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Neuroprotective Potential of Polyphenol-Rich Artichoke Waste Extract: Exploring Antioxidant and Immunomodulatory Activities in Cell Models

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ABSTRACT

Artichoke waste (AW) constitutes a significant amount of discarded material, but it might be considered a sustainable source of phenolic compounds with antioxidant activity. This study aims to investigate the neuroprotective capacity of AW extract, due to its high content of polyphenolic compounds. In particular, the quali-quantitative characterization by HPLC and LC-MS/MS analysis revealed a notable presence of chlorogenic acid and flavonoids such as luteolin derivatives. The AW extract exhibited an antioxidant/antiradical potential demonstrated by its low IC_{50} values in DPPH and ABTS assays (0.14 and 0.073 mg/mL respectively). The AW extract was then evaluated in neuroinflammation processes on human neuroblastoma SH-SY5Y and THP-1 macrophage cell models, where it reduced the H_2O_2-induced oxidative stress and enhanced cell viability, by dropping the production of ROS at 25 μg/mL. Furthermore, the AW ability in modulating inflammation was tested on THP-1-derived macrophages, demonstrating that the AW extract facilitated a shift in macrophage polarization from the pro-inflammatory M1 to the anti-inflammatory M2 phenotype. These findings underscore the potential of AW extract for neuroprotection and inflammation modulation, highlighting the value of AW as a source of bioactive compounds and paving the route for further research into its mechanisms and clinical relevance. Furthermore, our results emphasize the significance of utilizing agricultural by-products.

1 | Introduction

The agri-food industry constantly meets a perennial challenge: the generation of millions of tonnes of waste annually, leading to significant environmental issues in waste management (Galanakis 2012). Vegetable waste stands out as a rich source of bioactive compounds, including antioxidants and dietary bioactive substances. Consequently, repurposing food waste for the development of functional foods, additives, or nutraceuticals is increasingly crucial and driven by the growing demand for natural alternatives and superior bio-products. Key to this point is the extraction of these beneficial additives from raw materials without compromising the integrity or safety of the resulting products.

Artichoke (*Cynara cardunculus* L. var. scolymus) is a perennial plant, endemic to the Mediterranean region, extensively cultivated for its edible flower heads. Artichoke is also known for its high content of polyphenols, such as chlorogenic acid (CGA) and flavonoids, and has been widely studied for their health-promoting effects, providing potential cardioprotective, neuroprotective and antioxidant effects (Iglesias-Carres et al. 2023). Less attention has been paid to exploring the broader range of polyphenols found in the entire artichoke plant, particularly in the waste material. Artichoke Waste (AW), which includes the leaves, stems, and outer parts of the artichoke plant, are generated during the harvesting and processing stages and it is rich in phenolic compounds, including CGA and a variety of flavonoids. Historically, these by-products have been discarded or relegated to secondary uses such as animal feed. However, this disposal not only poses environmental challenges, but also represents a missed opportunity to recover and repurpose valuable bioactive compounds for various applications.

Artichoke waste (AW) is a veritable goldmine of dietary fiber, minerals, vitamins, and an array of phenolic compounds, recognized for their multifaceted health benefits and integral to plant defense and adaptation (Lavecchia et al. 2019; Zayed and Farag 2020). AW presents a rich variety of phenolic compounds, including hydroxycinnamic acids (e.g., chlorogenic acid - CGA - and cynarin) and flavonoids (e.g., luteolin and apigenin glycosides) (Jiménez-Moreno et al. 2019). Notably, CGA is the most abundant polyphenol and in the last years it has garnered attention for its ability to modulate numerous cellular pathways implicated in oxidative stress, inflammation, apoptosis, signal transduction, and even the composition and activity of the gut microbiota (Naveed et al. 2018). In particular, several studies have reported the antioxidant properties of CGA, exerting cardiovascular protection (Mubarak et al. 2012), antitumor activity (Zeng et al. 2021), and neuroprotective effects (Kwon et al. 2010). Interestingly, previous studies have shown that CGA lowers the risk of developing neurodegenerative conditions, such as Alzheimer's and Parkinson's diseases, through its inhibitory effects on Aβ40 aggregation (Heitman and Ingram 2017; Tajik et al. 2017). Several evidence suggest that consumption of coffee, a beverage highly rich in CGA, is associated with the mitigation of cognitive decline and reduction of the risk of developing Parkinson's and Alzheimer's diseases (Ascherio et al. 2001; Cropley et al. 2012; Hoelzl et al. 2010; Kwon et al. 2010). Moreover, a study highlighted the neuroprotective effect of CGA on human neuroblastoma SH-SY5Y cells injured by H_2O_2, a model system utilized for studying neuronal cell death induced by oxidative stress and for investigating the neuroprotective effects of compounds (Gao et al. 2021). Notably, CGA mitigated these morphological changes and reduced the incidence of cell apoptosis in a dose- and time-dependent manner (Gao et al. 2021). Although the pharmacological activities of artichoke (Feiden et al. 2023) and CGA are deeply explored, less is known regarding the potential nutraceutical activity of AW, in particular as a neuroprotective agent.

Considering the evidence regarding the neuroprotective effects of CGA and its high content in AW, this study aims to investigate the neuroprotective potential of AW by evaluating its phenolic composition and antioxidant/antiradical activity, as well as anti-inflammatory capacity. Furthermore, SH-SY5Y cells and THP-1-derived macrophages were used to evaluate the protective effect of AW against H_2O_2-induced oxidative stress. Moreover, the AW ability to modulate macrophage polarization was investigated by immunocytochemistry. The results were compared with those obtained in the presence of its main polyphenol component, CGA. Our results are in trend with recent investigation on the reduction of the environmental impact considering the potentiality of agri-food waste. Thus, exploring new opportunities to develop value-added AW-based products with health benefits as a source of bioactive compounds could be an innovative and thrilling goal.

2 | Materials and Methods

2.1 | Chemistry and Reagents

Solvents used for extraction procedures and high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (LC-MS/MS) analyses, such as water LC-MS grade and HPLC grade, methanol (MeOH) LC-MS and HPLC grade, acetonitrile (ACN) LC-MS and HPLC grade, 2-propanol LC-MS grade, *n*-hexane HPLC grade, dichloromethane HPLC grade, ethanol (EtOH) HPLC grade, ethyl acetate HPLC grade, acetic acid (AcOH, 100%) HPLC grade and formic acid LC-MS grade (FA, ≧98%) were all purchased from Sigma Aldrich-Merck (Merck srl, Milan, Italy The commercial analytical standards of pinoresinol, apigenin-7-glucoside, luteolin-7-glucoside, luteolin-7-rutinoside, rutin, narirutin, 1,5-dicaffeoyilquinic acid (1,5-CQA), verbascoside and trolox were purchased from Merck (Merck srl, Milan, Italy); chlorogenic acid, tyrosol, hydroxytyrosol, caffeic acid, vanillic acid, ferulic acid, p -coumaric syringic acid, and vanillin were purchased from TCI (Zwijndrecht, Belgium). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), catechin, nitroetrazolium blue chloride (NBT), dihydrorodamine (DHR) and sodium hypochlorite (NaOCl) were purchased from Sigma-Aldrich, Germany. Dimethylformamide (DMF), disodium (Na_2HPO_4) and monopotassium phosphate (KH_2PO_4) were obtained from Merck, Germany. β-nicotinamide adenine dinucleotide (NADH) and phenazine methosulfate (PMS) were purchased from Sigma-Aldrich, India, and USA, respectively. Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX-I, fetal bovine serum (FBS), streptomycin, penicillin and amphotericin B were from

Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). Triton X-100 was purchased from Sigma Chemical Co. (St. Louis, USA), while dimethylsulfoxide (DMSO) was obtained from AppliChem (Darmstadt, Germany).

2.2 | Sample Collection

AW is constituted by artichoke external leaves from *cultivar Terom* collected in Tuscan area in February 2022.

2.3 | Samples Preparation

After collecting, the sample was dried at 25°C in the dark for 60 days. The dried AW was crushed to obtain a homogeneous powder and stored at −20°C until analysis. The polyphenolic extraction was performed according to the procedure by Lavecchia et al. [28]. Briefly, 4 g of dried leaves was macerated with an EtOH/H_2O solution (80:20 v/v), stirring for 2 h at room temperature (rt). Then, the suspension was centrifuged, the supernatant was filtered by 45 μM diameter filter and finally evaporated. The resulting extract was dissolved in 1 mL of MeOH:H_2O (1:1 v/v) solution and injected for HPLC analysis.

2.4 | Analysis of Phenolic Compounds by HPLC

Complete quantitative analysis of samples was performed using a Shimadzu HPLC Nexera series (model CBM-40D) instrumentation equipped with a binary pump (LC-40D XR), a degassing unit (DGU-405), and a diode array detector (SPD-M40) (Shimadzu, OR, USA); the chromatograms were analyzed by the Shimadzu LabSolutions software version 5.111. A Phenomenex Gemini reverse-phase C18 column (250 × 4.6 mm, 5 μM particle size; Phenomenex, Castel Maggiore, Italy) was used as stationary phase. HPLC analysis of samples was performed using a slightly modified method previously reported (Lavecchia et al. 2019). The mobile phase was a mixture of H_2O:AcOH (98:2 v/v) (A) and ACN 100% (B). Gradient elution was applied with the linear gradient progressing from 5% (B) to 25% (B) in 30 min; then to 50% (B) in 10 min (40 min total time); and then in 10 min it changed to 100% (B) (50 min total time). Finally, 5 min re-equilibration was applied (60 min total time) to reach its initial 5% (B) composition. The flow rate was 1 mL/min, and the injected volume was 20.0 μL. Linear calibration curves for CGA, 1,5-CQA, caffeic acid, lutelolin-7-glucoside, luteolin-7-rutinoside and narirutin were carried out in triplicate, at the above established conditions, in the range 250–100 μg/mL; good linearities with correlation coefficients (R^2) of at least 0.998.

2.5 | Analysis of Minor Polyphenols by LC-MS/MS Analysis

Concentrations of minor polyphenols were measured using a sensitive, optimized LC-MS/MS method, based on a previously developed analytical approach (Cuffaro et al. 2023).

The instrumental setup consisted of an Agilent 1290 UHPLC system (Santa Clara, CA, USA), including a binary pump, a column oven set to 40°C, and a temperature-regulated autosampler. This system was coupled to a Sciex QTRAP 6500+ mass spectrometer (Concord, ON, Canada), configured in triple quadrupole mode and equipped with an IonDrive Turbo V source performing an electrospray ionization (ESI). Chromatographic separation was performed using an Agilent Zorbax SB-C18 StableBond Analytical column (4.6 mm × 150 mm, 5 μM particle size) The mobile phases used were (A) MeOH and (B) H_2O, both containing 0.025% CH_3COOH. A gradient elution method was employed at a flow rate of 800 μL/min, with the following conditions: 0.0–1.0 min (B) 95%, 12.0–14.0 min (B) 5%, 15.0–18.0 min (B) 95%. The injection volume was set to 10 μL. Instrument control and data acquisition were handled using Sciex Analyst software version 1.7.3, while Microsoft 365 Excel (Albuquerque, NM, USA) was used for data analysis. A mass spectrometry selected reaction monitoring (SRM) method was operated in negative ion mode. For each compound three transitions were considered for analysis. One transition was used for quantification (Q), while the other two were used as qualifiers (q). The list of optimized transitions, along with their corresponding retention times, declustering potential (DP), entrance potential (EP), collision energy (CE), and collision exit potential (CxP) potentials, can be found in Supporting Information S1: Table S1. Further operative parameters were gas source 1 (GS1), 40 arbitrary units; gas source 2 (GS2), 40 arbitrary units; ion spray voltage (ISV), −4.0 kV; source temperature (TEM), 650°C; Curtain gas (CUR), 20 arbitrary units; and collision gas (CAD) N2—operative pressure with CAD gas on, 2.80 mPa.

A stock solution of each standard compound, including the internal standard, was prepared in MeOH at a concentration of 100 μg/mL and stored at −20°C. Calibration curves were freshly prepared in pure LC-MS water as previously described. AW- extracted samples, kept at −20°C, were thawed at rt, resuspended in 500 μL of pure LC-MS grade water and 500 μL of LC-MS grade MeOH and then vortexed and sonicated for 5 min to ensure complete resuspension. An aliquot of the resuspended sample was added with the appropriate amount of the internal standard (IS) to achieve the same final concentration (500 ng/mL) in both the submitted samples and the curve calibration levels. The specificity and selectivity of the method was determined by repeatedly injecting standard analytes and observing their retention times to confirm clear distinction from other components and possible interferents in the samples. Linearity was assessed across the previously defined calibration curve range. Accurate and precise quantification of analytes was achieved by including an internal standard in each sample and calibration curve point.

2.6 | Determination of Total Phenolic Content (TPC)

The TPC was determined with the Folin–Ciocalteu (FC) assay, by following a procedure reported in the literature, with slight modifications.(Esposito Salsano et al. 2022) The AW extract (10 mg) was dissolved in 5 mL of MeOH. Then, 0.25 mL of FC reagent and 1.5 mL of Na_2CO_3 (20% w/v) were added to 1 mL of

the methanolic solution in a volumetric flask, after which distilled water was added, reaching the final volume of 10 mL. The resulting mixture was kept for 45 min at the controlled temperature of 25°C, on a stove (Heraeus). Spectrophotometric analysis was performed at $\lambda = 725$ nm using a Molecular Devices SPECTROstarNano UV/Vis spectrophotometer (San Jose, California). Each analysis was executed in triplicate, and the TPC of the extract was expressed as μg of gallic acid equivalent (GAE)/mg of AW extract.

2.7 | Determination of Total Flavonoid Content (TFC)

The TFC of the AW extract was determined using the colorimetric aluminum chloride assay, following a modified version of the method already described (Unuofin et al. 2018). Briefly, 1 mL of AW extract (2 mg/mL in 80% methanol) was mixed with 2 mL of distilled water and 0.15 mL of 5% sodium nitrite. The mixture was incubated for 5 min, followed by the addition of 0.15 mL of 10% aluminum chloride ($AlCl_3$), and allowed to react for another 5 min. Afterward, 1 mL of 1 M sodium hydroxide and 1.2 mL of distilled water were added. The absorbance was measured at 420 nm using a Molecular Devices SPECTROstarNano UV/Vis spectrophotometer. A control solution containing 0.5 mL of distilled water instead of the extract or standard was used as the blank. Each analysis was executed in triplicate. The TFC was calculated using a quercetin calibration curve, with results expressed as μg of quercetin equivalents (μg QE) per mg of extract.

2.8 | Determination of Total Chlorogenic Acid Content (TCGAC)

The TCGAC was determined with a recently reported method by Cuffaro et al. (Cuffaro et al. 2025). The AW extract (1 mg) was dissolved in 1 mL of Tris buffer (10 mM, pH 9). The absorbance of the resulting solution was measured at 360 nm using a Molecular Devices SPECTROstarNano UV/Vis spectrophotometer. Each analysis was executed in triplicate. The TCGAC was calculated using a CGA calibration curve, with results expressed as micrograms of CGA equivalents (μg CGAE) per mg of extract.

2.9 | Antiradical Activity

2.9.1 | 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The antiradical activity of AW and CGA was evaluated by the free radical scavenging of DPPH, using the protocol reported by Cuffaro et al. 2023. Briefly, 100 μL of DPPH solution in methanol (40.0 μg/mL) was added to 100 μL of sample solution in methanol. After 45 min of incubation at rt and in the dark, the absorbance was read at 517 nm in a Molecular Devics SPECTROstarNano (200–1000 nm) UV/Vis spectrophotometer. Methanol was used as blank, and DPPH• solution as negative control. Trolox was employed as positive control and was treated under the same conditions as the samples using a different concentration range (0.5–10 μg/mL, $IC_{50} = 6.8$ μg/mL). The percentage of antioxidant activity (%AA) was calculated according to the following equation:

$$\%AA = (AbsDPPH - Abssample)/AbsDPPH \times 100$$

AbsDPPH = the absorbance of the DPPH solution

Abssample = the absorbance of the DPPH solution containing the test compound.

The results were expressed as inhibitory concentration of 50% (IC_{50}). All experiments were performed in triplicate.

2.9.2 | ABTS Assay

The free radical scavenging activity of samples was determined by ABTS radical cation discoloration assay using the protocol reported by Cuffaro et al. (Cuffaro et al. 2023). Briefly, the ABTS solution was prepared mixing 7 mM of aqueous solution of ABTS with 2.45 mM potassium persulfate in a 1:1 ratio. The solution was incubated for 12 h in the dark at rt and, afterwards, diluted with ethanol to obtain an absorbance of 0.7 at 750 nm. 180 μL of ABTS solution were mixed with 10 μL of sample in ethanol and the solution was incubated 5 min at rt; then, the final absorbance was read at 734 nm. The % scavenging ability was calculated as follow:

$$\%\ \text{scavenging ability} = (AbsABTS - Abssample)/AbsABTS \times 100$$

AbsABTS = the absorbance of the ABTS solution

Abssample = the absorbance of the ABTS solution containing the test compound.

The percentage of scavenging ability was calculated against the sample concentration to obtain the inhibitory concentration at 50% (IC_{50}). Trolox was employed as positive control and was treated under the same conditions as the samples using a different concentration range (100–3 μg/mL, $IC_{50} = 13.4$ μg/mL). All experiments were performed in triplicate.

2.10 | Cell Culture and Treatment

Human neuroblastoma SH-SY5Y cells (A.T.C.C. Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, 1 mM glutamine, and 1% penicillin-streptomycin solution. Cell cultures were kept in a humidified atmosphere at 37°C, 5% CO_2, and grown until they reached 90% confluence. SH-SY5Y cells were plated on glass coverslips in 12-well plates at 150,000 cells/well density. Twenty-4 h after plating, the cells were treated with different concentrations of AW (5, 10, 20, and 25 μg/mL) for 24 h or with different concentrations of CGA (0.5 and 0.6 μg/mL, corresponding to the amount of CGA contained in 20 and 25 μg/mL of AW, respectively).

2.11 | Flow Cytometry Analysis of Cell Viability

Following the treatment with different AW concentrations (section 2.10), the cells were incubated with 1:1000 Live-or-Dye

Fixable Dead Cell dye 640/662 (Biotium, Inc. CA, USA) in fresh medium for 30 min at rt, according to the manufacturer's instructions. Cells were then harvested and washed with PBS. The BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit (BD Biosciences, Lake Franklin, NJ, USA) was used for cell fixation and permeabilization, according to the manufacturer's instructions. After the final washing step with 1× BD Perm/Wash buffer, the samples were analysed on a BD Accuri C6 flow cytometer (BD Biosciences, Lake Franklin, NJ, USA). Data were processed using the free Flowing software (Cell Imaging and Cytometry Core, Turku Bioscience Centre, Turku, Finland). The cells were identified by side-scattered (SSC) and forward-scattered (FSC) light. Cell viability was evaluated by the median fluorescence intensity (MFI) of the cellular population labeled with the fluorescent dye.

2.12 | Flow Cytometry Analysis of Intracellular Reactive Oxygen Species (ROS)

Following the treatment with different AW concentrations or with different CGA concentrations (Section 2.10), cells were exposed to 350 μM H_2O_2 for 2 h, to induce the production of Reactive Oxygen Species (ROS). Subsequently, the CellROX Green Reagent (ThermoFisher Scientific) was added at 5 μM for 30 min at 37°C, according to the manufacturer's instructions. Then, cells were harvested, washed with PBS, fixed and permeabilized using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution kit. After the final washing step with 1× BD Perm/Wash buffer, the samples were analysed on a BD Accuri C6 flow cytometer as described above. Intracellular ROS production was evaluated by the MFI of the cellular population labeled with the fluorescent dye.

2.13 | Immunocytochemistry on THP-1-Derived Macrophages

To assess the anti-inflammatory effect of AW, the protein expression of CD163, marker of the anti-inflammatory M2 phenotype, was evaluated by immunocytochemistry on THP-1 cells, previously differentiated to pro-inflammatory M1 macrophages (Gabbia et al. 2023). THP-1 cells were maintained in a RPMI complete medium, containing L-glutamine (1%), streptomycin/penicillin (1%) and 10% FBS, at 37°C with 5% CO_2 in humified atmosphere. To induce their differentiation into M1 macrophages, THP-1 cells were first seeded on glass coverslip into a 24-well plate (10^5 cells/mL) and treated with 320 nM PMA for 24 h (obtaining M0 macrophages) and then treated with 25 ng/mL LPS (Sigma Aldrich, USA) for 48 h. After the complete differentiation to pro-inflammatory M1 macrophages, they were treated with increasing concentrations of AW (5, 10, 20 and 25 μg/mL) or of CGA (0.5 and 0.6 μg/mL). After 24 h of incubation, cells were fixed with 4% PFA, PBS-washed and permeabilized for 5 min with 0.1% Triton X-100. Fixed cells were incubated with a rabbit anti-CD163 primary antibody (1:500 dilution) at 4°C overnight and then, with an AlexaFluor647 goat anti-rabbit IgG secondary antibody (1:200 dilution) in the dark for 1 h at rt. Hoechst33342 (1:400) was used as a nuclear counterstain. THP-1-derived M2 macrophages to be used as positive controls were obtained from M0 macrophages after 48-h-treatment with 25 ng/mL of IL4 and with 25 ng/mL of IL13 (Santa Cruz Biotechnology). The images were acquired using a Zeiss LSM800 confocal microscope and quantified with the Image J software. Mean fluorescence intensity was plotted and analyzed with the GraphPad Prism software ver. 10.2 (San Diego, CA, USA) by one-way ANOVA followed by the Newman Keuls post hoc test for multiple comparisons.

2.14 | Measurement of ROS in THP-1 Cells by Flow Cytometry

THP-1 cells were seeded on 6-well plate with a density of 10^5 cells/mL and treated with AW or CGA for 24 h. Then, cells were incubated with 2′−7′-Dichlorodihydrofluorescein diacetate (25 μM, DCFH-DA, Sigma Aldrich, USA) in the dark at 37°C. After 30 min, THP-1 cells were harvested, centrifuged for 5 min at 1500 × *g* and resuspended in BSA solution (1 mg/mL in PBS). Cells were further exposed to 1 mM H_2O_2 for 5 min and immediately analyzed by means of the flux cytometer BD FACSAria III Cell Sorter (BD Bioscience, San Jose, CA, USA). Intracellular ROS concentration was expressed as mean difluorescein fluorescence intensity (MFI) values.

2.15 | Statistical Analysis

Statistical analysis was performed using the KaleidaGraph software or GraphPad Prism software ver. 10.2. At last, 5000 cells were analyzed for each MFI dot. A p value lower than 0.05 was considered statistically significant. The single (*), double (**), and triple (***) asterisks refer to p-values lower than 0.05, 0.01, and 0.001.

3 | Results and Discussion

3.1 | Determination of Polyphenolic Compound of AW Extract by Colorimetric Detection

The AW extract was preliminarily analyzed using three different colorimetric assays: the FC method to determine the TPC, the TFC assay, and a recently published colorimetric method for quantifying CGA and its derivatives (TCGAC). The results, reported in Table 1, provide insights into the polyphenolic compound profile of the extract.

The TPC value of 169 μg/mg indicates a high presence of phenolic compounds in the extract, among them the TFC value of 65 μg/mg highlights the amount of flavonoids. Additionally, in our recently published work (Cuffaro et al. 2025), we developed

TABLE 1 | Colorimetric analysis of the main polyphenol (μg/mg) as TPC, TFC, and TCGAC of AW.

	μg/mg
TPC (μgGAE/mg)	169.4 ± 6.2
TFC (μgQE/mg)	65.0 ± 0.69
TCGAC (μgCGAE)	32.1 ± 3.0

Note: Values are expressed as mean ± standard deviation ($n = 3$, independent experiments).

a colorimetric method for the rapid and sustainable detection of CGA and its derivatives in the AW extract using alkaline Tris buffer (10 mM, pH 9). Applying this technique to the AW matrix we revealed that CGA derivatives collectively constitute approximately 32 μg/mg.

These results demonstrate that the extract contains a significant amount of polyphenols, including flavonoids and chlorogenic acids.

3.2 | Determination of Polyphenolic Compound of AW Extract by HPLC and MS Analysis

The extraction method for AW required a maceration process, using a mixture of EtOH and water, facilitating a high recovery of phenolic compounds. By HPLC analysis performed following a slightly modified method previously reported by Lavecchia (Lavecchia et al. 2019), we identified the main polyphenolic compounds present in AW extract (Figure 1), further confirmed by Q1 scan mass spectrometry analysis.

The quantification of phenolic composition of the AW extract, characterized by flavonoids and hydroxycinnamic acids, is reported in Table 2.

Among hydroxycinnamic acids, CGA (also known as 5-O-caffeoylquinic acid) is the most abundant polyphenol in the AW extract, with a concentration of 24.9 μg/mg, consistent with previous reports (Jiménez-Moreno et al. 2019; Pandino et al. 2011). 1,5-dicaffeoylquinic acid is present in lower amount (6.00 ± 0.95 μg/mg), while caffeic acid is poorly detected in the sample (0.863 μg/mg). The scarce presence of caffeic acid aligns with previous findings by Schütz et al. (Schütz et al. 2006), who attributed its formation to the hydrolysis of CGA and 1,5-dicaffeoylquinic acid that in AW extract are clearly present in the non-hydrolyzed form. Flavonoids in the extract are mainly represented by luteolin-7-rutinoside, the most abundant at 20.8 μg/mg, followed by luteolin-7-glucoside (14.5 ± 3.4 μg/mg), in accordance with literature data (Jiménez-Moreno et al. 2019; Pandino et al. 2011). Narirutin is present in lower concentrations (4.73 ± 0.47 μg/mg) compared to other flavonoids.

Further characterization of minor polyphenols (< 0.4 μg/mg) was performed using a previously validated LC-MS/MS method (Cuffaro et al. 2024). The results are presented in Table 3. The most abundant compounds in the waste extract were apigenin-7-glucoside, pinoresinol, and syringic acid, with concentrations slightly above 0.1 μg/mg. Tyrosol, hydroxytyrosol, verbascoside, vanillic acid, flavonoid glycoside rutin, and the hydroxycinnamic acid p-coumaric acid were detected at concentrations between 0.010 and 0.1 μg/mg, whereas ferulic acid and vanillin were only found in trace amounts (0.003 μg/mg).

TABLE 2 | HPLC analysis of the main polyphenol (μg/mg) of AW.

Polyphenols	μg/mg	λ max
Hydroxycinnamic acids		
CGA (5-O-caffeoylquinic acid)	24.9 ± 3.06[a]	330
Caffeic acid	0.863 ± 0.13[c]	280
1,5-dicaffeoylquinic acid	6.00 ± 0.95[c]	330
Flavonoids		
Luteolin- 7-O-glucoside	14.5 ± 3.4[b]	280
Luteolin-7-O-rutinoside	20.8 ± 3.1[a,b]	280
Narirutin	4.73 ± 0.47[c]	280

Note: Values are expressed as mean ± standard deviation ($n = 3$, independent experiments). Different letters ([a, b, c]), in the same column indicate significant differences between mean values ($p < 0.05$).

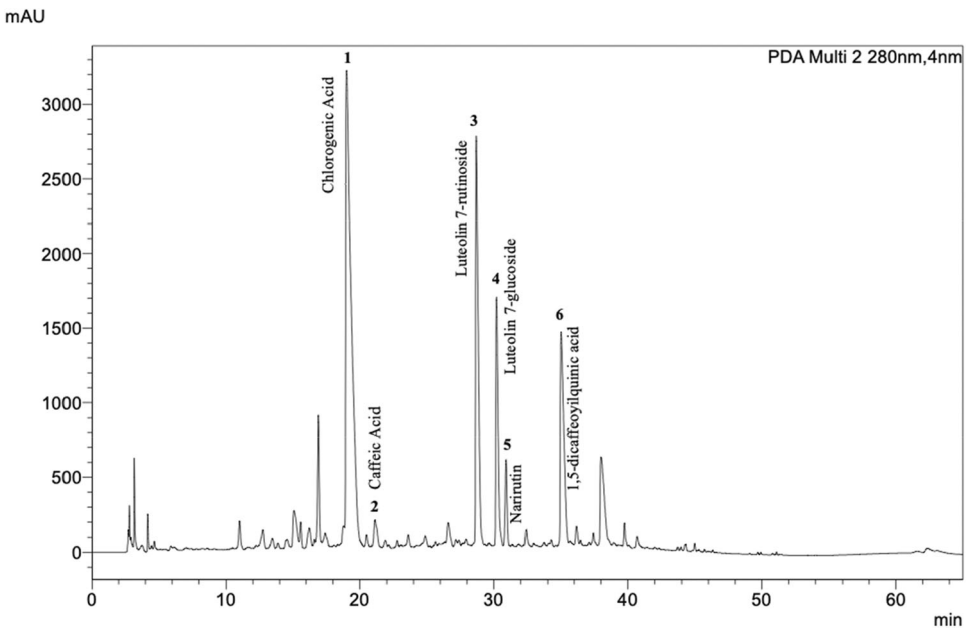

FIGURE 1 | HPLC chromatograms of AW. 1. CGA. 2. Caffeic acid. 3. Luteolin-7-rutinoside. 4. Luteolin-7-glucoside. 5. Narirutin. 6. 1,5-dicaffeoyilquinic acid. Values are expressed as mean ± standard deviation ($n = 3$, independent experiments).

TABLE 3 | Quantification of minor polyphenols using LC-MS/MS.

Polyphenols	μg/mg
Tyrosol	0.053 ± 0.002
Syringic acid	0.148 ± 0.005
p-Coumaric acid	0.039 ± 0.001
Ferulic acid	0.003 ± 0.001
Vanillic acid	0.024 ± 0.001
Vanillin	0.003 ± 0.001
Verbascoside	0.052 ± 0.001
Pinoresinol	0.323 ± 0.002
Apigenin-7-glucoside	0.128 ± 0.006
Rutin	0.052 ± 0.002
Hydroxytyrosol	0.063 ± 0.002

Note: Values are expressed as mean ± standard deviation ($n = 3$, independent experiments).

TABLE 4 | Antioxidant/antiradical activity of AW and CGA expressed as IC_{50}.

Sample	DPPH (mg/mL)	ABTS (mg/mL)
AW	0.14 ± 1.0	0.073 ± 1.3
CGA	0.006 ± 0.004	0.012 ± 0.007
Positive control		
Trolox	0.007 ± 0.001	0.003 ± 0.0004

Note: Values are expressed as mean ± standard error ($n = 3$ independent experiments).

3.3 | Evaluation of Antiradical Properties

Chronic oxidative stress has been implicated in the development and progression of various diseases, including cardiovascular diseases, neurodegenerative disorders, diabetes, and cancer. The inflammation resulting from oxidative stress is a major contributor to the pathogenesis of these chronic diseases. In this context, polyphenolic compounds, abundant in fruits, vegetables, and certain plant-based extracts, have attracted attention for their dual antioxidant and anti-inflammatory properties. Polyphenols are known to act as potent antioxidants by scavenging free radicals, thereby reducing oxidative damage, exhibiting a multitude of biological and pharmacological activities. (H. Zhang and Tsao 2016). In particular CGAs is able to reduce oxidative stress and the associated harmful effects resulting from an imbalanced intracellular redox state (Liang and Kitts 2016). Additionally, the phenolic compounds, and specifically CGA, are thought to play a protective role in the central nervous system against oxidative and excitotoxic stress, conditions that can contribute to the onset of neurodegenerative disorders, including Alzheimer's, Parkinson's and Huntington's diseases (Althagafi 2024; Yan et al. 2022; Ye et al. 2025; Zhang and Wang 2024). Therefore, with the aim to explore the neuroprotective application for AW, in this study, we investigated the antioxidant potential of AW, rich in flavonoids and hydroxycinnamic acids. Based on the chemical reactions involved, various assays are usually employed to estimate the antioxidant capacities of phytochemicals, such as ABTS and DPPH assays. In this study, the antioxidant activity of the AW extract was assessed using both assays, and data are presented in Table 4.

The AW extract exhibited significant antioxidant activity in both DPPH and ABTS assays, as evidenced by AW IC_{50DPPH} values of 0.14 ± 1.0 mg/mL and IC_{50ABTS} 0.073 ± 1.3 mg/mL, respectively, consistent with already reported data (Mejri et al. 2020). Moreover, AW extract demonstrated a higher radical scavenging activity compare to other similar extracts of various parts of Greek artichoke, as reported by Kollia et al. (Kollia et al. 2017), that showed DPPH scavenging activities with IC_{50} values of 1.13 mg/mL for bracts and 1.17 and 1.18 mg/mL for heads and floral stems, respectively. Furthermore, the antioxidant activity of our extract exceeds the ones reported by Carpentieri et al. (Carpentieri et al. 2022) for artichoke extract, which indicated IC_{50} values of 2.4 ± 0.5 mg/mL and 2.0 ± 0.2 mg/mL for DPPH and ABTS, respectively. Comparatively, CGA, the phenolic compound most abundant in artichoke waste, exhibited lower IC_{50} DPPH values of 0.006 ± 0.004 mg/mL and IC_{50} ABTS 0.012 ± 0.007 mg/mL in the DPPH and ABTS assays, compared to AW extract. This is consistent with previous studies demonstrating the strong antioxidant activity of CGA. The superior antioxidant potency of CGA compared to AW extract underscores its significance as a key compound to understand its influence in the AW activity in the following cell studies here reported. Moreover, it is important to note that AW extract represents a natural and sustainable source of antioxidants, produced through a simple maceration process that avoids the costly and complex purification.

3.4 | AW Shows Antioxidant Properties on Human Neuroblastoma Cells

In a recent study, the neuroprotective effects of CGA were investigated (Gao et al. 2021). A cell model consisting of human neuroblastoma SH-SY5Y cells exposed to H_2O_2 was selected to study neuronal cell death induced by oxidative stress and to explore the neuroprotective properties of CGA. In fact, the SH-SY5Y neuroblastoma cell line is a largely immortalized cell line and is widely used in in vitro models of neurological disorders, such as Alzheimer's disease (AD), neurotoxicity, ischemia, and amyotrophic lateral sclerosis (ALS). Importantly, CGA alleviated morphological alterations and decreased the incidence of cell apoptosis in a dose- and time-dependent manner following H_2O_2-induced injury. Moreover, several polyphenolic compounds, including CGA, proved to be able to across the blood-brain barrier, which is essential for their neuroprotective effects (Almeida et al. 2016; Ito et al. 2008). Following the observation that AW showed interesting antioxidant activity both with DPPH and ABTS assays, we extended our analysis to a cellular system. Specifically, we investigated the effect of AW on SH-SY5Y cells, the same model used for CGA, which is widely employed as a neuronal model for studying neurotoxicity and neuroprotection related to neurodegenerative disorders (D'Aloia et al. 2024; Xie et al. 2010). First, we tested different concentrations of AW, ranging from 5 to 25 μg/mL, to assess the effect of the extract on the viability. After 24 h treatment, cells were incubated with the Live-or-Dye Fixable Dead Cell dye 640/662 and analysed with flow cytometry (Figure 2A). The cells

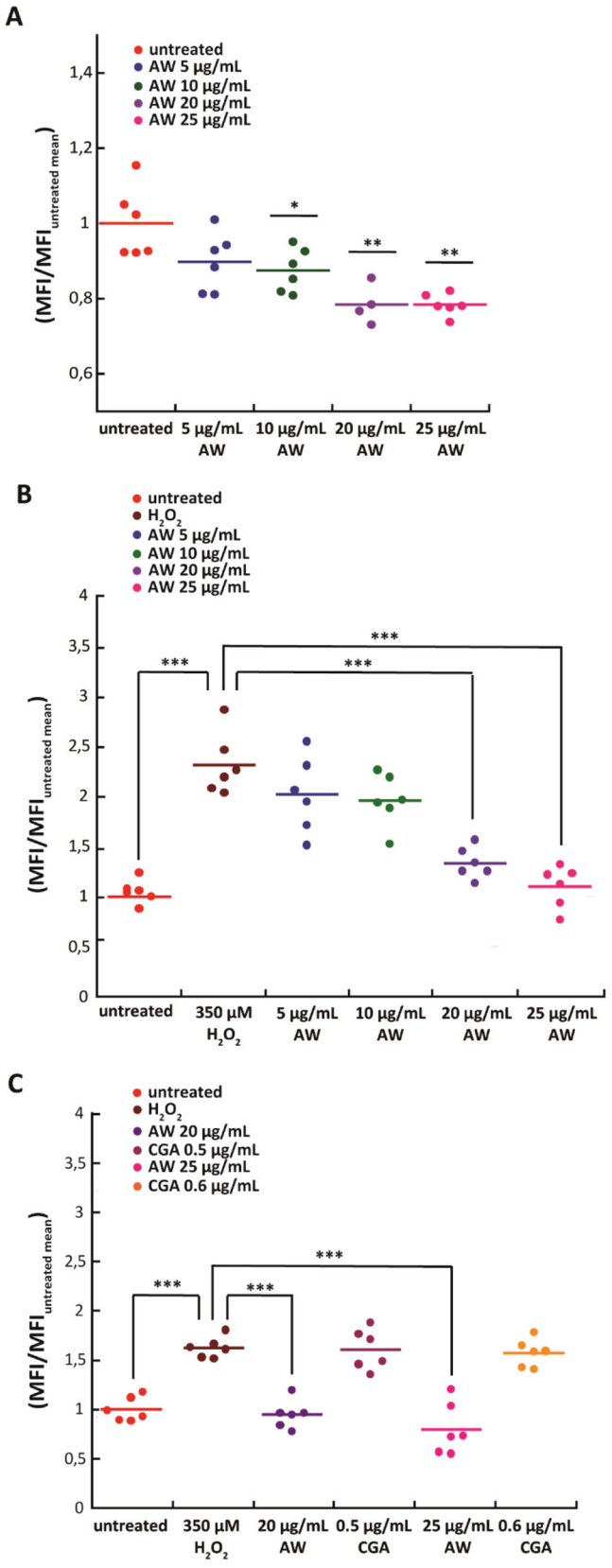

FIGURE 2 | Effect of AW treatment on cell viability and ROS production. (A) Flow cytometry analysis of cell viability of samples treated with different concentrations of AW (5, 10, 20, and 25 µg/mL) for 24 h and then stained with the Live-or-Dye Fixable Dead Cell dye 640/662, fixed, permeabilized and analysed through flow cytometry. > 5000 cells were analyzed for each MFI dot. Student's *t*-test $*p \leq 0.05$, $**p \leq 0.01$ with respect to untreated. (B, C) Flow cytometry analysis of ROS production in cells pretreated (B) with different concentrations of AW (5, 10, 20, and 25 µg/mL) or (C) with 20 and 25 µg/mL AW or with 0.5 and 0.6 µg/mL CGA for 24 h, and then incubated with 350 µM H_2O_2 for 2 h. Samples were then stained with the CellROX Green Reagent before flow cytometry analysis, fixed, permeabilized and analysed through flow cytometry. In all cases, $MFI/MFI_{untreated\ mean}$ values were obtained by dividing the MFI of a distribution by the mean MFI obtained from the untreated. > 5000 cells were analyzed for each MFI dot. Student's *t*-test $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

were discriminated from debris by FSC and SSC scatter gating, and the MFI values used to compare the data. The changes in fluorescence intensity distribution were quantified by normalizing the MFI values of treated and untreated samples to the mean MFI value from the untreated sample ($MFI/MFI_{untreated\ mean}$). The graph shows that the viability of cells treated with AW increases along with the increase of AW concentration, even statistically significantly already at 10 µg/mL, suggesting that the extract exerts a beneficial effect on the cells.

Subsequently, an evaluation on AW ability to protect cells from oxidative stress was conducted. The in vitro oxidative stress was induced with H_2O_2. Cells were treated with the above-mentioned AW concentrations for 24 h and then incubated with 350 µM H_2O_2 for 2 h. As a positive control of oxidative stress, a sample of cells was incubated solely with H_2O_2. The samples were then incubated with the CellROX Green Reagent and analysed with flow cytometry similarly as reported above (Figure 2B). Treatment with H_2O_2 significantly increased ROS production by ca. 2.4 fold with respect to the untreated sample ($p \leq 0.001$). However, cells pretreated with AW showed a reduction in ROS production in a dose dependent manner compared to H_2O_2 only, which became statistically significant at 20 and 25 µg/mL of AW (Figure 2B). These results corroborated the antioxidant properties of AW extract, additionally proving the ability of AW to reduce intracellular ROS production. Interestingly, the total ROS production observed at the highest AW concentration (i.e., 25 µg/mL) was similar to that in the untreated cells, suggesting that the original physiological environment was fully preserved (Figure 2B).

To establish if the positive effect of AW was attributable mostly to the already demonstrated antioxidant effect of CGA contained in AW (Gao et al. 2021), we performed a similar experiment in which cells were pretreated with 0.5 and 0.6 µg/mL of CGA, which correspond to the CGA in 20 and 25 µg/mL of AW extract, respectively. Flow cytometry results indicate that the pretreatment with both CGA concentrations did not prevent ROS production unlike the corresponding AW concentrations (Figure 2C). These results demonstrate that the antioxidant effect of AW cannot be attributed solely to the presence of CGA; rather, it is due to the copresence of diverse and essential polyphenols. After all, the synergistic interactions of dietary phytochemical combinations are amply demonstrated and several papers highlighting the synergistic antioxidant effects of phenolic and flavonoid compounds (X. Chen et al. 2022; Hajimehdipoor et al. 2014; Li et al. 2022).

3.5 | AW Shows Antioxidant Properties on THP-1 Monocytes

Elevated ROS production in circulating monocytes has been observed in different diseases, (Kim et al. 2010a; Restaino et al. 2017). More interestingly, this increase has been associated to ROS release in neuronal pathways, known to be pivotal in neurodegenerative processes (Balon and Wiatrak 2021; Klegeris et al. 2008; Owona et al. 2024; Wiatrak et al. 2022). To further investigate the beneficial properties of AW on the immune response, its effect on monocytes was evaluated in the THP-1 cell line. These cells were chosen due to their relevance in studying immune responses in the CNS during neurodegeneration. Accordingly, they have been widely used to investigate neuroinflammatory processes and oxidative stress in neurodegenerative diseases (Jin et al. 2024). Furthermore, they are easy to handle and could be easily differentiated into different macrophagic phenotypes involved in inflammatory-related diseases (Chanput et al. 2014).

Notably, an increase of ROS production in circulating monocytes has been observed in different diseases, (Kim et al. 2010b; Restaino et al. 2017) but more interesting it has been associated to ROS release in neurological pathways (Balon and Wiatrak 2021; Klegeris et al. 2008; Owona et al. 2024; Wiatrak et al. 2022). Therefore, to deepen the antioxidant characteristics of the AW, its effect on monocytes was evaluated by measuring ROS production in THP-1 cells stimulated with H_2O_2.

As reported in Figure 3, AW pretreatment was able to reduce significantly the ROS intracellular overload at all tested concentration. At variance with what previously observed in neuroblastoma cells, a similar effect was observed also after pretreatment with CGA, indicating that CGA may be pivotal for the antioxidant effect exerted by AW in these cells. This differential effect of CGA and AW extract observed in neuroblastoma cells and THP-1 monocytes is likely to suggest a cell-specific mechanism of action and it might be attributed to the presence of other compounds in AW extract that may synergize with CGA in neuronal cells. This phenomenon has already been demonstrated for other plant extracts (Daglia et al. 2014). Further investigation into these cell-specific responses could be useful for elucidating these targeted effects of AW extract and CGA in neurodegeneration.

3.6 | AW Modulates Inflammation by Polarizing THP-1-Derived Pro-Inflammatory M1 Macrophages Towards and Anti-Inflammatory Phenotype

Chronic inflammation is a fundamental cause of many (Zhang and Tsao 2016) chronic conditions, including degenerative diseases. The anti-inflammatory effects of dietary polyphenols are attributed to their role as antioxidants and their interference with oxidative stress signaling, but also to their suppression of pro-inflammatory signaling pathways (Zhang and Tsao 2016). Moreover the role of brain macrophages and their phenotypic changes in the health and disease of the central nervous system is gaining increasing attention (Silvin et al. 2023). Therefore, the anti-inflammatory properties of AW were tested on THP-1-derived macrophages, after their differentiation to the pro-inflammatory M1 phenotype, by assessing its effect on the switch towards the anti-inflammatory M2 phenotype, characterized by an increase of the surface marker CD163. As reported in Figure 4, AW significantly increased the expression of CD163 with respect to untreated control cells ($p < 0.001$) at the highest concentration tested, suggesting a possible anti-inflammatory effect of AW.

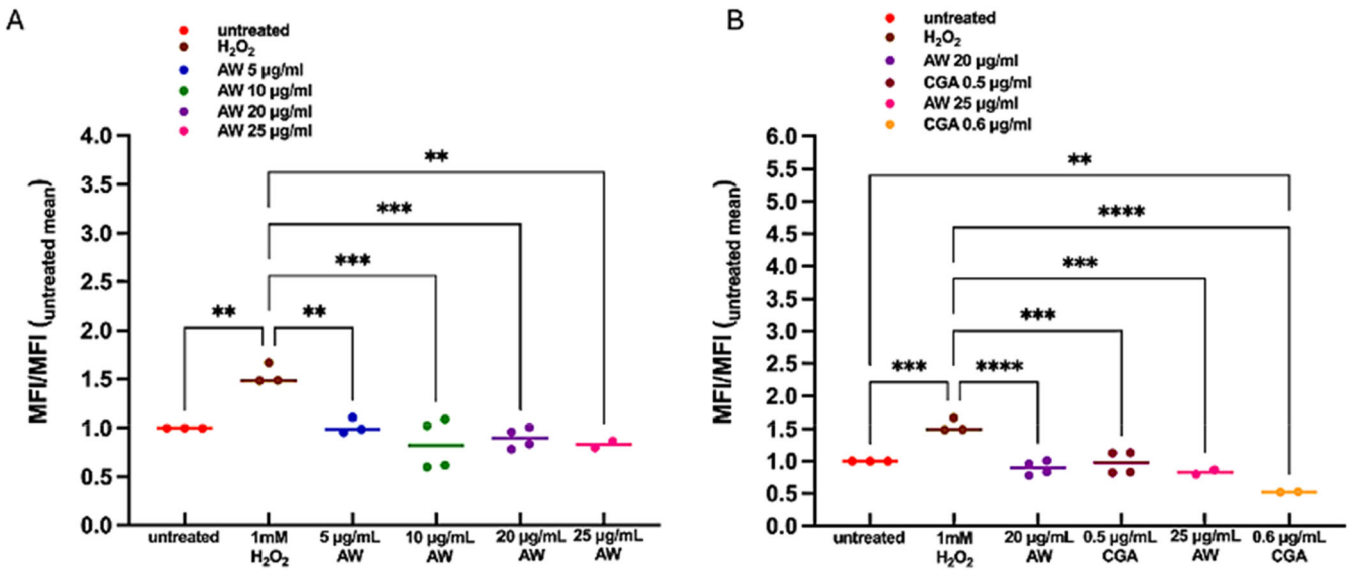

FIGURE 3 | Effect of AW treatment on ROS production in THP-1 monocytes. Flow cytometry analysis of ROS production in THP-1 cells pretreated for 24 h with different concentrations of AW (5, 10, 20, and 25 µg/mL, (A) or with 20 and 25 µg/mL AW or with 0.5 and 0.6 µg/mL CGA (B) and then incubated with 1 mM H_2O_2 for 30 min. In all cases, $MFI/MFI_{untreated\ mean}$ values were obtained by dividing the MFI of a distribution by the mean MFI obtained from the untreated. At least 5000 cells were analysed for each MFI dot. MFI: mean fluorescence intensity. Data are reported as mean ± SEM of independent experiments, each reported as single dot in the graph and performed in triplicate. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$ versus untreated cells.

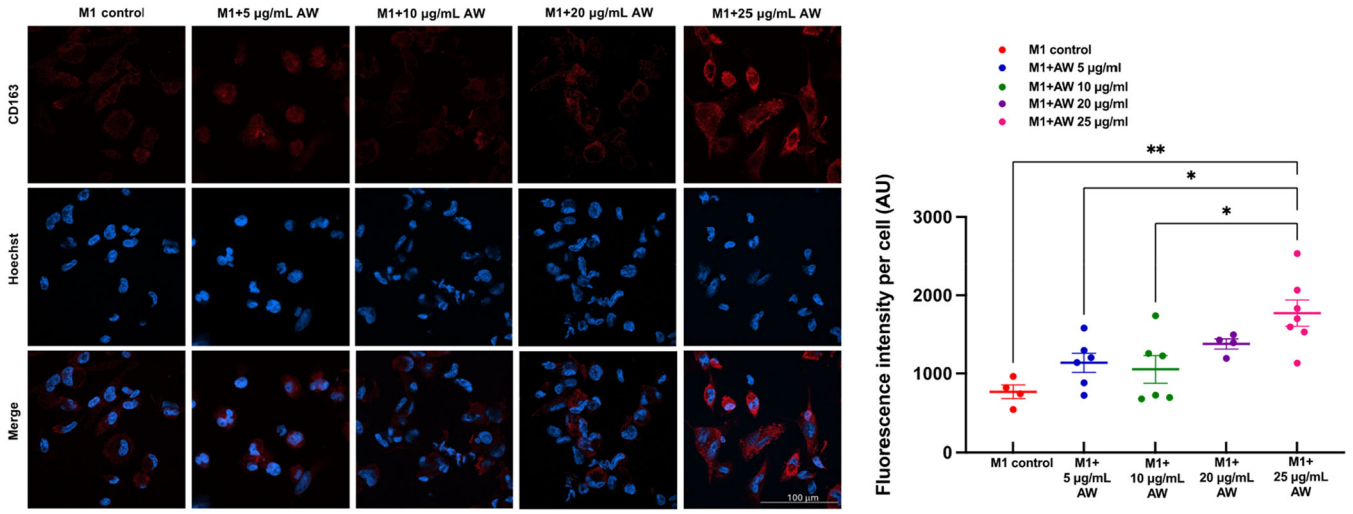

FIGURE 4 | Effect of AW on macrophage polarization. In vitro effect of AW exposure on THP-1-derived M1 macrophages, assessed as variation of CD163 protein expression by the ICC. Confocal images of THP-1-derived M1 macrophage treated for 24 h with increasing concentration of AW (5, 10, 20, and 25 μg/mL) stained with Hoechst 33342 (nuclei, blue) and anti-CD163 (red). Quantification of CD163 protein expression is reported as AU (arbitrary unit) of fluorescence intensity per cell. Data are reported as mean ± SEM of independent experiments, each reported as single dot in the graph and performed in triplicate. *$p < 0.05$, **$p < 0.01$ versus M1 untreated control cells.

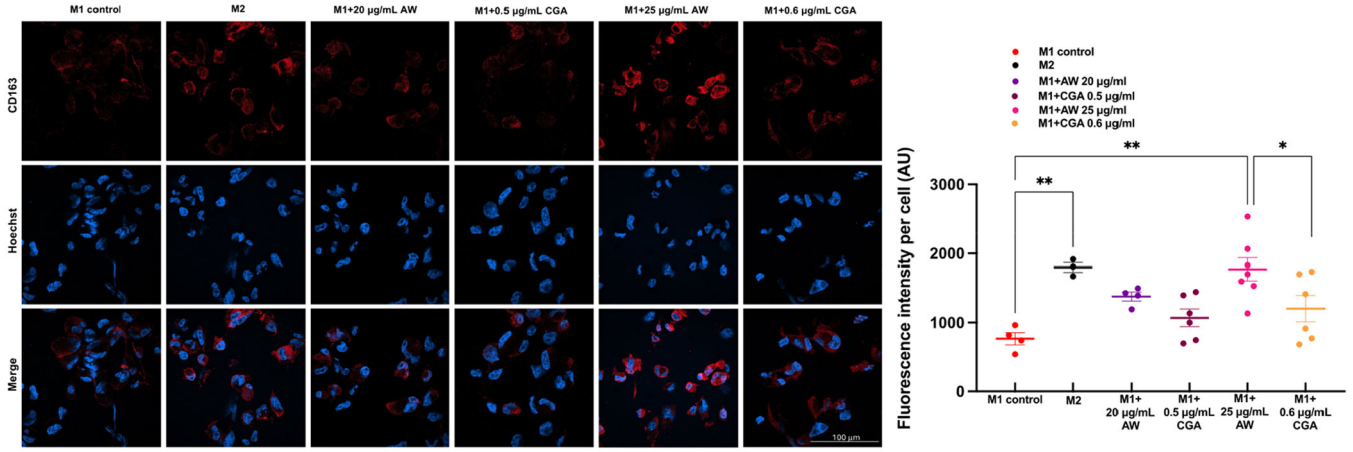

FIGURE 5 | Effect of AW and GCA on macrophage polarization. In vitro effect of AW exposure on THP-1-derived M1 macrophages, assessed as variation of CD163 protein expression by the ICC. Confocal images of THP-1-derived M1 macrophage treated for 24 h with 20 and 25 μg/mL AW or with 0.5 and 0.6 μg/mL CGA for 24 h stained with Hoechst 33342 (nuclei, blue) and anti-CD163 (red). Quantification of CD163 protein expression is reported as AU (arbitrary unit) of fluorescence intensity per cell. Data are reported as mean ± SEM of independent experiments, each reported as single dot in the graph and performed in triplicate. *$p < 0.05$, **$p < 0.01$ versus M1 untreated control cells.

Interestingly, the highest tested concentration of AW (25 μg/mL) significantly enhanced the expression of CD163 to levels similar to those of anti-inflammatory M2 macrophages, further confirming the M1 to M2 shift and the anti-inflammatory effect of AW (Figure 5). Moreover, this effect was not observed when M1 macrophages were treated with the same concentration of CGA alone, suggesting that the observed anti-inflammatory effect is likely attributable to the sum of different bioactive compounds present in AW, rather than only to CGA.

Further investigations are warranted to unravel the possible mechanisms of action of the AW extracts, that are likely to act on multiple cell pathways involved in neuroinflammation. An in vitro study investigating the anti-inflammatory properties of CGA in murine RAW 264.7 macrophages observed that it is able to inhibit cyclooxygenase-2 (COX-2) expression induced by LPS, decrease the activation of two key inflammatory signaling pathways, nuclear factor kappa B (NF-κB) and c-Jun N-terminal kinase/activator protein-1 (JNK/AP-1) and reducing pro-inflammatory cytokine production (Shan et al. 2009). Indeed, NF-κB plays a central role in neuroinflammation (Fornari Laurindo et al. 2023).

Another potential pathway through which AW may exert its effects could be the modulation of the Nrf2 signaling cascades. The Nrf2 pathway is crucial for cellular defense against oxidative stress and age-related neurodegenerative disorders (Fornari Laurindo et al. 2023). Recent studies have shown that polyphenols can activate Nrf2, leading to increased expression of antioxidant enzymes (Nabavi et al. 2016; Siswanto et al. 2022; Tang et al. 2025). Future studies should examine

how AW extract influences these pathways and their downstream targets. Additionally, exploring specific markers of M2 microglial polarization, could provide insights into AW effect on promoting anti-inflammatory phenotype in microglia (Orihuela et al. 2016).

4 | Conclusions

In conclusion, the data presented in this study highlight the neuroprotective effect of the AW extract, demonstrating its antioxidant and anti-inflammatory properties. The detailed analysis of AW extract using HPLC and LC-MS/MS has revealed a significant polyphenolic profile, recovering a variety of phenolic compounds with CGA being the predominant component. A preliminary screening of AW antiradical/antioxidant capacity demonstrated considerable free radical scavenging abilities as shown by its IC_{50} values in both DPPH and ABTS assays. Moreover, AW extract effectively enhanced cell viability and reduces oxidative stress in neuroblastoma cells, with significant reductions in ROS production observed at 25 μg/mL. This antioxidant effect of AW was even more marked compared to that of CGA at similar AW concentration, indicating a potential synergistic impact of the various polyphenolic compounds present in AW. Additionally, the antioxidant and antinflammatory activity exerted by AW extract in THP-1 monocytes and macrophages underscores its broader therapeutic potential in neuroprotection, since the detrimental role of microglia and macrophages pro-inflammatory activation in neurodegenerative disorders is well outlined (Mammana et al. 2018). It is important to note that these findings represent preliminary results, and further research is needed to fully elucidate the therapeutic potential of AW extracts. A key consideration is the physiological relevance of the concentrations used. Since significant effects were observed at 25 μg/mL of AW extract, it is crucial to investigate whether similar concentrations can be achieved in vivo, thus further investigations should focus on the bioavailability and pharmacokinetics of AW extract and its components in animal models and eventually in humans (Chen et al. 2024). However, data are available regarding the pharmacokinetics and bioavailability of CGA, including its distribution inside the brain. Notably, a study performed in rats (Kumar et al. 2019) reported plasma and brain concentrations much higher to those used in our study, after the administration of an intravenous 10 mg/kg dose. Moreover, it should be noticed that the oral bioavailability of CGA is not optimal and extremely variable, since it is strictly related to microbiome metabolism (Gonthier et al. 2003). Several technological approaches have been proposed to enhance CGA bioavailability, including liposomes (J. Zhang et al. 2024), and phytovesicles (Trivedi and Puranik 2023). This suggests that the CGA-dependent beneficial effects we observed in vitro can be recapitulated in vivo, with appropriate formulations. However, we cannot exclude the possibility that other components of the AW extract, beside exerting beneficial effects on their own, can impact on CGA pharmacokinetics, as already observed for other extracts (Qi et al. 2011), indicating that further studies are needed to obtain a full picture of the pharmacodynamics of AW extract in animal models and in humans after oral administration. Additionally, dose–response studies and long-term safety assessments will be essential to establish the optimal therapeutic concentrations and potential side effects.

The results suggest that AW's diverse bioactive components collectively offer a promising avenue for mitigating oxidative stress and inflammation associated with neurodegenerative and metabolic disorders. This study highlights the untapped potential of agricultural waste as a source of bioactive compounds. Moreover, the valorization of AW as a sustainable resource aligns with the growing interest in green chemistry and the circular economy, where agricultural by-products are repurposed for their health-promoting potential. The bioactive compounds derived from AW could pave the way for new therapeutic strategies, particularly in the field of neurodegenerative diseases, which remain a significant challenge in modern medicine.

Future research should focus on exploring the limitations of this study by investigating various fundamental aspects. Specifically, the molecular and cellular mechanisms underlying the effects of AW extract should be examined, including the activation of antioxidant enzymes, inhibition of pro-inflammatory cytokines, and modulation of specific signaling pathways (e.g., NF-κB, MAPK). These investigations could provide deeper insights into its neuroprotective potential. The next step would involve evaluating the bioavailability of polyphenols and developing appropriate delivery systems, such as oral formulations, to enhance the bioavailability and effectiveness of the active compounds. Additionally, in vivo studies are necessary to assess the safety and efficacy of AW extract, particularly in animal models of neurodegenerative diseases (e.g., Alzheimer's, Parkinson's) or neuroinflammation, to confirm its neuroprotective effects. By addressing these limitations and pursuing these future directions, researchers can gain a more comprehensive understanding of the full potential of AW extract as a source of bioactive compounds and its potential clinical applications.

Author Contributions

Doretta Cuffaro: conceptualization, data curation, methodology, formal analysis, validation, investigation, and writing – original draft. **Costanza Ceni:** data curation, formal analysis, methodology, investigation and writing-original draft. **Capitini Claudia:** data curation, formal analysis, methodology, investigation, validation and writing –original draft. **Daniela Gabbia:** data curation, formal analysis, methodology, investigation, validation and writing – original draft. **Ilaria Zanotto:** data curation, formal analysis, methodology and investigation. **Sara De Martin:** data curation, validation, supervision and writing – review and editing. **Massimiliano Mirabeni:** data curation, formal analysis, methodology and investigation. **Simone Bertini:** supervision and writing – review and editing. **Andrea Bertolini:** methodology, investigation, data curation, formal analysis, validation and writing – original draft. **Alessandro Saba:** supervision and writing – review and editing. **Martino Calamai:** supervision and writing – review and editing. **Maria Digiacomo:** conceptualization, data curation, methodology, validation, investigation, supervision and writing – review and editing. **Marco Macchia:** conceptualization, funding acquisition, supervision and writing – review and editing. All authors have read and approved the final manuscript.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data sets analyzed during the current study are available from the corresponding authors upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.