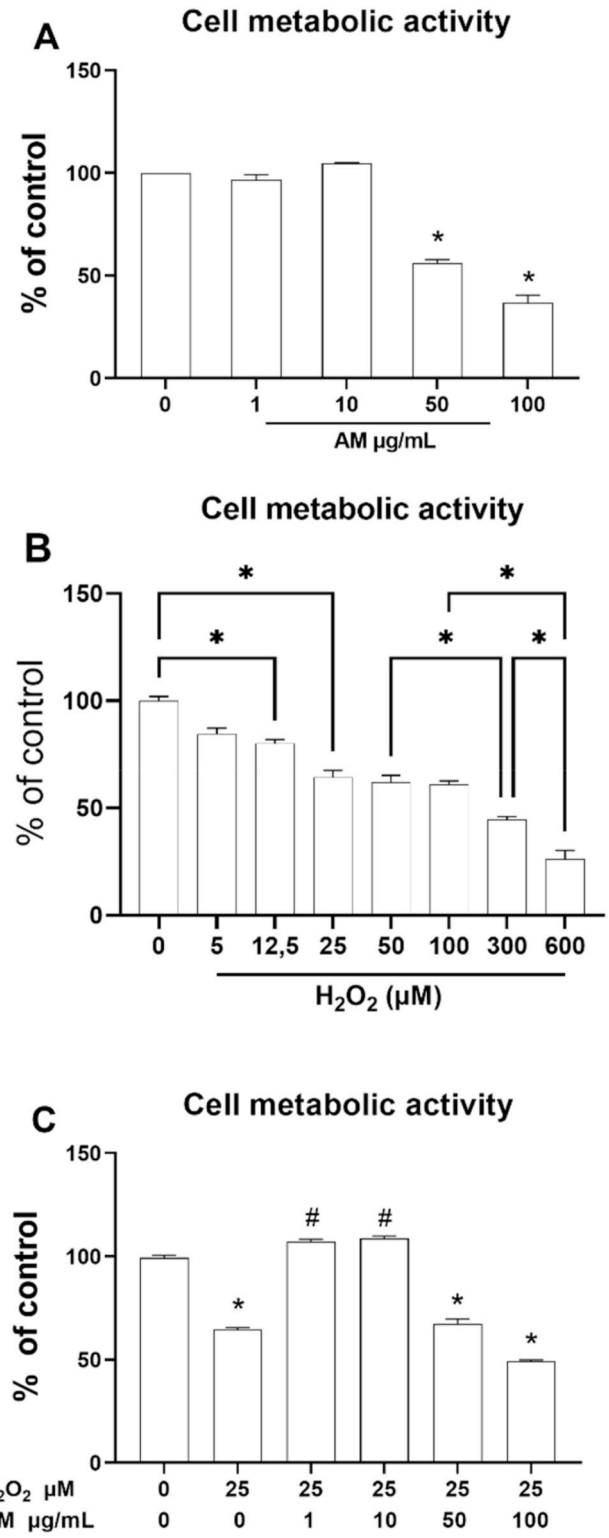

Fig. 2. Effect of H_2O_2 and *Ambrosia maritima* L. (AM) extract on cells metabolic activity. *A) AM extract dose-dependently affects cells' metabolic activity*. Cells were incubated for 24 h with a complete medium containing the indicated concentrations of AM extract and then processed for the cell metabolic activity assessment as reported in the material and methods. *B) Dose-response effect of H_2O_2 on cell metabolic activity.* Cells were cultured for 24 h in a complete medium. In this experiment, 2 µL of the indicated H_2O_2 concentrations were added 2 h before the end of the experiment and then cells were processed for the cell metabolic activity assessment as reported in the material and methods *C) AM extract effect on the cell metabolic activity of oxidatively stressed cells.* Cells were preincubated for 24 h with the indicated concentrations of AM extract. The extract was then removed, and cells were cultured for additional 24 h in a complete medium plus the addiction of 25 µM H_2O_2 2 h before the end of the experiment. At the end of the experiment period, cells were processed for the cell metabolic activity assessment as reported in the material and methods. A-C) Results are expressed as percent of untreated control cells and calculated as means ± SD of three different experiments. AM, *Ambrosia maritima* L.; AM = 0 µg/mL, control untreated cells; H_2O_2, Hydrogen peroxide; *, significantly different from untreated control cells; #, significantly different from 25 µM H_2O_2.

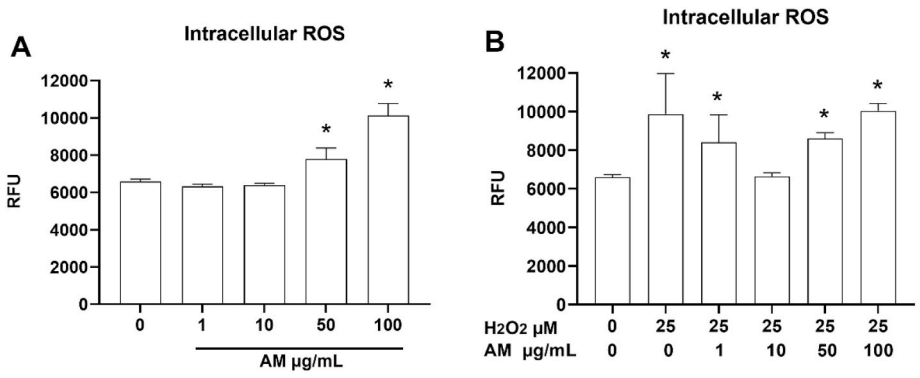

Fig. 3. Dose-dependent effect of *Ambrosia maritima* L. (AM) extract on the ROS levels of unstressed and oxidatively-stressed cells *A) Dose-dependent effect of AM on unstressed cells intracellular ROS levels of.* Cells were loaded with the ROS probe and then incubated for 14 h with a complete medium containing the indicated concentrations of AM extract and then processed for the ROS assessment as reported in the material and methods. B) *Dose-dependent effect of AM extract on oxidatively-stressed cells intracellular ROS levels of.* Cells were loaded with the ROS probe and then incubated for 14 h with a complete medium containing the indicated concentrations of AM extract. 2 hrs before the end of the experiment, 25 µM of H_2O_2 was added to each well to mimic cellular oxidative stress, then cells processed for the ROS assessment as reported in the material and methods. A-B). Results are expressed as the means ± SD of the Relative Fluoresce Units (RFU) obtained by the reading of three different experiments. AM, *Ambrosia maritima* L.; AM = 0 µg/mL, control untreated cells; H_2O_2, Hydrogen peroxide; *, significantly different from untreated control cells.

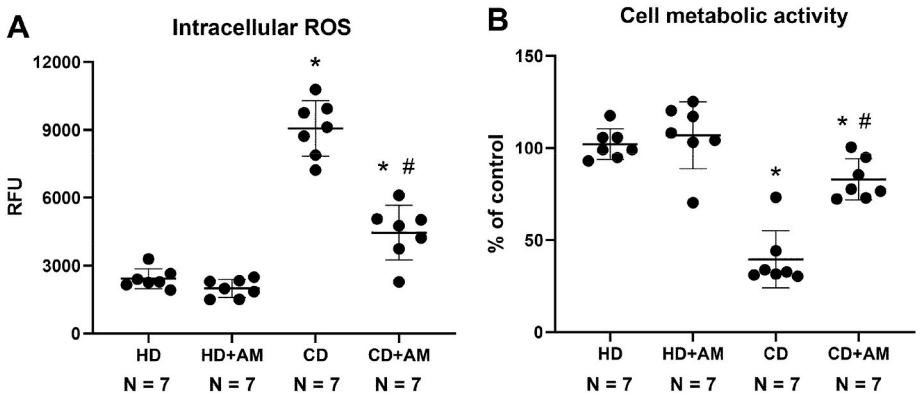

Fig. 4. *Ambrosia maritima* L. (AM) extract counteracts CD sera-induced oxidative cell damage: *A) AM counteracts CD-induced ROS increase.* HD, cells were loaded with the ROS probe and then incubated for 14 h with 5% (v/v) of sera from the healthy donors; CD, cells were loaded with the ROS probe and then incubated for 14 h with 5% (v/v) of sera from patients with Crohn's Disease; CD + AM, cells were preincubated for 24 h with 10 µg/mL of AM extract, the extract was then removed, and cells were cultured for additional 14 h with 5% (v/v) of sera from patients with Crohn's Disease. HD + AM, cells were preincubated for 24 h with 10 µg/mL of AM extract, the extract was then removed, and cells were cultured for additional 14 h with 5% (v/v) of sera from healthy donors. Cells were processed for the ROS reading as reported in materials and methods. Results are expressed as the means ± SD of the Relative Fluoresce Units (RFU) obtained by the reading of three different experiments. *B) AM counteracts CD-induced cell death.* HD, cells were incubated for 24 h with 5% (v/v) of sera from the healthy donors; CD, cells were incubated for 24 h with 5% (v/v) of sera from patients with Crohn's Disease; CD + AM, cells were preincubated for 24 h with 10 µg/mL of AM extract, the extract was then removed, and cells were cultured for additional 24 h with 5% (v/v) of sera from patients with Crohn's Disease. HD + AM, cells were preincubated for 24 h with 10 µg/mL of AM extract, the extract was then removed, and cells were cultured for additional 14 h with 5% (v/v) of sera from healthy donors. Cells were processed for the MTT assay as reported in materials and methods. Results are expressed as the percentage of untreated healthy control cells (HD) and calculated as means ± SD of three different experiments. AM, *Ambrosia maritima* L.; HD, sera from the healthy donors; CD, sera from patients with Crohn's Disease; *, significantly different from HD; #, significantly different from CD.

Parallelly, we aimed to set a convenient *Ambrosia maritima* L. extract concentration with antioxidant potential to be safely employed in the next experiments. For this purpose, we challenged the cell with increasing extract concentrations and measured their effect on the ROS intracellular levels. Cells treated with low doses of *Ambrosia maritima* L. showed steady levels of ROS, similar to those detected in untreated cells, while higher *Ambrosia maritima* L. concentrations significantly increased ROS levels when compared to untreated cells (Fig. 3A). However, in the presence of H_2O_2, as previously observed for the CMA, low doses of *Ambrosia maritima* L. (1–10 µg/ml) elicited an antioxidant effect with the best effect observed at 10 µg/mL, while the higher doses (50–100 µg/mL) generated increased ROS levels similar to the one detected in H_2O_2 treated cells (Fig. 3B). Based on the observed findings, the prepared *Ambrosia maritima* L. extract can elicit both antioxidant and prooxidant effects depending on the dosages employed on HUVECs. Our data indicate that *Ambrosia maritima* L. has a dose-dependent dual behavior, being able to counteract oxidative stress and oxidative-mediated cell death when employed at a relatively low dosage while becoming pro-oxidant and harmful to the cells if used at relatively high concentrations.

Our observations are in agreement with previous results concerning the naturally occurring antioxidant Resveratrol and other phenolic compounds with antioxidant characteristics (Calabrese et al., 2010; Carocho and Ferreira, 2013; Giordo et al., 2013; Pasciu et al., 2009; Posadino et al., 2013, 2015, 2019; Shaito et al., 2020a). According to these results, we selected the concentration of 10 µg/mL for the successive experiments.

We next hypothesized that sera from Crohn's disease patients (CD) may generate an imbalanced intracellular redox state and may ultimately lead to cell damage and death. To gain insight into this hypothesis, we tested the ability of CD sera to promote oxidative stress and cell death, along with the capacity of *Ambrosia maritima* L. to counteract these two phenomena. To this end, HUVECs were challenged with CD sera in the presence or absence of a pre-treatment with a single dose of *Ambrosia maritima* L. (10 mg/mL). Cells treated with healthy donors (HD) were utilized as a control. As expected, CD sera were able to generate an increase in ROS levels compared to HD sera (Fig. 4A), a phenomenon that was significantly counteracted by the *Ambrosia maritima* L. pre-treatment (Fig. 4A).

In line with the observed CD-induced oxidative stress, cell treatment with the pathological sera also significantly increased cell mortality

(Fig. 4B). Also in this case, *Ambrosia maritima* L. pre-treatment significantly reduced CD-induced cell mortality to values close to HD (Fig. 4B). On the other hand, AM treatment failed to induce any significant variation in HD-treated cells in terms of ROS and CMA (Fig A and B). The current findings indicate that *Ambrosia maritima* L. extract (10 μg/mL) can counteract CD sera-induced oxidative stress and associated cell death providing a protective effect against pro-oxidant factors present in the sera of CD patients.

4. Discussion

It is well recognized that CD is a non-specific and diffuse inflammatory pathology with a high disability rate. Its complex therapy includes conventional treatments based on steroids and salicylic acid derivatives besides other biological agents that lack effectiveness and have well-established side effects on CD patients (Bourgonje et al., 2019). To date, a positive correlation between oxidative stress and Crohn's disease is believed to exist. Indeed, tissue injury and fibrosis formation associated with CD have been proven to be accompanied by an imbalance between increased ROS levels and decreased antioxidant defenses within the affected tissues (Alemany-Cosme et al., 2021; Alzoghaibi, 2013; Fernanda Silva Basílio and Santos Branco, 2021). For this reason, among the rising demand for alternative treatments with better induction and maintenance of CD remission, antioxidant therapy is being seen as one of the promising approaches. In this context, alternative medicines, particularly herbal therapies, are attracting attention. Up to 21%–60% of IBD patients resort to medicinal plants due to their remarkable natural efficiency in managing the disease using various mechanisms, including immune system regulation and antioxidant activity. (Ganji-Arjenaki and Rafieian-Kopaei, 2019). In this regard, several species from the *Asteraceae* family have been extensively investigated for their antioxidant potential (Didier et al., 2011). In the present work, we evaluated the phenolic profile of an *Ambrosia maritima* L. hydroethanolic extract, a plant from the Asteraceae family, by assessing its antioxidant behavior and capability to counteract H_2O_2- and CD sera-elicited oxidative stress on a primary human endothelial cells model.

Extraction methods and the procedures applied to them have been reported to be a pivotal point in the effectiveness and quality of natural antioxidants' extracts, influencing their potential bioactivity (Altemimi et al., 2017; Sasidharan et al., 2011; Zhang et al., 2018). Indeed, temperature, solvent type and concentration employed are influential factors playing an important role in extraction efficiency (Xu et al., 2017). With the intent of preventing thermolabile molecules degradation, here we used both low pressure and temperature to carry out the extraction process in a hydroethanolic mixture (60%). The obtained phenolic extract had an extraction yield of 17.66%, and most of the obtained phenolic compounds were hydrosoluble antioxidants in polar and medium polar solvents, such as water, ethanol, methanol, and their aqueous mixtures, which are commonly used in the extraction procedures (Xu et al., 2017).

Polyphenols and their phenolic acids have been considered a focal point of a great deal of research because of their broad distribution in plants, wide spectrum of biological activities, and positive impact on health (Havsteen, 2002; Posadino et al., 2013, 2018b, 2021). In the Asteraceae family, these compounds have long been characterized and studied for their antioxidant capacity (Didier et al., 2011). In our hands, the *Ambrosia maritima* L. extract chemical profile assessment revealed a considerable level of phenolics with 110,74g ± 1,06 mg of gallic acid equivalent/g of dry weight. Similar results were reported in a previously study with an amount of 188.06 mg gallic acid equivalent obtained from the leaf powder of *Ambrosia maritima* L., suggesting this plant as potential source of phenols (Said et al., 2018).

The fingerprint analysis of bioactive molecules from plant extracts using high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry has captured a great interest especially in the separation and isolation of flavonoids and their glycosides. Indeed, this technique has the ability to distinguish flavonoids *O*-glycosides, *C*-glycosides and *O,C*-glycosides as well as the substitution position even without a previous separation or purification from the complex (Chen, G. et al., 2016). Using this technique, here we reported for the first time the detection of three dicaffeoylquinic acid isomers in *Ambrosia maritima* L.: 3,4 Dicaffeoylquinic acid, 3,5 Dicaffeoylquinic acid and 4,5 Dicaffeoylquinic acid. In this context, plants belonging to the Asteraceae family have been reported to contain important amounts of flavonoids and caffeoylquinic acid derivatives (Didier et al., 2011; Marksa et al., 2020; Salehi et al., 2019). In particular, our findings align with those of Tamura et al. stating that 3,5 Dicaffeoylquinic acid is an abundant compound in *Ambrosia artemisiifolia* L., a species of the same genus of *Ambrosia maritima* L. (Tamura et al., 2004).

Two flavonoids were also detected from the *Ambrosia maritima* L. extract, Apigenin 8*C*-(-6-*O*-Feruloyl)-glucopyranoside and Kaempferol -7- *O*-glucoronide. Kaempferol and its derivatives have commonly been isolated from many species belonging to the Asteraceae family (M. Calderon-Montano et al., 2011). In this regard, numerous biological activities and a variety of health benefits of flavonoids, especially those against chronic human diseases and oxidative damage, are correlated to their antioxidant properties, which are due to their phenolic nucleus and the conjugated C3-side chain (Shahidi and Chandrasekara, 2010). Moreover, several *in vitro*, *in vivo* and epidemiological studies reported that ferulic acid bioactivities and health benefits, particularly against oxidative damage-associated chronic diseases, are linked to its higher antioxidant capacities, which are associated with its phenolic nucleus, conjugated C3 side chain and hydroxyl group (Shahidi and Chandrasekara, 2010). It is also common for naturally occurring components to bear 1 or more biologically active feruloyl moieties in their skeleton easily recognizable by mass spectrometry. It has been also stated that ferulated compounds may be even stronger antioxidants that the acid itself. Notably, a wide range of sugar monomers substitutions linked to oxygen at carbons in various skeleton positions, including glycosyl, also occur in ferulated flavonoids. (de Oliveira Silva and Batista, 2017; Shahidi and Chandrasekara, 2010). Sugars and their derived acids are common in plant-derived flavonoids, attributing beneficial health properties to these compounds. The above-mentioned conjugation has also been found to influence their cellular uptake and anti/pro-oxidant properties since the conjugated forms of some flavonoids exhibit a stronger antioxidant activity than the parent flavonoid (Boersma et al., 2002). In the regard, our LC/MS fingerprint analysis showed the presence of both glycosylated and glucoronated flavonoids: Apigenin 8*C*-(-6-*O*-Feruloyl)-glucopyranoside and Kaempferol -7- *O*-glucoronide, respectively.

The *Ambrosia maritima* L. extract antioxidant activity was chemically tested *in vitro* using five different methods with the lowest IC_{50} recorded for the GOR test with a value of 14.33 ± 3.86 μg/mL. Using the DPPH test, similar results have been previously shown for some *Ambrosia maritima* L. purified fractions, which demonstrated a remarkable antioxidant activity ranging between 80% and 86% (Nazik et al., 2020). It is widely accepted that applying several tests allows antioxidant activity assessment of different targets within the matrix, which is why five various tests were employed. For example, the ABTS technique is based on decolourisation in presence of antioxidants (like carotenoids, phenolic compounds and derivatives), while the DPPH technique relies on the fact that antioxidants behave as a hydrogen donor. Moreover, Cu (II) can also be used as an oxidant in the determination of one compound's antioxidant potential in the CUPRAC assay (Carocho and Ferreira, 2013). In line with our results, accumulated literature data reports polyphenols such as caffeoylquinic acid and derivatives as major antioxidant metabolites (Didier et al., 2011; Kim and Lee, 2005; Mijangos-Ramos et al., 2018). Furthermore, antioxidant properties have also been widely linked to flavonoids as they fulfill the main criteria of antioxidant properties, such as ROS scavenging and activation of the cellular antioxidant system. Finally, several studies related the variation

of flavonoids' antioxidant properties to their degree of hydroxylation, methoxylation, and glycosylation of A, B, and C rings which results in derivative structures of the parent flavonoid (Pietta, 2000). Also, in agreement with our results, a significant body of data reported the antioxidant activity of Apigenin and derivatives, which appears related to their capacity to upregulate the expression of antioxidant enzymes (Chen, W. et al., 2016). Several other studies reported that Kaempferol antioxidant activity is attributed to its degree of conjugation and hydroxylation, responsible for the electrons' delocalization (Chen, W. et al., 2016; Pietta, 2000). Nonetheless, cytotoxic effects of *Ambrosia maritima* L. leads have been reported in the literature (Dirar et al., 2017; Ibrahim Dirar et al., 2014), in line with our current data.

In the last years, the ever-growing interest in polyphenols as potent antioxidants was controverted by evidence showing that they exhibit biphasic concentration-dependent effects, acting as antioxidants at low concentrations and as prooxidants at high concentrations. Such an aspect seems associated and influenced by the antioxidant type, dosage, and redox environment, which are emerging pivotal determinants in affecting the equilibrium between antioxidants beneficial and deleterious effect (Calabrese et al., 2010; Carocho and Ferreira, 2013; Giordo et al., 2013; Pasciu et al., 2009; Posadino et al., 2013, 2015, 2019; Shaito et al., 2020a). Particularly, flavonoids and phenolic acids are good example of such dual behavior, noticeably quercetin, myricetin, kaempferol, resveratrol and caffeic, chlorogenic and ferulic acids (Bouayed, 2012; Kung et al., 2021). Moreover, a number of works reported the ability of some individual dietary polyphenols to induce mitochondrial-mediated cell death and apoptosis in a dose-dependent manner (Galati and O'Brien, 2004; Posadino et al., 2015; Posadino et al., 2013). Consistent with the literature, our current results revealed a dual behavior of the *Ambrosia maritima* L. phenolic extract, both cytoprotective and cytotoxic, when challenged with primary HUVECs. This peculiar extract behavior has been further confirmed by assessing its effect on the intracellular ROS levels under the same experimental conditions, confirming its low concentration-associated antioxidant effect and high dosage-associated pro-oxidant properties. Such extract observed phenomenon can be presumably justified by the presence of chlorogenic acid and kaempferol derivatives already recognized as polyphenols with a dose-dependent dual redox behavior (Bouayed, 2012; Giordo et al., 2013; M. Calderon-Montano et al., 2011). On the other hand, for what concern Apigenin, it was found to be safe exerting no toxicity even in high doses (Salehi et al., 2019).

Several studies have reported the implication of oxidative stress in the onset and development of IBD (Bourgonje et al., 2019, 2020; Khaloian et al., 2020; Luceri et al., 2019). In our study, low doses of *Ambrosia maritima* L. phenolic extract were able to counteract oxidative damage and cell death induced by sera from CD patients. The protective effect of several polyphenols and their derivatives against gastrointestinal IBD disorders like CD, ulcerative colitis (UC), and irritable bowel syndrome (IBS) have been investigated, and their antioxidant, anti-inflammatory and immunomodulatory properties have been demonstrated *in vivo* and *in vitro* experiments using cell cultures (Chiu et al., 2021; Kaulmann and Bohn, 2016; Martin and Bolling, 2015). Interestingly, experiments performed with kaempferol, hydroxycinnamic acids and derivatives showed that their consumption in an *in vivo* colitis model was able to significantly lower the disease index scores (Larrosa et al., 2009; Park et al., 2012). However, concerning the phenolic compounds dose-dependent dual redox behavior, many studies highlighted that their adverse effects may be enhanced in the presence of free metal ions like copper or iron when the tissue is damaged, all common aspects of the IBD pathology (Pallone and Monteleone, 2001). Conversely, when applied in considerable yet physiologically relevant doses, phenolic compounds are capable of reducing oxidative stress in cells oxidatively stressed or inflamed (Giordo et al., 2013; Kaulmann and Bohn, 2016). Finally, it is important to state that experimental trials (*in vitro* and *in vivo*) have their own limitations concerning CD investigation since they have been performed with native components without taking into consideration the effect of the digestion and the eventual interference with microbial metabolism which may reduce their bioavailability in the gastrointestinal system. Besides, the monolayer cell culture model shows limitations like the absence of immune cells and microbiota interactions, short-term exposure to stress conditions and the possible changes of the phenolic profile due to the digestion process (Kaulmann and Bohn, 2016). Hence, a well-established and designed complex model is required to better study the effect of natural compounds, especially polyphenols, on the amelioration of the CD redox state. In particular, the environmental redox state, the microbial interactions and immune system modulation aspects need to be considered to better understand the phenolic compounds bioactivity in the context of this pathology.

5. Conclusion

Taken together, our study findings support the use of *Ambrosia maritima* L. in folk medicine. Di-caffeoylquinic acid isomers were identified for the first time in *Ambrosia maritima* L. Moreover, to our knowledge, the potent *in vitro* antioxidant activity, paralleled with the cytoprotective effect elicited against H_2O_2- and sera from CD patients-elicited oxidative stress and cell death is reported for the first time at low doses for this species. However, the biphasic redox comportment of its phenolic extract falls in line with phenolic compounds' overall behavior. Thus, it is evidently discernible that *Ambrosia maritima* L. extract is a potential source of antioxidants that may have applications in ameliorating the redox state impairment associated to CD, and consequent benefit for pathology overall status. Nevertheless, further large-scale trials are required to better understand the mechanism of action of this extract, including *in vitro, in vivo,* and clinical-based experimentation to better understand their bioavailability and their interference with this pathology's different stages.

Institutional review board statement

This study was approved by the Ethical Committee ASL 1 Sassari Prot 2149/CE/2.

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CRediT authorship contribution statement

I.R. is the leading author, who developed the main ideas and wrote the research paper. A.M.P supervised the whole experiments and contributed to the technical and academic realization of this work during the internship in her lab. Y.S, A.B, D.H and G.D provided help and technical guidance during simulations and experiments. G.P. is one of the two corresponding authors and provided with S.L.A. the necessary technical tools for the realization of this work and followed the writing of the paper and its revision with S.Z. the supervisor of this work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data used are presented in the article

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