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Dual protection of aqueous garlic extract biomolecules against hemolysis and its oxidation products in preventing inflammation

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Abstract

Garlic (Allium sativum) is recognized as functional food, rich in bioactive compounds that can combat diseases associated with oxidative stress. This study aims to investigate the protective potential of aqueous garlic extract against hemolysis and oxidation. Despite being caused by membrane fragility, hemolysis can lead to inflammation through the oxidation of its products, and in some cases, even exacerbate it in certain pathological contexts. Supplementation with antioxidant molecules can improves oxidative status, in this study, we selected garlic, an excellent functional food, and targeted its effects using aqueous extract and pure molecules. The aqueous garlic extract was prepared under safe conditions and subjected to toxicity on human neutrophils and red blood cells before experimentation. The results indicate that aqueous garlic extract significantly reduces hemolysis with a maximum protection of 98.74 ± 1.08 % at a concentration of 5µg/ml. Additionally, experiments were conducted with pure compounds found in garlic such as quercetin, gallic acid, and caffeic acid. The outcomes show that quercetin reduces hemolysis of RBC with a maximum protection of 88.8 ± 2.89 % at 20 µM followed by caffeic acid and gallic acid. The action mechanism of the extract was tested on human neutrophil cells, the extract significantly reduced luminol-amplified chemiluminescence of PMA-stimulated neutrophils up to 50 % at 10 µg/ml in addition to its ability to directly scavenge hydrogen peroxide. Our results suggest that aqueous garlic extract exerts promising anti-inflammatory activity in vitro. Through its dual protection against hemolysis and Ros production, garlic may indirectly prevent inflammation reducing the oxidation of hemolysis products. These abilities make garlic aqueous extract promising candidate for improving cardiovascular health, reducing oxidative stress and modulating immunity.

Keywords: Aqueous garlic extract, Quercetin, Gallic acid, Caffeic acid, Hemolysis, Neutrophils, Chemiluminescence, ROS.

1. Introduction

Many diseases are thought to be linked to eating habits, prompting growing interest in functional foods. Functional food is not a dietary supplement in tablet or capsule form, nor a drug, but a substance with curative or preventive properties against a lot of diseases in addition to their basic nutritional properties. Garlic is considered as a good example of functional food, consumed for centuries by various communities and many civilizations have relied on its ability to cure various diseases. Indeed, garlic has several modes of action: it acts as an immunostimulant and immunosuppressant and also appears to be very useful in preventing the generation of free radicals by neutrophils. This could be effective in pathological conditions associated with inflammation, and provide powerful protection against oxidative stress [1–5]. It is also known to reduce the incidence of diet-related diseases including anti-hypertensive effect [6] and anti-cancer activity [7, 8].

Garlic contains a variety of bioactive compounds, including organosulfur compounds like allicin, as well as higher levels of phenolic compounds compared to many common vegetables. The notable phenolic compounds found in garlic are gallic acid, rutin, as well as quercetin and caffeic acid…[1, 8, 9]. These compounds can act individually or in synergy through complex mechanisms [10, 11].

Hemolysis is an adverse consequence of the deformation of red blood cells (RBCs), which can have several origins, including immune responses and immune regulation [12], infectious from bacterial endotoxins [13]or fragments of their often pro-inflammatory cell walls. Patients with renal insufficiency present delicate clinical cases due to their repeated exposure to micro-infections and deficiencies in the blood cell environment during dialysis. Moreover, intravascular hemolysis can rarely occur in cases of hemodialysis complications. This type of hemolysis is believed to result from osmotic factors, influenced by the electrolyte composition of the dialysate, as well as

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being thermally, chemically, or toxically induced by chloramines [13, 14].

To address these deficiencies, adjuvants such as macronutrients and micronutrients are employed, but it is also equally important to correct the oxidative status of the serum. It's crucial to recognize the interconnection between renal insufficiency and cardiovascular disease, as oxidative status can create a conducive environment for their onset and exacerbation [13].

Furthermore, cellular-origin microparticles including platelets, leukocytes, endothelial cells, and red blood cells may be present in the plasma and contributeto oxidative stress and initiating inflammation even exacerbating inflammation-specific pathological context [15]. The role of leucocytes, notably neutrophils, in inflammation is well known. Their actions are manifested through the production of reactive oxygen species (ROS), degranulation and in some cases the formation of neutrophil extracellular traps (nets). Hypochlorous acid is enzymatically generated by myeloperoxidase using peroxide hydrogen as a substrate during infection or inflammation, often via neutrophils or monocytes. H_2O_2 is produced by NADPH oxidase [13, 16] in infection-related or inflammatory stimuli[17]. Both hypochlorous acid and peroxidehydrogen freely diffuse across the red blood cells and oxidize intracellular targets[17, 18]. The process of ROS production by NADPH oxidase is intricately involved in the inflammatory mechanism.

RBCs being the most abundant cells in the human body possess desirable physiological and morphological characteristics [19]. They are very practical cell model, easily accessible for *in vitro* testing and experimental settings particularly in the evaluation of biomembrane behavior concerning biomolecules and xenobiotics, as well as assessing the effects of various substances on the membrane.

Exposure of RBCs to harmful substances, such as oxidizing agents, heavy metals, heat and hypotonic solutions can lead to membrane lysis and hemolysis. This is accompanied by specific signs, including biochemical changes such as reduction in enzymatic content, metabolic slowdown, and loss of membrane lipids, oxidative processes, and disturbances of ion exchange [20]. Morphological alterations may lead to a reduction in membrane surface and hyperhydration affecting red blood cell deformability [21].

In this context, the aim of this study red blood cells (RBCs) and Neutrophils as models is to demonstrate the potential benefits of introducing an aqueous extract of garlic, together with its phenolic components, as adjuvants to improve the oxidative state of the blood environment prevent inflammation and over-inflammation induced by the oxidative products of hemolysis, which can lead to the incidence and exacerbation of pathologies linked to oxidative stress.

2. Materials and methods

2. 1. Chemicals

Hanks' balanced salt solution (HBSS), 4-phorbol-12-myristate-13-acetate (PMA), Luminol (5-amine-2,3-dihydro-1,4-phtalazinedione, HRPO, Ascorbic acid, Caffeic acid, Gallic acid, Quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA), $AlCl_3$, Trypan blue, phosphate-buffered saline (PBS), Bovin Serum Albumin (BSA), calcium chloride $CaCl_2$, $CuSO_4 5H_2O$, Dithiothreitol (DTT), Dextran, DNS, Ethanol, Trichloroacetic acid (TCA), Tris, Ficoll, Folin Ciocalteu, HCl, H_2O_2, Na_2CO_3, NaOH, NaCl, Ortho-dianisidine, Saponin 0.2 %, Glucose solution, Sodium potassium tartrate, Lysis buffer, hexadecyl trimethyl ammonium bromide (HTAB).

2. 2. Plant material and extraction procedures

Fresh garlic bulbs of the "purple" variety were selected for the study. The extraction was performed using a slightly modified method of Diao et *al.* [22]. 5 g of plant material was homogenized with 30 ml of extraction buffer Tris-HCl (50 mM, pH 7. 3) containing $CaCl_2$ (0. 5 M) and DTT (5 mM) at 4°C for 1 h. The supernatant collected after centrifugation for 30 minutes at 4000*g* and 4°C was filtered with a sterile syringe filter (pore size 0.22 µm), and the filtrate was placed in frozen storage (-20 °C) until analysis.

2. 3. Chemical analysis

Total protein concentration in different extracts was determined by the Lowry method [23]. Concentrations are expressed in grams per 100 grams of fresh matter (g/100 g) using the regression equation obtained with bovine serum albumin (BSA). The content of phenolic compound was determined spectrophotometrically according to Boizot and Charpentier [24] using the Folin-Ciocalteu reagent and the concentrations were expressed as mg of gallic acid equivalents per 100 g of fresh matter (mg GAE /100g FM) by using a standard curve of gallic acid. The flavonoid content was determined colorimetrically using aluminum trichloride [25] and the concentrations were expressed as mg quercetin equivalents per 100g of fresh matter (mg QE/100g FM) by using a standard curve of quercetin. The vitamin C content was determined according to the colorimetric method of Jagota and Dani [26] updated by Senani et *al*. [27] and the concentrations were expressed as mg/100 g of fresh matter by referring to the ascorbic acid calibration curve [28].

2. 4. Evaluation of the potential toxicity of aqueous garlic extract on human erythrocytes and neutrophils

2. 4. 1. Cytotoxicity of fresh garlic extract on isolated human neutrophils

Neutrophils were isolated by the dextran/ficoll method. The separation of blood components was based on the principles of sedimentation by density gradient and centrifugation [29, 30]. The toxic effect of the extract was determined by the trypan blue exclusion test; where isolated human neutrophils were incubated with increasing concentrations of garlic extract (5, 10, 20, 40, 80, 100, and 200 µg/ml) at 37 °C for 1 h. The percentage of cell viability was determined by counting viable neutrophils that remained transparent.

2. 4. 2. Cytotoxicity of aqueous garlic extract on human red blood cells

Human blood was collected from healthy volunteers in heparinized tubes and centrifuged for 15 min at 1400 *g* and 4 °C. The red cell pellet recovered after removal of the supernatant was washed with a 0.9 % NaCl solution by centrifugation for 10 min at 1400 *g*. The washing was repeated three times under the same conditions. In order to evaluate the cytotoxicity of garlic extract on human red blood cells (RBCs), a test for the hemolytic effect of the extract was performed. The test consists of incubating hu-

man red blood cells with garlic extract at increasing concentrations (5, 10, 20, 40, 80, 100, and 200 µg/ml) for 30 min at 37°C under gentle agitation. The positive control (total hemolysis) was induced with 0.2 % NaCl. After centrifugation at 300 *g* at 4 °C for 10 min the absorbance of the supernatants was measured at 540 nm by a spectrophotometer against the negative control (0.9 % NaCl).

2. 5. Determination of anti-hemolytic activity of fresh garlic extract

2. 5. 1. Hemolytic molecule screening

Hemolysis can be induced by two different mechanisms: osmotic hemolysis and membrane solubilization. The first mechanism occurs through a hypotonic solution, while solubilization is possible with surfactant agents [31]. Here, erythrocyte suspension was exposed to a series of hemolytic agents, including a hypotonic solution (0.2 % NaCl), distilled water, lysis buffer, saponin, and HTAB, to assess the hemolytic potential of each and to select a positive control for this study. The mixtures were incubated at 37 °C for 30 min with gentle stirring. After centrifugation at 300 *g* at 4 °C for 10 min, the absorbance of the supernatants was measured at 540 nm using a spectrophotometer against the negative control.

2. 5. 2. Protective effects of the garlic extract against 0.2 % NaCl-induced hemolysis

1 ml of garlic extract at increasing concentrations (5, 10, 20, 40, 80, and 100 µg/ml) and 40 µl of RBC suspension were pre-incubated for 10 min at room temperature, and then 1 ml of 0.2% NaCl was added to induce hemolysis. The positive control contained 40 µl of RBC suspension and 2 ml of 0.2% NaCl, while the negative control contained 40 µl of RBC suspension and 2 ml of 0.9% NaCl.

The mixtures were incubated at 37 °C for 30 min with gentle stirring. After centrifugation at 300*g* at 4 °C for 10 min, the absorbance of the supernatants was measured at 540 nm by a spectrophotometer against the negative control. The protection rate was calculated using the following equation:

% of protection = $[(A_C - A_e)/ A_C] \times 100$

Where A_e is the absorbance of the test sample (in the presence of the extract) and A_c is the absorbance of the positive control (without the extract).

2. 5. 3. Macroscopic and microscopic observation of red blood cells

After centrifugation, red blood cells are subjected to osmotic stress with or without the extract, and the appearance of the supernatants under all the experimental conditions was visualised with the naked eye and photographed. Red blood cell morphology was observed under a light microscope at x10 magnification in RBCs treated with a hypotonic solution (0.2 % NaCl) in the absence and presence of fresh garlic extract at different concentrations.

2. 5. 4. Protective effect of quercetin, gallic acid, and caffeic acid against 0.2 % NaCl-induced hemolysis as individual molecules

Red blood cells were also treated with pure molecules of quercetin, gallic acid, and caffeic acid at 20 µmol concentration. The red blood cell suspensions were pre-incubated for 10 min at room temperature, and then 0. 2 % NaCl was added to induce hemolysis. The positive control contained red blood cell suspension and 0.2% NaCl, while the negative control contained red blood cell suspension and 0.9 % NaCl. The mixtures were incubated at 37 °C for 30 min with gentle stirring. After centrifugation at 300g at 4 °C for 10 min, the absorbance of the supernatants was measured at 540 nm by a spectrophotometer against the negative control. The protection rate was calculated using the following equation:

% of protection = $[(A_C - A_e)/ AC] \times 100$.

Where A_e is the absorbance of the test sample (in the presence of pure molecule) and A_c is the absorbance of the positive control (without the pure molecule).

2. 6. Antioxidant effect of aqueous extract garlic

2. 6. 1. Total ROS production by luminol-amplified chemiluminescence in PMA-induced neutrophils

Neutrophils (5×10^5/0.5 ml) were suspended in HBSS in the presence of or not of increasing concentrations of garlic luminol (10 µM) for 10 min at 37 °C. Cells were then stimulated with PMA (100 ng/ml), with HRPO (5mU), Chemiluminescence was evaluated with a luminometer (Auto Lumat LB953 model, EG & G Berthold), where light emission was recorded in c. p. m (counted photons per minute) during 30 min at 37 °C [32].

2. 6. 2. Scavenging test of hydrogen peroxide (H_2O_2)

The direct reaction of aqueous garlic extract on peroxide was performed with incubating increasing concentration of the extract with or without H_2O_2 (0,003%) in the presence of luminol (10µM) and HRPO (5mU). Change in chemiluminescence was measured during 20 min [29].

2. 6. 3. Peroxidase activity assay

The peroxidase activity (oxidoreductase EC1. 11. 1. 7) was measured by spectrophotometric determination of the absorbance at 470 nm using o-dianisidine as a reducing substrate according to the protocol described by Bradley et *al*. [33] and modified by Bedouhene et *al*. [34]. The reaction mixture contained 775 µl of PBS pH 6, 100 µl of ortho-dianisidine (2 mg/2 ml), and 25 µl of the extract. The reaction was initiated by the addition of 100 µl of H_2O_2 (2/1000).

In the control, the extract was replaced by PBS buffer. Measurements were carried out within 10 min at 1 min intervals. The enzyme activity unit was defined as the amount of enzyme responsible for changing absorbance by 0.001/min [35]. The results were expressed in units per minute per gram of fresh matter (U/min/g of FM).

2. 7. Statistical analysis

All analyses were carried out in triplicate and all the results were expressed as mean ± standard deviation. The data analyses were performed using GraphPad Prism version 5.00 for Windows. The anti-hemolytic activity test results were statistically analyzed using the ANOVA variance test.

3. Results

3. 1. Content of biochemical and bioactive components in fresh garlic extract

The results of the quantitative analysis of biochemical and bioactive compounds in fresh garlic extract performed by spectrophotometric methods showed that this

extract contains a level of 2.58±0.11g/100g MV of protein and 1.14±0.04g/100g MV of reducing sugars, is a good source of bioactive compounds including antioxidants, and contains 94±3.33mg EAG/100g MV of total polyphenols, 7.21±0.28mgEQ/100g MV of flavonoids, and 66.7±1.93mg/100g MV of vitamin C [27].

3. 2. Potential toxicity of garlic extract on human erythrocytes and neutrophils

The results of the toxicity test performed prior to testing the activity of garlic extract on isolated neutrophils, expressed as a percentage (%) of the viability in relation to the extract concentration (Figure 1A), indicate a 100 % neutrophil viability rate across the entire range of garlic extract concentrations (5, 10, 20, 40, 80, 100, and 200 µg/ml). This means that the extract has no significant cytotoxic effect on human neutrophils. Before testing the anti-hemolytic activity of garlic extract, a toxicity test is necessary to determine the concentrations to be used. The results of the evaluation of the hemolytic activity of the aqueous garlic extract at different concentrations (5, 10, 20, 40, 80, 100, and 200 µg/ml) show a complete absence of hemolysis, and it clearly shows that the extract has no cytotoxic effect on the red blood cells for the tested concentration range. Erythrocytes remain stable even at high concentrations of the extract as shown in Figure 1B, there is no difference observed between RBCs in NaCl 0.9% and those treated with garlic extract.

3. 3. Hemolytic molecule screening

The results of hemolytic effect for different molecules (hemoltytic agents), including saponin 2%), distilled water, lysis buffer, hypotonic solution (0.2% NaCl), H2O and HTAB 2% tested in order to select a positive control (Figure 2) show that all molecules have considerable hemolytic power, with the highest value observed for NaCl at 0.2 %. Consequently, we employed a hypotonic condition with 0.2 % of NaCl to prevent membrane solubility which can occur with both of HTAB and Saponin.

3. 4. Protective effects of the garlic extract against 0.2 % NaCl-induced hemolysis

The results of the effect of fresh garlic extract at different concentrations on suspended red blood cells treated with 0.2 % NaCl, expressed as percent protection (Figure 3B), show that there is no hemolysis over the entire concentration range of garlic extract (5, 10, 20, 40, 80, and 100 µg/ml). The 5 µg/ml concentration of the extract leads to a significant protection rate of 98.74± 1.1% which is comparable to the control (0.9 % NaCl) and remains stable for all concentrations tested, thus reflecting a very significant anti-hemolytic effect exerted by the extract towards osmotic lysis induced by the hypotonic 0.2 % NaCl solution. The study of hemolysis as a function of time revealed that a time of 3 hours provides the same level of protection that lasts for 24 hours.

3. 5. Result of macroscopic and microscopic observation of red blood cells

As can be seen in Figure 3A, the positive control tube has a red supernatant due to red blood cell hemolysis and hemoglobin release. While the tubes of RBC_S treated with 0.2% NaCl and in the presence of various concentrations of fresh garlic extract (5, 10, 20, 40, 80, and 100 µg/ml), as well as the negative control tube, show transparent supernatants and RBC pellets, this is due to the complete

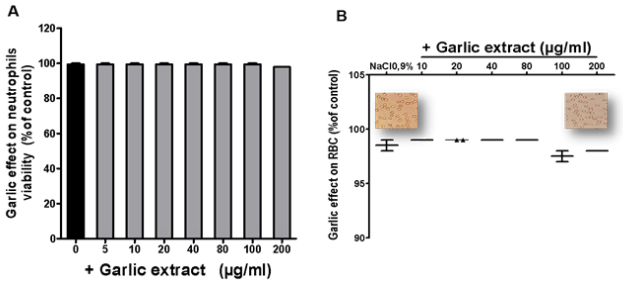

Fig. 1. Effect of fresh garlic extract. A: on neutrophils viability. B: on red blood cells hemolysis, Neutrophils and red blood cells were incubated with garlic extract at different concentrations (5, 10, 20, 40, 80,100, and 200µg/ml). Results are expressed as mean +/- SEM, n=3, *p < 0.05%.

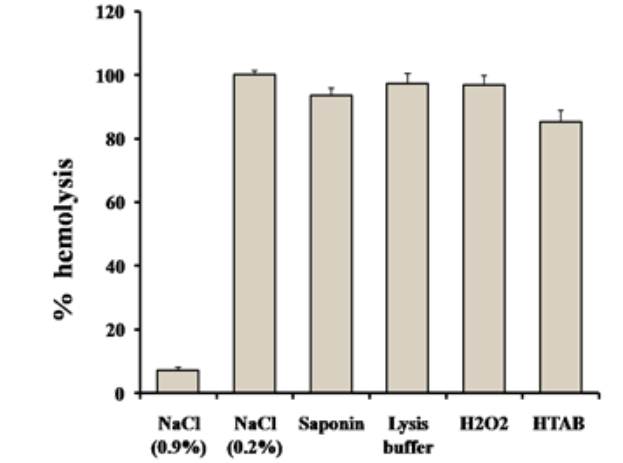

Fig. 2. Rate of hemolysis induced by different agents.

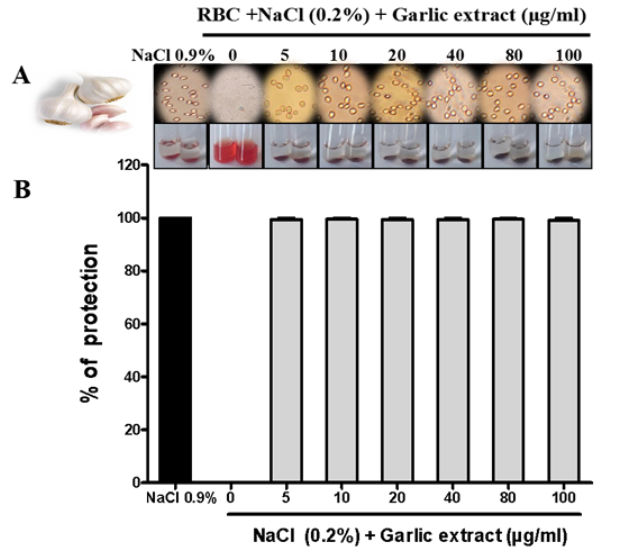

Fig. 3. Effect of fresh garlic extract on 0.2% NaCl-induced hemolysis. A: Protective effect of fresh garlic extract on the morphology of human red blood cells. The magnification was ×10 and the Release of hemoglobin during the hemolysis process. B: Percent protection of human red blood cells. Human red blood cells were incubated with garlic extract at different concentrations (5, 10, 20, 40, 80, and 100 µg/ml). 0.2% NaCl (+) and 0.9% NaCl (−) were used as positive and negative controls, respectively. Results are expressed as mean +/- SEM, n=3, *p < 0.05%.

absence of hemolysis.

Light microscopic observations at ×10 magnification of RBCs treated with hypotonic solution (0.2% NaCl) in the absence and presence of fresh garlic extract at different concentrations (Figure 3A) show that RBCs treated with 0.2% NaCl without garlic extract are completely lysed. Pretreatment of RBCs with different concentrations of garlic extract (5, 10, 20, 40, 80, and 100 µg/ml) prevented osmotic lysis of cells. The morphology of the RBCs membranes was not altered, and their appearance is comparable to that of control cells.

3. 6. Result of protective effect of quercetin, gallic acid, and caffeic acid against 0.2 % NaCl-induced hemolysis as individual molecules

The results of individual effect of quercetin,gallic acid, and caffeic acid, each at a concentration of 20 µmol, on red blood cells in suspension treated with 0.2% NaCl expressed as percent protection as shown in Figure 4. These findings demonstrate that quercetin, gallic acid, and caffeic acid present powerful anti-hemolytic activity against osmotic stress within the tested concentration range.

Quercetin and caffeic acid at a concentration of 20µmol present maximum inhibition of 0.2% NaCl-induced hemolysis rates, which is of the order of 88.9±4.93% for quercetin and 78.53±2.64% for caffeic acid. While at the same concentration gallic acid exhibits a minimum protection rate of 43.75±8.29%. According to the results, the protection rate remains relatively stable over almost the entire quercetin concentration range tested (20, 40, 80, and 100µmol), indicating a potent anti-hemolytic effect of quercetin against osmotic stress. The anti-hemolytic effect decreases with increasing concentrations of caffeic acid. The percent of protection decreases to 65.69±2.16% at a caffeic acid concentration of 100µmol. In contrast,this protection increases with increasing concentrations of gallic acid. The level of protection increases to 57.44±1.49% with a gallic acid concentration of 100µmol. In addition, Gallic acid exhibits low anti-hemolytic activity as compared to quercetin and caffeic acid.

3. 7. Antioxidant effect of aqueous extract garlic

3. 7. 1. Total ROS production by luminol-amplified chemiluminescence in PMA-induced neutrophils

To evaluate the effect of garlic aqueous extract on total ROS production in PMA-stimulated neutrophils, we pre-treated cells with increasing concentrations of the garlic extract. We used luminol to detect total ROS, mainly superoxide anion, hydrogen peroxide, and hypochlorous acid. As shown in Figure 5A, chemiluminescenceexpressed as (CPM), was strongly reduced by lower concentration (5 µg/ml) compared to the control (PMA), and the ROS production was totally inhibited at 20 µg/ml. The panel B represents the mean of all results. Here we observed a significant inhibitory effect on ROS production in dose-dependent manner, with 50% of inhibition occurring at 10 µg/ml. This result suggests that the Aqueous garlic extractacts by scavenging ROS or inhibiting NADPH oxidase which is responsible for their production.

3. 7. 2. Scavenging test of hydrogen peroxide « H_2O_2 »

The same test as the previous one with luminol was conducted in a cell-free system, targeting a single form of free radical, hydrogen peroxide (H_2O_2). Increasing concentrations of garlic extract were directly exposed to hydrogen peroxide in the presence of luminol. In figure 6A, the graph illustrates the kinetics of H_2O_2 radical scavenging compared to the control that was not treated with the extract. The chemiluminescence peaks reach their maximum intensity and subsequently decline rapidly, starting from the lowest concentration of 5 µg/ml. This trend, coupled with the reaction's specificity, enables us to confirm that garlic aqueous extract exhibits substantial antioxidant potential, particularly in its capacity to counteract hydrogen peroxide. In panel B, the mean of resultsis summarized in histogram form. These results in substantial scavenging activity, notably starting at 10 µg/ml concentration, where radical neutralization reaches approximately 75%.

3. 7. 3. Peroxidase enzymatic activity

In the figure 7, the kinetics of peroxidase activity as a function of time are shown for different concentrations of the aqueous garlic extract. This activity is proportional to the concentration of the extract. The described extraction process is a very simple procedure to obtain a peroxidase-rich extract. The result of the peroxidase activity assay

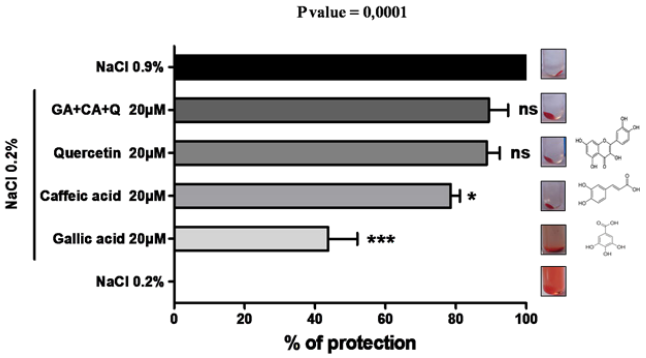

Fig. 4. Effect of phenolic compounds on 0.2% NaCl-induced hemolysis. Protective effect of quercetin (Q), gallic acid (GA), and caffeic acid (CA). Human red blood cells were incubated with phenolic compounds at different concentrations (20; 40; 80, and 100µmol). 0.2% NaCl (+) and 0.9% NaCl (−) were used as positive and negative controls respectively.

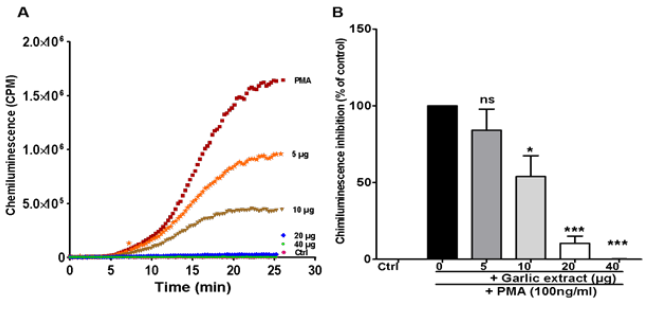

Fig. 5. Effect of garlic aqueous extract on luminol-amplified chemiluminescence in PMA stimulated neutrophils. A: Neutrophils ($5x10^5$/0.5ml) were incubated with garlic aqueous extract at different concentrations (0,5,10,20 and 40 µg/ml). Cells were then stimulated with PMA (100 ng/ml) in the presence of luminol and HRPO (5mU). This result represents an example of ROS production. Luminol-amplified chemiluminescence was measured for 30 min. B: Histogramme represents ROS production as calculated by mean of the total area under the chemiluminescence curve. Results are expressed as mean +/- SEM, n=3, * $p < 0.05\%$.

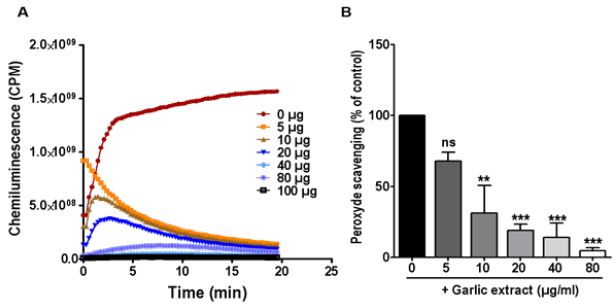

Fig. 6. Effect of garlic aqueous extract on luminol-amplified chemiluminescence in acellular system. A: H_2O_2 was incubated with increasing concentrations of garlic aqueous extract (0; 5; 10; 20, 40 and 80 µg/ml) in the presence ofluminol (10µM) and HRPO (5mU). This result represents an example of ROS production. Luminol-amplified chemiluminescence was measured for30 min. B: The histogramrepresents ROS production as calculated by mean of the total area under the chemiluminescence curve. Results are expressed as mean +/- SEM, n=3, * p < 0.05%.

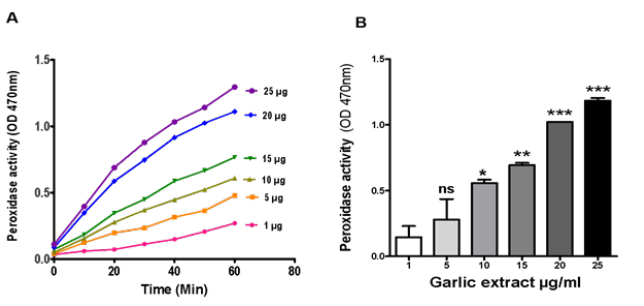

Fig. 7. Peroxidase activity of garlic extract aqueous. It was measured in the presence of increasing concentrations of garlic extract aqueous, H_2O_2 and orthodianizidine. A: This is an example representing the profile of peroxidase activity as a function of garlic extract concentration. B: Histogram represents mean of all experiments. Results are expressed as mean ± SEM, n=3, * p < 0.05%.

(oxidoreductase EC1. 11. 1. 7) shows that the garlic extract has a peroxidase activity of 309. 1±2. 53 U/min/g of FM.

4. Discussion

The aqueous extract of garlic has gained significant attention in recent years due to its potential health benefits, not forgetting to mention that garlic is an excellent functional food. In this study, we attempted to demonstrate the direct and indirect anti-inflammatory effects via antihemolytic action of the aqueous extract of garlic and some biomolecules present in the extract.

Red blood cells (RBCs) are often used as a model in various types of experiments, including for studying the behavior of biological membranes. Investigating the effects of polyphenols on the properties of erythrocyte membranes is an interesting research topic. Several studies conducted on plant extracts have revealed the ability of phenolic compounds to provide protection to red blood cell membranes against hemolysis. This action could be either direct, by binding to the membrane, or indirect, by scavenging free radicals [36–38].

The aqueous extract of garlic was prepared in soft conditions and the mentioned garlic extract prepared was subjected to biochemical characterization; the results are already published [27]. The aqueous extract of garlic was rich in very interesting molecules that can be considered as antioxidants, including enzymatic compounds such as peroxidase (309.1 ± 2.53 U/min/g of PM) and non-enzymatic compounds such as polyphenols (94 ± 3.33 mg GAE/100g of PM), flavonoids (7.21±0. 28 mg QE/100g of PM), and vitamin C (7.21±0.28 mg QE/100g of PM); its inocuity was verified on human neutrophils and red blood cells before experimentation. Results showed that aqueous garlic extract reduced strongly RBCs hemolysis under hypotonicity. The synergistic action of each of quercetin, caffeic acid, and gallic acid seems to have shown its maximum effect at low doses (20 µM) at equal concentrations. To explain the molecular mechanism of protection, we demonstrated the extract's ability to directly scavenge hydrogen peroxide *in vitro*, on the other hand, we evaluated the total ROS (Reactive Oxygen Species) production in PMA-stimulated neutrophils using the luminol amplified chemiluminescence assay. Luminol detects multiple reactive oxygen species, mainly hydrogen peroxide, superoxide anion, and hypochlorous acid [32]. The extract aqueous garlic extract inhibits significant chemiluminescence of PMA-stimulated neutrophils at lower concentrations (to 50 % at 10µg/ml), adding to this its direct scavenging of peroxide.

Phorbol myristate acetate (PMA) is an activator of protein kinase C, which induces inflammation under *in vitro* conditions including ROS production in neutrophils. Based on the results obtained, the garlic extract would reduce inflammation by inhibiting protein kinase C. Quercetin, the predominant molecule, tested for peroxide scavenging showed a significant reduction in scavenging and production of ROS. Following Sankaranarayanan et *al*. [39] the same extract at higher concentrations showed inhibition of intracellular ROS under fMLP stimulated rat neutrophils using Dichloro fluorescein Di-acetate. The next step, prompted by these results, is to explore the molecular mechanisms of the biomolecules present in this extract in order to understand their role in protecting against hemolysis, a factor contributing to inflammation induction. Previous research has reported that the inflammatory process results from factors that may be at the origin of this protection, such as the presence of antioxidant biomolecules like flavonoids (quercetin), polyphenols (caffeic acid. . .), and enzymes with antioxidant properties, namely peroxidase [32]. Together, these compounds contribute to controlling the morphology of erythrocytes and protecting them from hemolysis. It is worth noting that peroxidase, with its substrate-neutralizing action on hydrogen peroxide, interferes with the oxidation of erythrocyte membrane components, particularly since it has the property of diffusing through biological membranes [40].

Despite the origin of the hemolysis, its incidence leads to the release of hemoglobin into plasma, as well as its derivate, including free heme, which could potentially be pro-inflammatory [13, 14, 41]. Indeed, the oxidized form of free heme and hemoglobin could stimulate and recruit innate immune cells, including neutrophils, responsible for the production of ROS and DNA traps [42, 43]. This is the mechanism that describes the involvement of hemolysis in the inflammatory process according to Sesti-Costa [43] and Nader et *al*. [15]. The production of pro-inflammatory cytokines during hemolysis in a vascular microenvironment is not exclusive to neutrophils and monocytes, but

they are recognized as the majority [44].

Based on evidence suggesting that hemolysis products can act as mediators of inflammation, it's easy to explain how molecules derived from garlic and its aqueous extract can modulate inflammation caused by hemoglobin derivatives. This attenuation is achieved by reducing the hemolysis of red blood cells and inhibiting the oxidation of hemoglobin products.

The protective role of some polyphenols on RBCs is linked to the ability of these compounds to interact and partition with the lipid membrane without interfering with ATPase activity [45]. On the contrary, this activity may be increased, allowing both environments to remain in balance [46]. Polyphenols, being amphiphilic in nature, can interact with membrane lipids at both the polar group level and within the bilayer. This interaction depends on solubility, polarity, hydrophobicity, lipid bilayer composition, the location of polar groups, molecule size, and concentration [47]. Such interactions can lead to alterations in membrane dimensions and/or the arrangement of membrane components. These changes are particularly noticeable in lipid rafts rich in cholesterol and sphingolipids and may affect the activity of proteins associated with rafts, which are involved in numerous key cellular processes [48, 49].

Polyphenols induce a change in the lipid phase of the RBCs membrane. Due to their amphiphilic nature, they primarily incorporate into the hydrophilic regions of lipids (packing of the lipid polar heads) in the outer layer of the RBC membrane, ensuring RBCs resilience under osmotic or oxidative stress conditions [50, 51].

Polyphenols, due to their distribution in cell membranes and the resulting restriction of membrane fluidity, could sterically hinder the diffusion of free radicals and thus reduce the kinetics of radical reactions [52]. Quercetin's strong affinity for the cell membrane would explain its potent protective effect on red blood cells, whereas Caffeic acid and Gallic acid distribute poorly within the lipid bilayer. According to studies on structure-activity relationships, compounds with a high affinity for the membrane are biologically active at low concentrations [53, 54]. Caffeic acid interacts with human RBCs, preferentially localizing in the outer monolayer, particularly in the hydrophilic region of the red blood cell membrane, and also specifically targeting sphingomyelin in ordered membrane domains. This mechanism of action of this dietary polyphenol plays a significant role in protecting the red blood cell membrane from damage [48, 55].

Quercetin also affects the polar region of the bilayer membrane, and its location near the membrane surface protects it from peroxidation. Due to the changes induced in the bilayer's structure, it can lead to alterations in its permeability[53]. Quercetin inhibits hypotonic hemolysis by modifying membrane permeability or by increasing the surface area-to-volume ratio of the cell [56]. Gallic acid possesses good antioxidant and anti-hemolytic properties against HClO-induced hemolysis[57, 58]. HClO molecules cause lateral expansion of the inner monolayer of RBC membranes, altering their biconcave shape. The localization of gallic acid in the outer monolayer of the membrane prevents this oxidizing agent from penetrating the lipid bilayer, which can hinder the insertion of HClO into the inner monolayer and thus neutralize its harmful effects [58].

5. Conclusion

In conclusion, our results suggest that aqueous garlic extract possesses significant antioxidant potential against various reactive species of oxygen (ROS) and offers protection to red blood cells against hemolysis. These two properties have the potential to provide preventive action against inflammation and over-inflammation in pathological contexts. The abundance of bioactive compounds ingarlic aqueous extract makes it a promising candidate for enhancing cardiovascular health, reducing oxidative stress and modulating immune responses. The aqueous extract, along with its active components, serves as an a natural anti-inflammatory agent, offering an alternative solution to conventional anti-inflammatory drugs. It may serve as an effective adjuvant to correct serum oxidative status, particularly in cases of renal insufficiency and cardiovascular diseases.

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Interest conflicts

The authors declare no conflicts of interest.

Consent for publications

The author read and proved the final manuscript for publication.

Availability of data and material

All data generated during this study are included in this published article

Authors' contribution

S. Bedouhene, T. Rekeb, N. Senani, M-D. Chabane performed the study design, the experiments and the data analyses including results and statical analysis, S. Dermeche and D. Messaoudi contributed to the analysis and results discussion. All authors contribute to the manuscript writing.

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Ethics approval and consent to participate

Venous blood was obtained from healthy volunteers after written informed consent had been obtained. The study protocol was approved by the Ethics Committee: Com-

Eth-DeonL2023/UMMTO-12.

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