



Anti-inflammatory and prolonged protective effects of *Artemisia herba-alba* extracts via glutathione metabolism reinforcement

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ABSTRACT

Artemisia herba-alba (AHA) is a medicinal plant known for its preventive capacities against chronic diseases. Inflammation is a naturally occurring cellular response to noxious stimuli, and both acute and chronic inflammation are involved in human disorders. Preventing and limiting the negative impact of inflammation by using phytochemicals has attracted a lot of attention. Here, we show that AHA extracts are potent anti-inflammatory products at low doses in white blood cells, treated with phorbol myristate acetate (PMA), a pro-inflammatory trigger. Regardless of the mode of extraction, i.e. maceration or microwave, all extracts reduced on dose-dependent manner reactive oxygen species (ROS) production induced by the pro-inflammatory compound PMA in white blood cells and leukemic Jurkat cell line. The induction of the anti-inflammatory effect was evaluated by the measurement of glutathione metabolism (glutathione reductase, transferase, and peroxidase), and protein-free thiols in Jurkat cells. Jurkat cells incubated with low to high concentrated AHA extracts, ranged from 10 to 100 g/mL, induced a fast cell response measured at one and six hours. Interestingly, extracts from the microwave showed a stronger induction, 5 to 10 fold changes at early time points, and maintained over 72 h. On the other hand, AHA extracts showed a moderate anti-proliferative effect in the leukemic Jurkat line. We concluded that AHA extracts enhance directly anti-inflammatory cell response, earlier via ROS induction. Later, however, vigorously maintained, independently from ROS, suggesting the intervention of the cellular metabolism in enhancing these effects. Considering the preventive and anti-inflammatory potential of AHA, regular consumption is advisable, and phytotherapeutic applications are encouraged to treat local inflammations.

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1. Introduction

Medicinal plants are essential resources for traditional medicine in treating pathological disorders, bacterial and fungal infections. Regular consumption of some medicinal plants is associated with a low risk for developing chronic disease, suggesting prophylactic and protective effects against aging and organ failures (Petrovska, 2012). Most bioactive phytoconstituents mediate protective mechanisms by specific molecular pathways (Craig, 1999). However, the benefit of medicinal plants was determined empirically and little is known about their mechanisms of action.

The genus *Artemisia* L. englobes small herbs and shrubs that belong to the important family Asteraceae and occurs throughout the northern half of the world (Quezel and Santa, 1962). The genus *Artemisia* encompasses more than 500 species presenting a huge phytochemical variability (Amri et al., 2013). From a large number of *Artemisia* species, produced essential oils are used in the cosmetics and pharmaceutical industry, with a profitable economic value. *Artemisia herba-alba* is widely distributed in the Mediterranean area, growing in semi-arid regions. The plant *Artemisia herba-alba* is employed in different forms to treat diseases based on empirical experiences and transmitted local knowledge. The essential oil extracted by distillation from the dried leaves and the thin branches of the plant are extensively used in traditional medicine, treating bronchitis, and local skin infections. Infusions of *Artemisia herba-alba* treat cough, intestinal pain, diabetes, and inflammations (Khelifi et al., 2013). The chemical composition and the fingerprint of *Artemisia herba-alba* may vary. Enriched bioactive single compounds are

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mainly volatile molecules. Among the dominant volatile constituents are the monoterpenes including two diastereomeric forms of thujone, 1–8 cineole and camphor (Younsi et al., 2018). Some reports investigated the composition of dominant compounds and analyzed a number of polyphenols, considered as the main antioxidants against reactive oxygen species (ROS) in organisms. These reports indicated a high enrichment for polyphenols as measured by different assays (Khlifi et al., 2013; Said Mel et al., 2016; Younsi et al., 2018). To characterize experimentally the biological properties of *Artemisia herba-alba* (AHA), reports focused on antibacterial and antifungal actions (Abu-Darwish et al., 2015; Saleh et al., 2006). Others demonstrated a reduction in pro-inflammatory cytokine productions (Messaoudene et al., 2011). However, it remains unclear whether the beneficial physiological properties of AHA extracts were associated only with the quantity and the number of polyphenols, and flavonoids present, which possess the scavenging potential that is seen *in vitro*, or/and with the functional modulation of molecular pathways, which safeguards cellular homeostasis in a more complex action.

Phenolics and flavonoids are ubiquitous in plants and possess antioxidant activities *in vitro*. They are obtained exclusively through the diet; their protective effect is direct and can neutralize regularly ROS by a high intake of rich foods of fruits and vegetables (Rice-Evans et al., 1995). Glutathione (GSH), however, is an intracellular antioxidant, responsible for cellular homeostasis (Ostergaard et al., 2004). GSH is synthesized by the rate enzyme gamma-glutamylcysteinylase and is maintained in the reduced form by glutathione reductase through regeneration of oxidized glutathione (GSSG). Glutathione is a cofactor for multiples detoxifying enzymes such as glutathione transferase (GST) and glutathione peroxidases (GPx). Both enzymes transfer GSH moiety to block reactive chemical groups. Therefore, it is well established that glutathione is the major redox buffer in the cell. Many human diseases are associated with phases of acute or chronic inflammatory episodes, which can lead to irreversible organ damages. The severity of the syndrome is proportional to ROS level and cell death occurring in the tissues. Indeed, increased ROS occurs early in such syndromes, and likely preclinical usage of antioxidants reduced inflammation and tissue injury. Of note is that the direct antioxidant activity of some compounds is associated with conflicting results (Booth et al., 2011). Hence, external modulation of GSH level became one of the chemopreventive strategies to reduce inflammation-mediated damage (Pereira et al., 2013).

The objective of our work is the molecular characterization of the anti-inflammatory effects of AHA extracts through the maintenance of the redox balance against pro-inflammatory triggers. We hypothesized that endogenous upregulation of glutathione metabolism by AHA extracts blocks PMA mediated inflammation, which is more relevant from the physiological point of view. Especially in the case of regular consumptions aiming for chemopreventive or therapeutic usage of AHA. Our work describes the modulation of glutathione metabolism and ROS level by AHA extracts, and provides a piece of clear evidence for the chemopreventive role of AHA against inflammation by redox buffering.

2. Materials and methods

2.1. Plant material

Artemisia herba-alba samples were collected from the region of Bejaia (located in the east of Algeria) in August 2017. The taxonomic identity of the plant was confirmed by comparing voucher specimen with a known identity already deposited in the herbarium (N° 02888). The species was identified by Dr. Hadjadj-Aoul Seghir and stored as a voucher specimen in the plant Herbarium of the department of Biology, University of Oran 1 Ahmed Ben Bella, Algeria. The harvested fresh aerial parts including stems plus leaves with no flowers were rinsed with distilled water, then oven-dried for 72 h at 40 °

C, the powder was passed through standard 250 μm sieve and only fraction with particle size < 250 μm was used. The powder was stored in amber glass jars until use. The extraction was performed using solvents with different polarity: distilled water, methanol/water (80:20, v/v), ethanol/water (80:20, v/v), acetone/water (80:20, v/v) and a mixture of methanol, ethanol, acetone and water (25:25:25:25, v/v/v/v).

2.2. Microwave assisted extraction

A modified microwave oven was used to perform the extraction under constant sample volume. The modified microwave (NN-S674MF, Samsung, Malaysia) with cavity dimensions of 22.5 cm $\times$ 37.5 cm $\times$ 38.6 cm was equipped with a digital control system for irradiation at 2450 kHz working frequency and microwave power (the latter linearly adjustable from 200 to 1000 W). Six grams of powder were added to 120 mL of 80% (v/v) extraction solvent. The mixture was heated using 200 W power for 25 min. Extracts were concentrated by rotary evaporation under vacuum until solidification and stored at $-80\text{ }^{\circ}\text{C}$. All stock solutions were prepared freshly before use at 10 mg/mL of each AHA extract in DMSO.

2.3. Conventional solvent extraction

For the conventional extraction, six grams of sample powder were placed in a volume of 120 mL of 80% (v/v) extraction solvent or a mixture of equal volumes of solvents. The mixture was subjected to magnetic stirring for 24 h at $10\text{ }^{\circ}\text{C}$ in the darkroom. Extracts were concentrated by rotary evaporation under vacuum and stored at $-80\text{ }^{\circ}\text{C}$. Except for total phenolic content measurements, all stock solutions were prepared freshly before use at 10 mg/mL of each AHA extract in DMSO.

2.4. Total phenolic contents (TPC)

Total phenolic contents were determined according to the Folin-Ciocalteu assay (Folin, 1929; Folin and Denis, 1912). The working solutions were obtained by the dilution of the stock solutions at 1:50 in DMSO. The final concentrations were 200 $\mu\text{g/mL}$.

In brief, 2 mL of working solutions from different *Artemisia herba-alba* extracts were mixed with 10 mL deionized water, 1 mL of Folin-Ciocalteu reagent (Carlo Erba Reagents, Val-de Reuil, France), and completed with 12 mL sodium carbonate of 1.5 M. After incubation at $25\text{ }^{\circ}\text{C}$ for 30 min, the absorbance of the samples was measured at 740 nm using a UV-Visible spectrophotometer. A standard calibration curve of gallic acid was performed ($y = 4.394x + 0.021$; $R^2 = 0.9979$). All analyses were performed in triplicate, and TPC were expressed as milligram of gallic acid equivalents per gram of dried extract (mg GAE/g).

2.5. Determination of free radical scavenging activity

The antioxidant activity of *Artemisia herba-alba* extracts was measured by bleaching of the purple-colored solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) following a previously reported method (Meda et al., 2017). A solution of 2.5 $\mu\text{g/mL}$ of AHA extracts in DMSO was used as a working solution. Ten microliters of *Artemisia herba-alba* working extract solution was mixed with 2.5 mL of DPPH (25 $\mu\text{g/mL}$ in methanol) incubated for 30 min at $37\text{ }^{\circ}\text{C}$, and the absorbance at 515 nm was measured. The antioxidant capacity was expressed as a percentage of inhibition of DPPH radical (% DPPH inhibition) calculated according to the following equation: Percentage of DPPH inhibition (%) = $[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100$. $\text{Abs}_{\text{control}}$ is the absorbance of DPPH radical and methanol; $\text{Abs}_{\text{sample}}$ is the absorbance of DPPH radical and sample extract at 30 min. All analyses

were done in triplicate and radical scavenging activities were indicated as IC₅₀.

2.6. Isolation of whole white blood mononuclear cells (WBCs) and cell culture

Blood buffy coats were collected from healthy human volunteers ($n = 3$ volunteers, Établissement Français du Sang, EFS, Clermont-Ferrand, France). They were carefully layered on a double gradient of Ficoll–Histopaque1119® and 1077® as previously described (Cholet et al., 2019, Gainche et al., 2020). After centrifugation ($400 \times g$, 40 min at 20 °C), the first layer of plasma was aspirated, reaching the monocytes and lymphocytes phase (WBCs) just above the 1.077 g/mL layer. Then, the Ficoll layer was aspirated, yielding a phase of polymorphonuclear cells (PMNs) corresponding to a density between 1.119 and 1.077 g/mL. The WBCs were washed with RPMI and centrifuged (5 min, $400 \times g$) twice and then suspended in 5 mL of supplemented RPMI (FBS10%, gentamicin 50 µg/mL, and glutamine 2 mmol/L). Meanwhile, the residual erythrocytes in the WBCs phase were lysed by hemolytic shock using ammonium chloride solution. Then, the WBCs were washed with RPMI, centrifuged, and suspended in 1 mL of supplemented RPMI.

Human lymphoid T cell lines (Jurkat) were obtained from ATCC cell collection and maintained in RPMI-1640 medium (Biovest, Nuailé, France) supplemented with 10% fetal calf serum L-glutamine (2 mmol/L), penicillin 50 mL and streptomycin (50 µg/mL) (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The cells (Jurkat and WBCs) were cultured at 37 °C in a humidified atmosphere with 5% CO₂. Cell preparations were adjusted to 10⁶ cells/mL for all assays. All glutathione metabolism parameters were measured in Jurkat cell lysates following incubation of the cells with the different concentrations of each AHA extract. The collections of cell lysates were performed according to typical protocols for each assay (details were listed for each assay hereafter).

2.7. Quantification of reactive oxygen species (ROS) production

ROS production assay was carried out using dihydrorhodamine 123 (DHR) 10 mM (Sigma-Aldrich, Saint-Quentin-Fallavier, France) that can passively diffuse through cell membranes where it can be oxidized by ROS to rhodamine 123, and detected as green fluorescence. Human white blood cells (WBCs) and Jurkat human cell lines were plated at a density of 1.10⁶ cell/mL in a 96-well plate. Human white blood and Jurkat cells were treated with *Artemisia herba-alba* diluted extracts starting with 10 mg/mL of stock solution concentration. Dilutions were made so that the final concentrations in the wells are 100, 50, 25, and 10 µg/mL. One part of the cells was plated with PMA (Sigma-Aldrich, Saint-Quentin-Fallavier, France) 1 µM and the others were left without PMA as control. ROS production was measured every 5 min during 2 h at 37 °C, using a microplate reader (Fluoroskan Ascent Microplate Fluorometer®, Thermoscientifique Waltham, MA) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Fluorescence was corrected for background for each well. All assays were in triplicates.

2.8. Enzymatic activities measurements

Total proteins amounts were measured using the bicinchoninic acid method (BCA from Pierce, Interchim, France), according to the manufacturer's instructions. Standard curve was obtained using a standard solution of bovine serum albumin (2 g/L).

Glutathione reductase (GR) activity was determined as previously reported (Arab, 2006b). The cell lysate was incubated with buffer reagent (100 mM Tris-HCl, 1 mM EDTA, 0.16 mM NADPH, and 4.6 mM oxidized glutathione (GSSG), pH 7.4) (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Kinetic NADPH oxidation was followed at

340 nm and 37 °C for 3 min in a microplate spectrophotometer reader. GR activity, normalized to the protein content, was in UI/g.

Glutathione peroxidase (GPx) activity resulted in the oxidation of GSH in the presence of tert-butyl-hydroperoxide. Secondly GR recycled GSSG in the presence of NADPH as previously described (Mahbouli et al., 2018). The cell lysate was incubated with buffer reagent (100 mM Tris-HCl, 1 mM EDTA, 22 mM Tert-butyl-hydroperoxide, 5 mM GSH, 0.1 IU/mL GR, 2 mM NADPH, pH 7.4). (Sigma-Aldrich, Saint-Quentin-Fallavier, France). NADPH oxidation was measured using a microplate spectrophotometer reader set at 340 nm and 37 °C. GPx activity was normalized to the protein cell lysate content and expressed as UI/g of proteins.

Glutathione S-transferase (GST) activity was quantified following the method described in (Arab, 2006a). The cell lysates were incubated with buffer reagent (50 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene, pH 7.4). (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The kinetic 1-chloro-2,4-dinitrobenzene-glutathinylation was followed at 340 nm and at 37 °C in a microplate spectrophotometer reader. The GST activity was normalized to the protein content was in UI/g.

2.9. Total glutathione and total protein thiols

Total glutathione was determined by the method of (Cereser et al., 2001). In brief, dithiothreitol reduced (DTT) cell lysate for 10 min at room temperature and glutathione ethyl ester was added as an internal standard. After protein precipitation, the supernatant was derivatized by adding ortho-phthalate-aldehyde (OPA) (Sigma-Aldrich, Saint-Quentin-Fallavier, France). The HPLC separation of GSH—OPA adducts used a UP3 HDO C-18 reversed-phase silica column (150 × 3.60 mm, particle size 3 µm) from Phenomenex (Interchim, Montluçon, France), maintained at 37 °C followed by fluorimetric detection at 420 nm after excitation 340 nm (Summit HPLC system, Dionex SA, Courtaboeuf, France). Derivatives eluted using a 10–15% acetonitrile gradient in a 25 mM phosphate buffer at pH6 for 5 min. The flow rate was 0.25 mL/min for an elution run of 20 min. Chromatograms were integrated using Chromeleon software from Dionex (version 6.80, Dionex SA, Courtaboeuf, France). GSH content, calculated using a standard curve plotted under the same conditions and expressed in µmol/g protein.

Protein thiols were assayed according to the method of (Himmelfarb et al., 2000). In brief, free thiol groups oxidized by dithiobis-2-nitrobenzoic acid (Sigma-Aldrich, Saint-Quentin-Fallavier, France) were measured at 405 nm on a microplate spectrophotometer reader. The cell lysate-free thiol concentration was expressed as a ratio to protein content in mmol/g.

2.10. Cell viability measurements

Cell viability was estimated by the resazurin assay using 7-hydroxy-3H-phenoxazin-3-one (Sigma-Aldrich, Saint-Quentin-Fallavier, France) as described in the manufacture protocol (Abcam, Paris, France). The oxidized, blue, non-fluorescent resazurin is reduced to resorufin a pink fluorescent dye by cellular activity, due to oxygen consumption through metabolism, which is therefore proportional to aerobic respiration.

In brief, human white blood mononuclear cells (PBMcs) or Human leukemic cancer cell lines (Jurkat) were plated at a density of 1.10⁶ cell/mL in a 96-well plate. WBCs and Jurkat cells were treated with *Artemisia herba-alba* diluted extracts starting with 10 mg/mL stock solution concentration. Dilutions were made so that the final concentrations in the wells are 100, 50, 25, and 10 µg/mL. A part of cells/extracts were plated with PMA (Sigma-Aldrich, Saint-Quentin-Fallavier, France) 1 µM, and the others were left without PMA as control. Resazurin oxidation was measured every 5 min during 2 h at 37 °C, using a microplate reader (Fluoroskan Ascent Microplate

Fluorometer®, Thermoscientifique Waltham, MA) set on an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Fluorescence was corrected for background for each well. All assays were carried out in triplicates.

2.11. Statistical analysis

Each experiment was performed in triplicate, the average values are treated as single data points, and the data are expressed as mean $\pm$ standard deviation. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). For all assays, the statistical comparisons were performed using ANOVA with one way, and the significances were indicated as *P*-values in comparison to the control as following: * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, or non-significant (ns) if *P*-value > 0.05. For multiples comparisons with continues data points, Mann-Whitney test was used, and the *p*-values was calculated following each serial data in comparison to the related control.

3. Results and discussion

To get more insight into how *Artemisia herba-alba* mediates protective effects against inflammation, and in preventing the initiation of chronic related disorders, we investigated *in vitro* and *in vivo*, the capacities of AHA extracts obtained from different extraction methods i.e. maceration or microwave. We tested AHA extracts in human white blood cells and leukemic derived cell line Jurkat treated with phorbol myristate acetate (PMA), a pro-inflammatory chemical compound. The enhancement of glutathione metabolism by AHA extracts will support the evidence of a chemopreventive role against inflammation through redox buffering.

3.1. Total phenolic contents and DPPH radical scavenging capacities of *Artemisia herba-alba* extracts

To optimize the effectiveness of the extractions, we carried out two extraction modes using different solvents. AHA dried samples were macerated or subjected to microwave-based extraction in different solvents (water, methanol, ethanol, acetone, or a mix of all solvents at equal volumes). Estimation of total phenolic contents (TPC) revealed that higher amounts were obtained using the microwave method, which yielded of about 20% more compared to the maceration method (Table 1). As expected, water in both extraction methods yielded lower TPC compared to organic solvents. In maceration mode, acetone extract contained the highest amount of TPC followed by methanol, and ethanol extracts. In microwave-assisted extraction, acetone, methanol and ethanol gave similar amounts of TPC. Of note, the combination of different solvents did not improve polyphenols extraction in both extraction modes (Table 1). To assess the relationship between TPC and *in vitro*, antioxidant capacities of AHA extracts we used DPPH free radical method to estimate radical scavenging capacities of AHA extracts. Similarly, with TPC evaluations, acetone, ethanol, and methanol extracts exhibited the highest antioxidant capacities in both modes of extraction, attested by their lower IC₅₀ values (Table 2). Again, no improvements in antioxidant capacities were observed for extracts with mixed solvents. Water extracts, which exhibited the weakest activities in both extraction methods (Tables 1 and 2). A good correlation was observed between TPC and radical scavenging effects of all extracts, determined by linear correlation ($R^2 = 0.804$), as similarly reported (Singleton., 1999). The radical scavenging properties observed *in vitro* are attributed to the phenolic constituents as suggested by the substantial correlation between TPC and DPPH scavenging activities of the extracts (Tables 1 and 2), concisely reported in by different studies (Abu-Darwish et al., 2015; Khelifi et al., 2013; Messaoudene et al., 2011). Polyphenols are potent reducing agents, acting as hydrogen donors and free radical

Table 1

Total phenolic content (TPC) of *Artemisia herba-alba* extracts obtained from microwave or maceration method. Data are an average of 3 replicates $\pm$ standard deviation (SD), and the statistical difference is significant if *P*-value < 0.05 or non-significant if *P*-value > 0.05 (ns).

Extraction mode	Solvents	TPC (mg GAE/g dry weight)	P-value
Microwave	Water	114.58 $\pm$ 0.022	–
	Methanol	167.47 $\pm$ 0.005	0.001
	Ethanol	177.32 $\pm$ 0.007	0.001
	Acetone	176.14 $\pm$ 0.031	0.001
	Equal volume mixture	158.08 $\pm$ 0.005	0.001
Maceration	Water	67.45 $\pm$ 0.010	–
	Methanol	140.09 $\pm$ 0.010	0.001
	Ethanol	139.31 $\pm$ 0.010	0.001
	Acetone	150.42 $\pm$ 0.43	0.001
	Equal volume mixture	131.53 $\pm$ 0.005	0.001

Table 2

DPPH radical scavenging capacities of *Artemisia herba-alba* extracts obtained from microwave or maceration method. Data are an average of 3 replicates $\pm$ standard deviation (SD), and the statistical difference is significant if *P*-value < 0.05 or non-significant if *P*-value > 0.05 (ns).

Extraction mode	Solvents	IC ₅₀ (μ g/ml)	P-value
Microwave	Water	38.14 $\pm$ 3.71	–
	Methanol	19.25 $\pm$ 2.62	0.002
	Ethanol	20.50 $\pm$ 4.73	0.007
	Acetone	18.64 $\pm$ 5.91	0.008
	Equal volume mixture	25.85 $\pm$ 3.60	0.015
Maceration	Water	114.52 $\pm$ 8.42	–
	Methanol	30.16 $\pm$ 3.23	0.001
	Ethanol	32.95 $\pm$ 5.9	0.001
	Acetone	23.12 $\pm$ 3.29	0.001
	Equal volume mixture	26.82 $\pm$ 4.21	0.001

scavengers. In our study, total phenolic contents in *Artemisia herba-alba* extracts were slightly higher compared to what was previously reported: TPC = 123.95 g GAE /kg dry weight (Khelifi et al., 2013), TPC = 87.5 mg GAE/g dry weight (Bourgou et al., 2017) and TPC = 13.06 $\pm$ 0.40 mg GAE/g dry weight (Djeridane et al., 2006). The choice of the solvent and the method of extraction remains the determining point. These are not the only parameters that can affect the phenolic yield. Indeed, climate conditions (hot temperatures high solar exposure, dryness, short growing season) may be the reason for the increase in phenolic content (Djeridane et al., 2006). Based our findings, we decided to focus further evaluations on methanol and acetone extracts which exhibited the highest radical scavenging properties in both microwave-assisted and maceration methods.

3.2. Evaluation of cytotoxicity effects of *Artemisia herba-alba* extracts

After selecting the most effective AHA extracts *in vitro* obtained using methanol and acetone by maceration and microwaves-assisted extraction methods, we evaluated their cytotoxicity and their protective effects against PMA treated human white blood cells (WBCs: monocytes and lymphocytes, and polymorphonuclear cells) and Jurkat cell line. We exposed cells to different concentrations (the same we used *in vitro* assays) of AHA extracts, alone or in the presence of PMA for up to 2 h post-treatment. Immediate treatments using AHA extracts and PMA or AHA extracts alone obtained from both extraction modes, did not affect the cell viability of human blood cells. However, we observed a moderate synergistic effect in reduction of cellular viability of about 20%, while incubating Jurkat cells with AHA extracts and PMA, suggesting toxic cooperative effects happening early (Fig. 1). In this cell cellular model, we appreciated the absence of cytotoxicity in WBCs (Fig. 1A), in contrast to the leukemic Jurkat cell line, which showed a growth reduction (Fig. 1B).

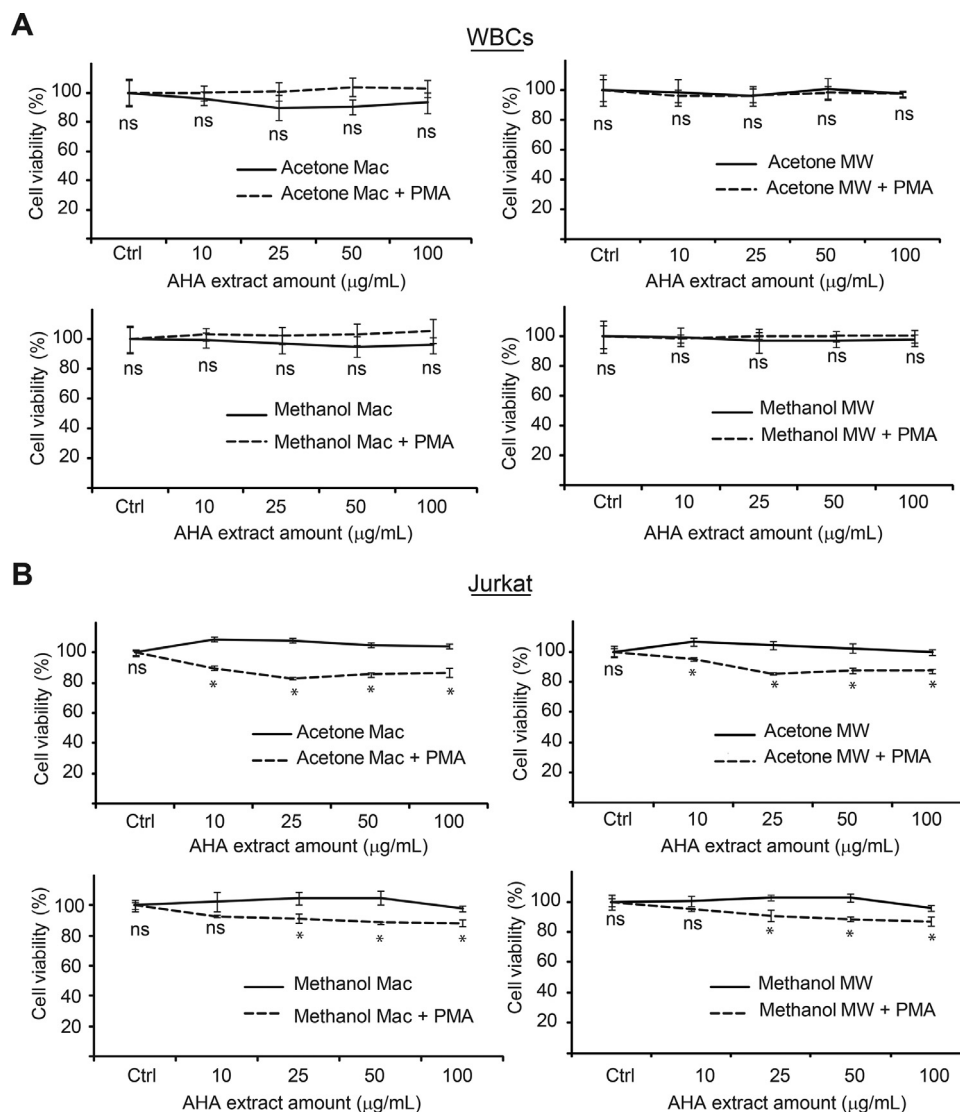


Fig. 1. Cell viability estimated by the resazurin assay in white blood cells (WBCs) and Jurkat cell line treated with *Artemisia herba-alba* extracts (AHA). (A) Cell viability of WBCs composed of monocytes and lymphocytes (1:1) treated with extracts alone or with phorbol myristate acetate (PMA). Upper panel, right: acetone maceration (Mac) extract; left acetone microwave (MW) extract. Lower panel, right methanol maceration extract (Mac); left methanol microwave (MW) extract. (B) cell viability of Jurkat cells treated with AHA extracts in same manner and shown as in A. Data are an average of 3 replicates $\pm$ standard deviation (SD), and the statistical difference is significant if P -value < 0.05 or non-significant if P -value > 0.05 (ns).

Concordantly, in other cancer cells, AHA extracts used as AHA essential oils induced DNA damage and cell death (Tilaoui et al., 2015).

3.3. *Artemisia herba-alba* extracts, in vivo inhibitors of reactive oxygen species production

We measured ROS production using dihydrorhodamine 123 fluorescence in WBCs and Jurkat cell line. In agreement with cytotoxicity results obtained using PMA and AHA extracts (Fig. 1), and to test similarly for ROS inhibition using AHA extracts in the presence of PMA, we measured ROS levels in time course manner (up to 2 h) to follow intrinsic ROS inhibitory capacities of AHA extracts. We used methanol and acetone extracts for testing based on their high TPC levels. Maceration and microwave extraction methods were tested in parallel. WBCs incubated with PMA led to a high induction of ROS levels compared to untreated cells, ROS levels being induced smoothly after immediate PMA spike-in. Interestingly, incubation of WBCs with acetone or methanol extracts in the presence of PMA delayed the induction and reduction of ROS production (Fig. 2A). In comparison to WBCs, PMA-mediated ROS induction in Jurkat was quicker and stronger before reaching a plateau (Fig. 2B). Similarly, in

WBCs, PMA ROS induction was inhibited by AHA extracts before ROS levels reaching basal values observed in PMA untreated cells. In both cell types, the inhibitory effect of AHA extracts was dose-dependent, demonstrated by the decrease in the p -values following an increase in the concentration of AHA extracts (Fig. 2 and Supplemental Table 1). Additionally, the rate of ROS inhibition did not differ between the maceration and microwave extraction modes. We concluded that AHA extracts could reduce and block ROS production at high doses in living cells, suggesting beneficial properties of AHA consumption in preventing inflammatory syndromes. These observations are in agreement with the empirical usage of AHA in traditional medicine, treating bronchitis, and bowel infections. Infusions of *Artemisia herba-alba* were used to treat cough, intestinal pain, and diabetes (Khelifi et al., 2013).

3.4. *Artemisia herba-alba* extracts exert an anti-inflammatory effect via the mobilization of glutathione metabolism

To understand the role of AHA extracts in reducing PMA related inflammation, we investigated the modulation of glutathione metabolism at early and late time points without PMA. The similar behavior

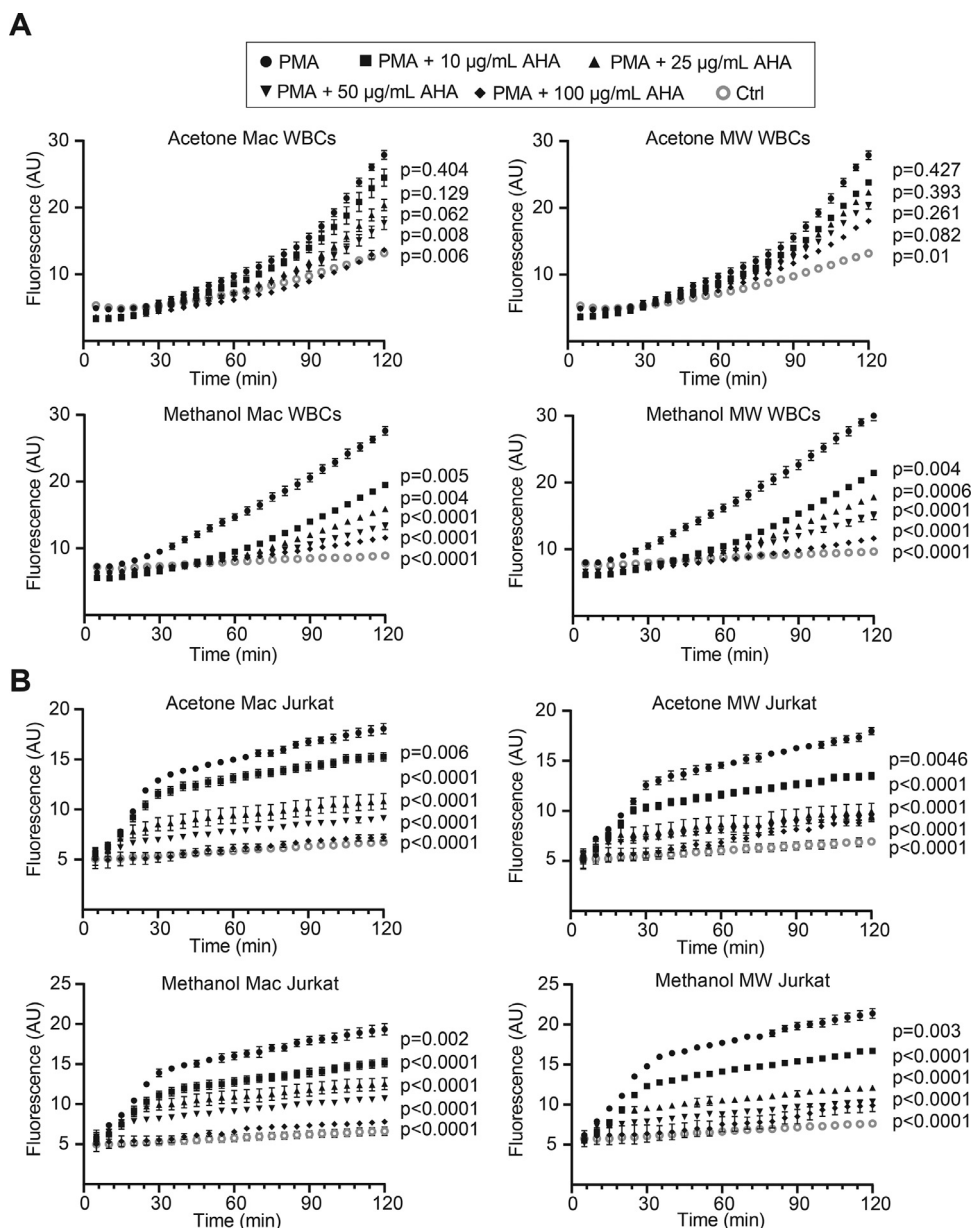


Fig. 2. Kinetic of reactive oxygen species (ROS) production in white blood cells (WBCs) and Jurkat cell line after treatments with different concentrations of *Artemisia herba-alba* extracts (AHA). (A) ROS level expressed as arbitrary units (AU) of fluorescence measured in WBCs composed treated with extracts alone or with phorbol myristate acetate (PMA). Upper panel, right: acetone maceration (Mac) extract; left acetone microwave (MW) extract. Lower panel, right Methanol maceration extract (Mac); left methanol microwave (MW) extract. (B) ROS level in Jurkat cell line treated with AHA extracts in same manner and as shown in A. Data are an average of 3 replicates $\pm$ standard deviation (SD). The statistical significances were calculated using all kinetic data points, and WBC + PMA as a control. Mann-Whitney test was applied to each AHA doses or untreated cells (WBCs), and *P*-values were shown accordingly. The difference is significant if *P*-value < 0.05 or non-significant if *P*-value > 0.05 (ns).

of WBCs and Jurkat cells in ROS inhibition in the presence of AHA extracts prompts us to use Jurkat cells to study the kinetics of the antioxidant response following AHA extracts exposure, due to the difficulty in maintaining WBC in culture. We followed cellular response up to 72 h post-treatment with AHA extract without any PMA pre-treatment. Parameters of glutathione metabolism were analyzed by: (1) glutathione levels (GSH) and glutathione reductase (GR) enzymatic activity for redox status homeostasis; (2) analysis of glutathione transferase (GST), glutathione peroxidase (GPx) enzymatic activities; (3) total free thiol-proteins for assessing ROS detoxifying cell capacities. Methanol and acetone extracts from maceration and microwave extraction modes were evaluated, accordingly. Interestingly, incubation of Jurkat cells with macerated methanol AHA extracts at different concentrations, elevated GSH levels shortly after 1 h post-treatment (Fig. 3A, left panel) and was maintained up to 6 h

(Fig. 3A, right panel). The induction of GSH levels correlated well with GR activity, reinforcing the pool of reduced glutathione and detoxifying capacities. GST, GPx, and free protein thiols followed the same induction trend for GSH and GR. The concentration of 25 g/mL was the most efficient (Fig. 3A). However, parameters of the glutathione metabolism were not fully correlated with amounts of methanol AHA extracts added to the cells. Acetone macerated extracts induced as well as GSH levels and GR activity on a dose-dependent manner, but with much higher levels, of about 2.5 fold increase compared to methanol macerated extracts at 1 h (Fig. 3B, left panel), and was maintained over 6 h (Fig. 3B, right panel). GSH levels reached high and equal levels for all concentrations of acetone maceration extracts at 6 h post-treatment (Fig. 3B, right panel). The Activities of GST, GPx, and the free thiol protein contents followed the same increasing trend at each extract concentration, corroborating the induction of

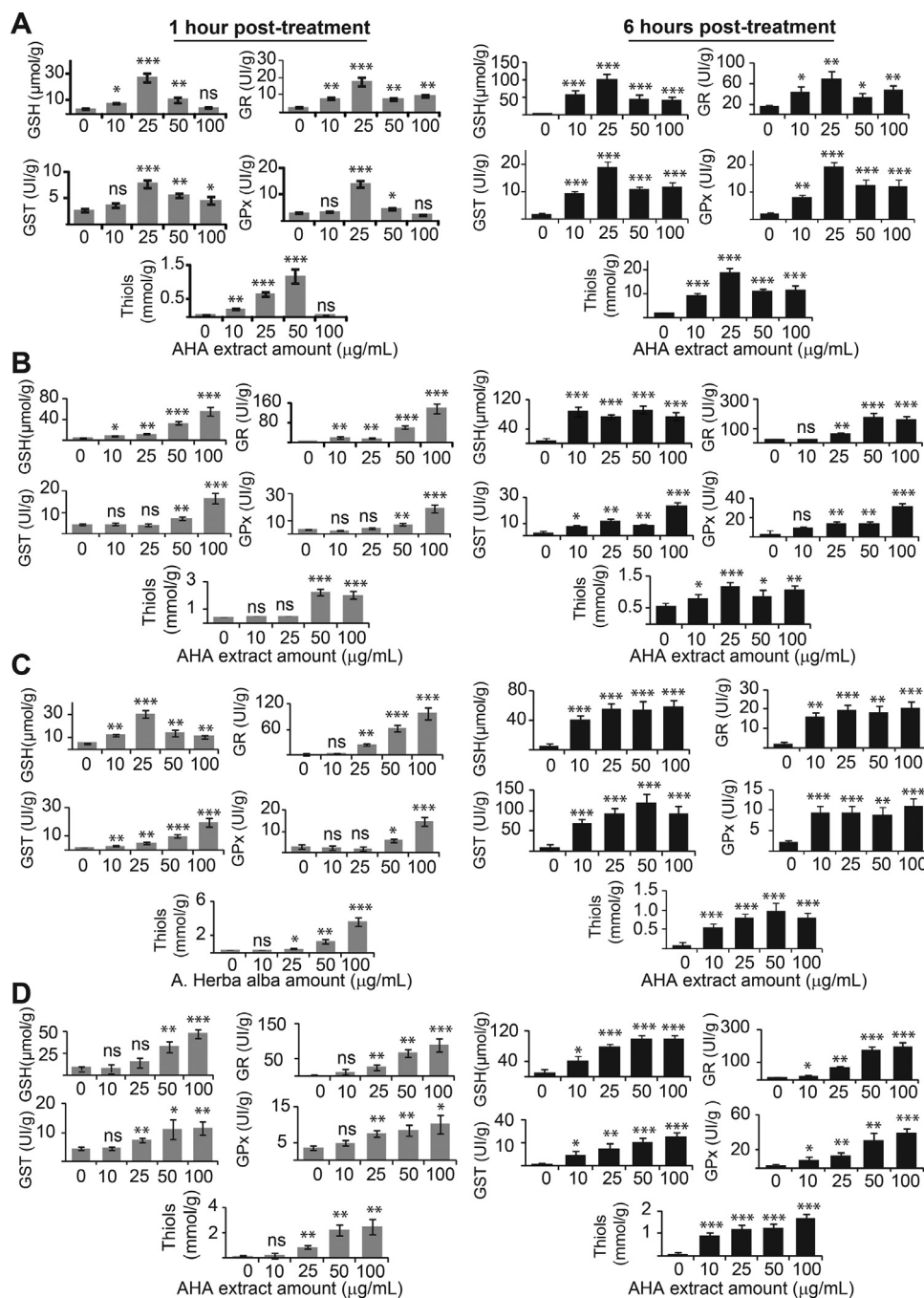


Fig. 3. Evaluation of glutathione metabolism in Jurkat cell line treated with different concentrations of *Artemisia herba-alba* extracts (AHA) for a short time period of 1 and 6 h post-treatments. (A) Redox status parameters of Jurkat treated with methanol maceration extracts; reduced glutathione (GSH) level, glutathione reductase (GR), glutathione transferase (GST), glutathione peroxidase (GPx) enzymatic activities and protein thiols (Thiols). Left panel, 1 h post-treatment (gray bars). Right panel, 6 h post-treatment (black bars). (B) Redox status of Jurkat treated with acetone maceration extracts, results as shown in A. (C and D) Redox status parameters of Jurkat treated with methanol and acetone microwave extracts for 1 and 6 h, results as shown in A and B, respectively. All measurements are an average of three replicates $\pm$ standard deviation (SD), normalized to the total proteins content. The statistical significances shown as P-values indicated as non-significant (ns), or significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$), calculated using AHA untreated cells as a control (0).

GSH levels, which remained similar to methanol extracts (Fig. 3B). After 6 h, acetone extracts in this case had a mild detrimental effect at high doses (100 g/mL), which changed moderately the free-thiol proteins pool (Fig. 3B, right panel). The concentration of 10 g/mL was enough to mobilize equal GSH content compared to higher concentrations. The discrepancy in cellular redox status parameters between methanol and acetone macerated extracts could be explained by the difference in extracts composition.

Taking in consideration these differences, we tested AHA microwave-assisted extracts from methanol and acetone for the

same parameters. Analysis of GSH levels and GR activities revealed that the concentration of 25 g/mL of methanol extract induces the highest values after 1 h incubation (Fig. 3C, left panel). Microwave methanol extracts of AHA led to a saturation effect of the cellular response for all parameters at 6 h of incubation following each concentration (Fig. 3C, right panel). Microwave acetone extracts induced a similar cell response as for acetone macerated extracts at 1 h with an increasing dose-effect after 6 h, without reaching saturation, for all redox status parameters (Fig. 3D). Both methanol and acetone microwave-assisted

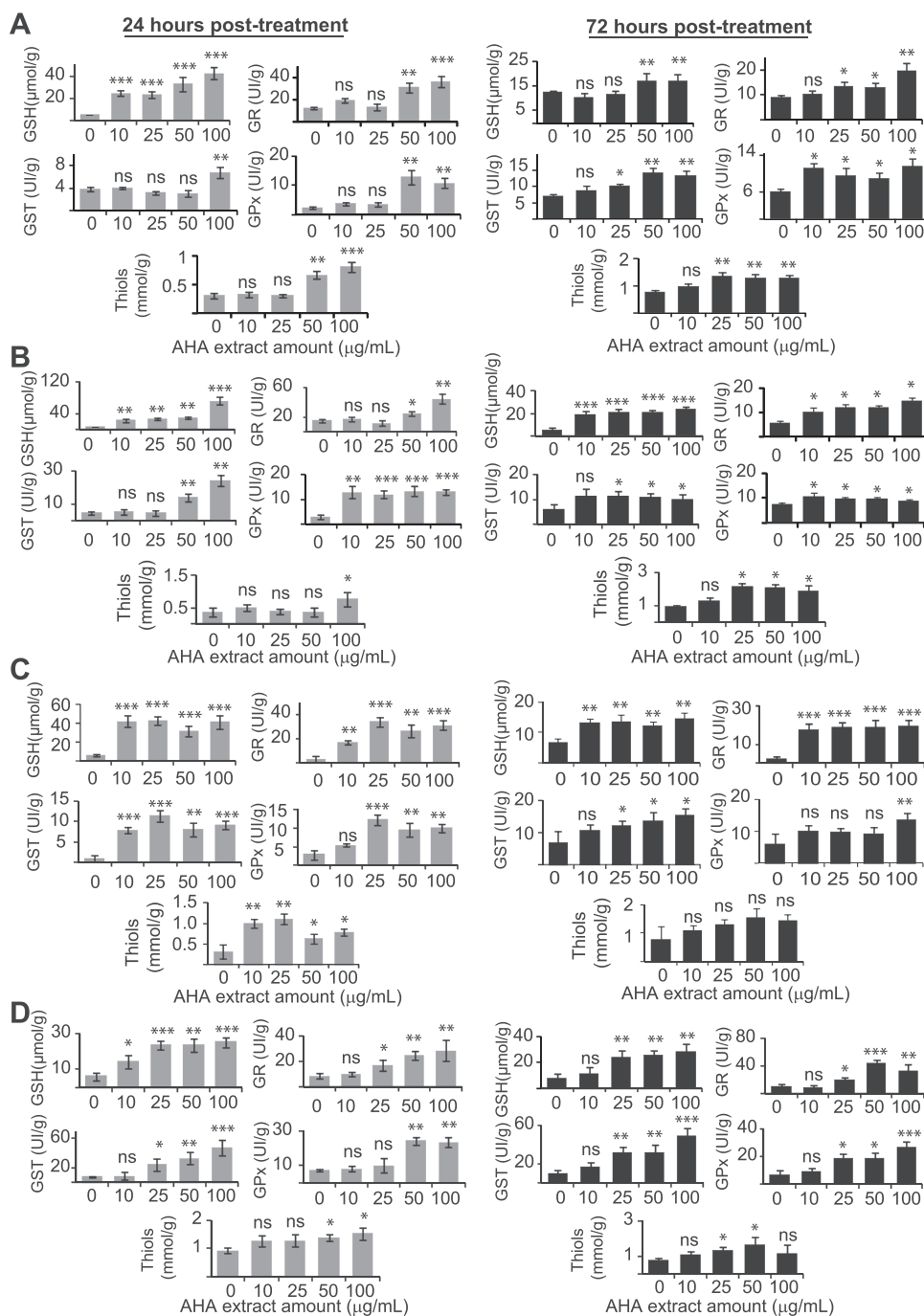


Fig. 4. Evaluation of glutathione metabolism in Jurkat cell line treated with different concentrations of *Artemisia herba-alba* extracts (AHA) for a longer period of 24 and 72 h post-treatments. (A) Redox status parameters of Jurkat treated with methanol maceration extracts; reduced glutathione (GSH) level, glutathione reductase (GR), glutathione transferase (GST), glutathione peroxidase (GPx) enzymatic activities and protein thiols (Thiols). Left panel, 24 h post-treatment (gray bars). Right panel, 72 h post-treatment (black bars). (B) Redox status parameters of Jurkat treated with acetone maceration extracts, results as shown in A. (C and D) Redox status parameters of Jurkat treated with methanol and acetone microwave extracts for 24 and 72 h, results as shown in A and B, respectively. All measurements are an average of three replicates $\pm$ standard deviation (SD), normalized to the total proteins content. The statistical significance is shown as *P*-values indicated as non-significant $p > 0.05$ (ns), or significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$), calculated using AHA untreated cells as a control (0).

extracts were more potent in maintaining cellular response than maceration extracts.

To evaluate the cellular response at late time points following exposure of Jurkat cells to macerated methanol and acetone extracts, we measured parameters of cell redox status at 24 and 72 h. Methanol macerated extracts still stimulated GSH production and its recycling in a dose-dependent manner (Fig. 4A, left panel), with acetone macerated extracts reaching high levels at 100 mg/mL (Fig. 4B). Detoxifying GSH related capacities increased following in the same

trend after 24 h post-treatment. The kinetics of the stimulation pursued and maintained through all doses of AHA extracts and remained dose-dependent, before reaching a plateau at 72 h (Fig. 4A, right panel). Similarly, acetone macerated extracts showed the same kinetic at 24 h but with higher GSH mobilization and detoxifying capacities (Fig. 4B, left panel). Acetone macerated extracts maintained high levels of all redox status parameters up to 72 h, fold changes ranged from 2 to 4 (Fig. 4B, right panel). In contrast, methanol microwave extracts produced a high antioxidant response at 24 h

and remained unchanged at 72 h whatever the used doses (Fig. 4C). As expected acetone microwave extracts constantly maintained high levels of cellular redox status parameters, which continued to increase after 24 h (Fig. 4D, left panel) showing a dose-dependent pattern, reaching the saturation plateau only at 72 h (Fig. 4D, right panel). Concomitantly, acetone microwave extract at 100 g/mL might trigger cytotoxicity as levels of GST, GPx further increased but were followed by a reduction in free thiol proteins and GR activity. At a high dose of 100 g/mL in acetone extracts, permanent increase in GSH content was probably not sufficient to rebalance the redox status of the cells, which could trigger alternative cellular damage via lipid peroxidation and DNA damage because of the recurrence of a permanent oxidative stress in leukemic Jurkat cells.

Analysis of *in vitro* antioxidant capacities of AHA extracts using DPPH assay confirmed previous findings (Bourgou et al., 2017; Khlifi et al., 2013), with a stronger potency for microwave extracts. Both, white blood cells and Jurkat line treated with microwave-assisted and maceration extracts induced, and later blocked entirely in a dose-dependent ROS production. The primary ROS blocking effect is attributable to the presence of several anti-oxidant compounds such as flavonoids and polyphenols (Younsi et al., 2018). However, glutathione metabolism stimulation, which reinforces cell capacities against inflammatory triggers, dominates. For the long term, in the Jurkat cell line, the anti-inflammatory effect, evaluated by parameters of glutathione metabolism, indicated that microwave-assisted extracts have a long-lasting effect, maintained over 72 h. The explanation may reside in the differences in the content of the extracts. Alternatively, some cellular metabolic steps may generate other bioactive compounds from the extract, triggering in a cascade the cellular response, independently from ROS induction, reinforcing the beneficial health effects of AHA. A possible explanation is that consumed AHA extracts may have several beneficial effects depending on the metabolism; the study of the metabolism of AHA extracts will bring more evidence in this direction.

4. Conclusion

Artemisia herba-alba plant is used widely in different forms to treat several pathological syndromes in humans, based on empirical experiences and transmitted local knowledge. Here, our work provided more evidence for how the preventative action of AHA against chronic disorders or inflammation is mechanistically happening. The reinforcement of glutathione metabolism by AHA extracts instead, condition the cells to react faster and more potently against ROS and inflammatory circuits, following severe infections or exposure to environmental toxins (Pereira et al., 2013). Future investigations are needed to dissect the entire molecular mechanisms, and the fingerprints of all AHA extracts for evaluating bioactive molecules singly, by which GSH metabolism remains stimulated for longer times in the cells, and whether the human metabolism contributes to the emergence of novel entities. Our work reinforces the evidence for beneficial properties of AHA consumption in the Mediterranean population and provides evidence for more specific usage of AHA extracts.

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Data availability

The datasets used and/or analysed during this study are available from the corresponding author upon request. All data generated or analysed during this study are included in this published article.

Declaration of Competing Interest

The authors declare no competing or financial interests.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2021.06.018.

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