

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELs), 2023

Volume 11, Pages 1-11

ICVALS 2023: International Conference on Veterinary, Agriculture and Life Sciences

Anti-Inflammatory and Antioxidant Activities of Cow's Milk Supplemented with Aqueous Extract of Malva Sylvestris

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Abstract: Cow's milk is known for its beneficial properties for health, added to this, scientific research is increasingly interested in molecules from medicinal plants. The objective of this study is to evaluate some of the biological activities resulting from the association of cow's milk and the aqueous extract of leaves of malva sylvestris L, a medicinal plant of the traditional pharmacopoeia of Algeria. The combination produced showed appreciable antioxidant activity. Its capacity to trap the DPPH radical, the OH radical and H₂O₂ was respectively estimated by an IC₅₀ of 5.215 ± 0.759 µg/ml, 381.44 ± 37.12 µg/ml and 205.52 ± 12.03 µg/ml. The milk associated with the plant presented an IC₅₀ for its total antioxidant capacity of 362.90 ± 7.04 µg/ml and of 211.02 ± 35.27 µg/ml for its reducing power of the ferric ion. The assessment of the antioxidant capacity of this association was also carried out by the β-carotene bleaching inhibition test where we recorded an inhibition of $83.93 \pm 4.82\%$ of the extract at a concentration of 1mg/ml and the ferrous ion chelation test estimated by an IC₅₀ of 506.84 ± 54.50 µg/ml. With regard to anti-inflammatory activity, the cow's milk studied showed a stabilizing effect on erythrocyte membranes against osmotic stress ($79.71 \pm 3.81\%$), oxidant stress induced by HOCL ($80.32 \pm 0.92\%$) and heat ($90.1 \pm 3.05\%$). The sum of the results obtained during this study clearly shows that the combination of cow's milk and the aqueous extract of *malva sylvestris* leaves has significant potential for the biological activities investigated. As a result, this association constitutes a potential source of bioactive molecules and thus constitutes a therapeutic alternative to the treatment of a number of pathologies initiated by oxidative stress.

Keywords: Cow milk, Malva sylvestris, Antioxidant, Anti-radical, Anti-inflammatory

Introduction

Throughout the world, plants have always been used as medicines and are a precious heritage for humans, offering a varied and variable source of biomolecules with widely recognized therapeutic potential. The renewed interest in biomolecules from medicinal plants is based on the numerous scientific studies highlighting the numerous merits of medicinal plants (Singh, 2015). Nowadays, the exploration of the antioxidant properties of plant sources has gained a considerable place in therapeutics, thus marking the decline in the use of synthetic antioxidants (Velioglu et al., 1998). This change of direction in scientific research is a logical response given the alarming implications of certain chemical antioxidants. As free radicals inflict often irreversible damage to biomolecules and to protect the integrity of biological systems, scientific research is relying on new antioxidant biomolecules, such as polyphenols, which have shown high antioxidant potential and could help maintain the

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oxidative balance of biological systems (Bhattacharyya et al., 2014). Polyphenols, known for their antioxidant properties, owe their biological functionality to their chemical structures, which allows them to be excellent electron donors and efficient hydrogen sensors (Leopoldini et al., 2004). *Malva sylvestris*, commonly called sylvan mallow, is an annual plant with weakly lobed leaves and purple flowers that bloom in spring. Native to Europe, North Africa and southwest Asia, this hardy plant is found in varied environments, from humid coastal areas to grasslands, ditches and river banks (Razavi et al., 2011). Long known for its use as a vegetable, *Malva sylvestris* is now renowned as a medicinal plant. Numerous scientific studies have confirmed its numerous virtues, particularly as an antioxidant, radical scavenging and antimicrobial agent (DellaGreca et al., 2009; Awwad et al., 2015). Considering the widespread use of aqueous extracts of *Malva sylvestris* leaves in traditional medicine, coupled with the use as feed for livestock animals (Hassan et al., 2015; KADIOĞLU et al., 2022) associated with this the fact that the cow's diet has a direct impact on the composition of the milk produced, this study aims to evaluate the antioxidant potential of the combination

Materials and Methods

Plant Collection

Malva Sylvestris leaves were collected in October 2022 from M'sila, Algeria. The plant was identified by Doctor Mahmoud Laribi, botanist at Mouloud Mammeri University of Tizi-Ouzou, department of vegetal biology, where a voucher specimen was deposited (FSBSA/MK/oct2722). The sample was dried and then ground to obtain a powder that was stored at room temperature and in the dark until extraction.

Extract Preparation

20g of powder are dissolved in 200ml of distilled water. After 24 hours of maceration at room temperature, the filtrate was lyophilized.

Milk Collection

Collected in October 2022, cow's milk samples are milks of small mixtures, resulting from herds of healthy cows, localized in the area of Tizi-Ouzou.

Skimming

Skimming of milk consists of removing fat from milk by centrifugation at 3500xg for 20 minutes and at 4°C. At the end of the centrifugation we note a phase separation with the formation of a layer on the surface corresponding to the cream of the milk, once this has been removed using spatula, the resulting skimmed milk is filtered through the glass wool. To ensure good skimming, the entire skimming operation is repeated two other times, thus eliminating any residual trace of fat for the rest of the isolation protocol.

Determination of DPPH Radicals Scavenging Activity

The free radical scavenging activity of the extract was measured using the stable free radical DPPH test according to the method described by (Sharma & Bhat, 2009; Santos et al., 2010). 250 µl of 0.8 mM DPPH in ethanol was mixed with 3.65 ml of extract and 100µl of cow milk. The reaction was carried out in triplicate and the absorbance was measured at 517nm after 30 minutes in dark. L-Ascorbic acid was used as reference standard.

Hydroxyl Radical Scavenging Assay

Scavenging activity of hydroxyl radical of the extract was measured according to the method of (Rajamanikandan et al., 2011) Three millilitres of the final reaction solution consisted of aliquots (500 µl) of various concentrations of the extract, 100µl of cow milk, 1ml FeSO₄ (1.5 mM), 0.7 ml hydrogen peroxide (6

mM) and 0.3 ml sodium salicylate (20 mM). The reaction mixture was incubated for 1 h at 37°C. L-Ascorbic acid was used as the standard. The colour development was measured at 560 nm against a blank.

Hydrogen Peroxide Radical Scavenging Activity

The scavenging ability of water extract of *Malva Sylvestris* on hydrogen peroxide was determined according to the method of (Serteser et al., 2009). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Cow milk (100µl) and extract in distilled water (3.4 ml) was added to a hydrogen peroxide solution (0.6 ml, 40mM). Absorbance of hydrogen peroxide at 230 nm was measured 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide.

Ferrous Ion Chelating Activity

Ferrous ion chelating activity was determined by inhibition of the formation of iron(II)–ferrozine complex, following the method of (Dinis et al., 1994; Nabavi et al., 2012). Briefly, 100 µl of 0.6 mM FeCl₂ was added to 500µl of different concentrations of the extract mixed with 100µl of cow milk or EDTA (positive control). The reaction mixture was adjusted to a final volume of 1.5ml with methanol, and then 100µl of 5 mM Ferro zine solution were added. The mixture was shaken vigorously and left to stand at room temperature for 5 min. Absorbance was determined at 562nm.

Ferric Reducing Power Assay

Reducing power was determined by the method described by (Oyaizu, 1986; Hazra et al., 2008). Different concentrations of extract and cow milk were mixed with 1.25 ml of 0.2 M, pH 6.6 sodium phosphate buffer and 1.25 ml of potassium ferricyanide (1%). The mixture was incubated at 50°C for 20 min. After incubation, the reaction mixture was acidified with 1.25 ml of trichloroacetic acid (10%) and centrifuged at 3000 rpm for 10 min. Finally, 0.5 ml of freshly prepared FeCl₃ (0.1%) was added to this solution, and the absorbance was measured at 700nm. Ascorbic acid at various concentrations was used as standard.

Total Antioxidant Capacity

Total antioxidant capacity was estimated by phosphomolybdenum assay (Prieto et al., 1999; Rao et al., 2010) The tubes containing extract and cow milk and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95°C for 90 min. Then the solution was cooled to room temperature and absorbance was read at 695 nm. Ascorbic acid was used as standard.

Antihemolytic Activity

Red Blood Cell Suspension

Blood was obtained by venipuncture from healthy volunteers collected in heparinized tubes and centrifuged at 2 000 r/min for 10 min at 4 °C. After removing the plasma, red blood cells (RBCs) were washed for three successive times using phosphate buffer saline (PBS) (0.9% NaCl). The study protocol was performed according to the Helsinki declaration and approved by Scientific Committee of the Faculty of Biology (CSFB). Informed written consent was obtained from Hospital Department of Hematology (University Hospital Nedir Mohamed of Tizi-Ouzou).

Hypotonic Solution Induced Hemolysis

Membrane stabilizing activity of extract and cow's milk was assessed using hypotonic solution induced hemolysis, and the method was described by de Freitas et al. (2008). In hypotonic solution, the test sample consisted of washed stock erythrocyte (RBC) suspension (40 mL) with 1 mL of hypotonic solution (0.1%,0.3%, 0.5%, 0.7%, 0.9% NaCl) in sodium PBS (pH 7.4) containing either of the different concentrations of goat's milk.

The mixture was incubated for 30 min at 37 °C under gentle stirring, centrifuged for 10 min at 2 000 r/min and the absorbance of the supernate was measured at 540 nm.

Heat Induced Hemolysis

Different concentrations of the extract mixed with cow's milk (mg/mL) or aspirin dissolved in isotonic PBS (pH 7.4) was mixed with 1 mL of 2% RBCs suspension. The reaction mixture was incubated in a water bath at 56 °C for 30 min. After incubation, the tubes were cooled under running tap water, then centrifuged at 2 000 r/min for 10 min and the absorbance of the supernatants was estimated at 560 nm (Sakat et al., 2010).

Oxidant Induced Hemolysis

One milliliter of RBC suspension (5%) in PBS (pH 7.4) was incubated for 15 mn at 37°C with 1 ml of the extract mixed with cow's milk at different concentrations. After preincubation, the mixture was centrifuged at 2 000 r/min for 10 min at 4°C, the supernatant was removed and packed RBCs were resuspended with 0.5 mmol/L HOCl in PBS. After this, the incubation was performed as previously described. The absorbance was determined at 540 nm (Suwalsky et al., 2007; Chandler et al., 2013).

Inhibition of Albumin Denaturation

A solution of 0.2% (w/v) of egg albumin was prepared in a PBS (pH 6.4). A volume of 50 µL the extract mixed with cow's milk or standard at different concentrations was added to 5 mL of this stock solution. The test tubes were heated at 72 °C for 5 min and then cooled. The absorbance of these solutions was determined at 660 nm (Karthik et al., 2013).

Results and Discussion

DPPH Scavenging Activity

Figure 1 shows the percentage inhibition of DPPH radical scavenging activity by the samples tested. Corresponding to the concentration of antioxidant required to trap 50% of DPPH radicals, the IC50 was used as a comparative value for the different samples studied. The IC50 value of ascorbic acid was $2.359 \pm 0.091 \mu\text{g/ml}$, which was relatively lower than the IC50 ($7.81 \pm 0.402 \mu\text{g/ml}$) of the aqueous extract. Despite this disparity between these two values, it remains significantly closer to that recorded for the mixture of plant extract and cow's milk IC50 ($5.215 \pm 0.759 \mu\text{g/ml}$).

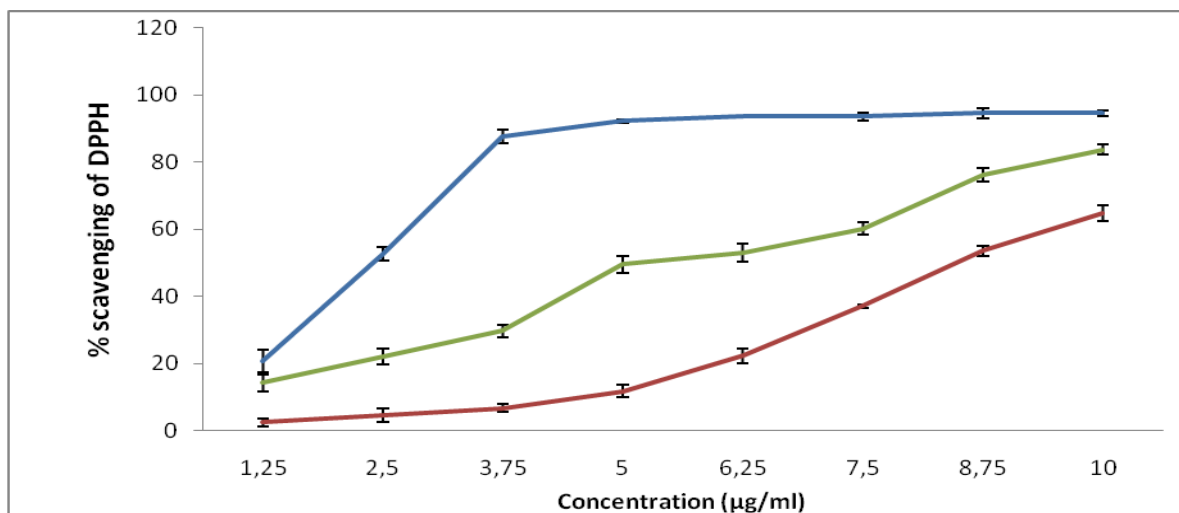


Figure1. DPPH radical scavenging activity of ascorbic acid, aqueous extract of *Malva Sylvestris* alone and combined with cow milk.

Hydroxyl Radical Scavenging

The ability of *Malva Sylvestris* aqueous extract alone and combined with cow milk to compete with salicylic acid for hydroxyl radicals is the principle applied to the evaluation of its ability to scavenging these radicals. As shown in Figure 2, Hydroxyl radical scavenging increased with increase in concentration. The ascorbic acid ($IC_{50} = 758.83 \pm 7.40 \mu\text{g/ml}$) showed more effective scavenging ability when compared to that of aqueous extract ($IC_{50} = 971.28 \pm 27.12 \mu\text{g/ml}$) and combined with cow milk ($IC_{50} = 381.44 \pm 37.12 \mu\text{g/ml}$).

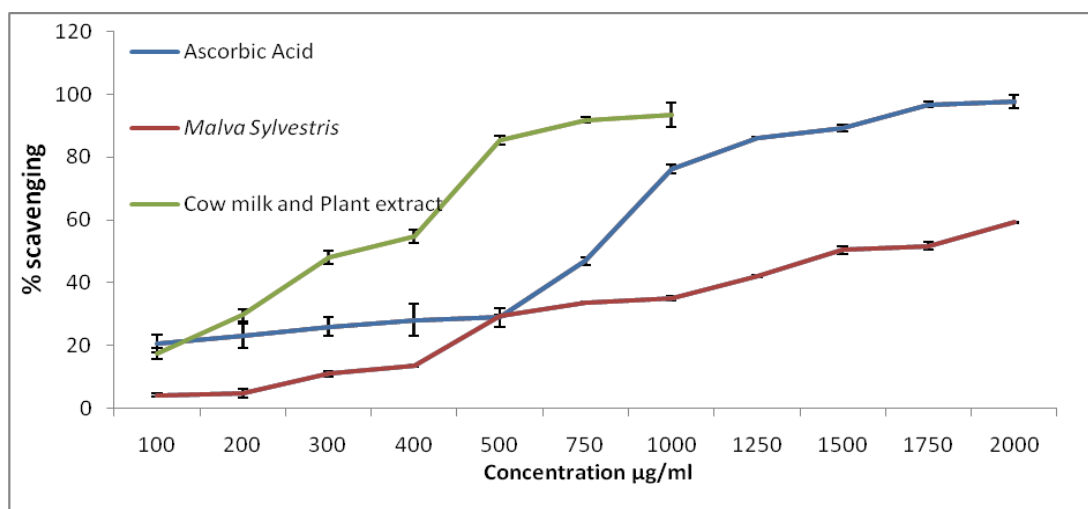


Figure 2. Hydroxyl radical scavenging activity of ascorbic acid, aqueous extract of *Malva Sylvestris* alone and combined with cow milk.

Hydrogen Peroxide Radical Scavenging Activity

Scavenging activity of hydrogen peroxide for extract alone, combined and ascorbic acid as reference compound in terms of effective concentration was remarkably different and shown to be 69.12%, 95.46%, 96.4% and respectively (Figure 3).

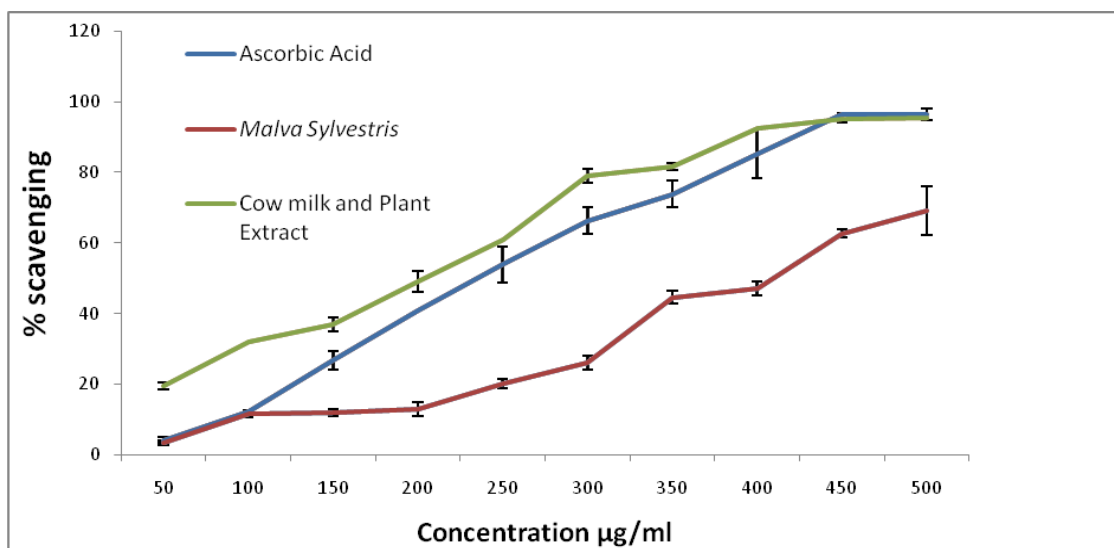


Figure 3. Hydrogen peroxide radical scavenging activity of ascorbic acid and aqueous extract of *Malva Sylvestris* alone and combined with cow milk.

According to the results, *Malva Sylvestris* showed an activity dependent on the concentration and the H_2O_2 scavenging IC_{50} was $431.06 \pm 11.72 \mu\text{g/ml}$, which indicates a distant effective scavenging potential referring to ascorbic acid IC_{50} ($259.95 \pm 9.33 \mu\text{g/ml}$). the data collected shows a rapprochement between this last value and that recorded for the combination of plant extract, cow's milk IC_{50} (205.52 ± 12.03).

Ferrous Ion Chelating Activity

Because of a possible secondary antioxidant activity, we have been interested in one of the most important mechanisms and that is the chelating of pro-oxidant metals such as Iron. The test put in place is based on the formation of a complex between Ferrozine and Fe²⁺ with a characteristic red color. In the presence of chelating agent, the complex formation is disrupted and the red color is decreased. Measurement of color reduction, therefore, allows the estimation of the chelating activity of the plant extract. The metal chelating effect of investigated extract and EDTA were dependent on concentration (Figure 4). EDTA (IC₅₀ = 57.21 ± 0.44 µg/ml) in this assay demonstrated relatively high activity in comparison to extract (IC₅₀ = 74.631 ± 1.19 µg/ml). However, this difference is accentuated when milk is added to the aqueous extract studied (IC₅₀ = 506.84 ± 54.50 µg/ml)

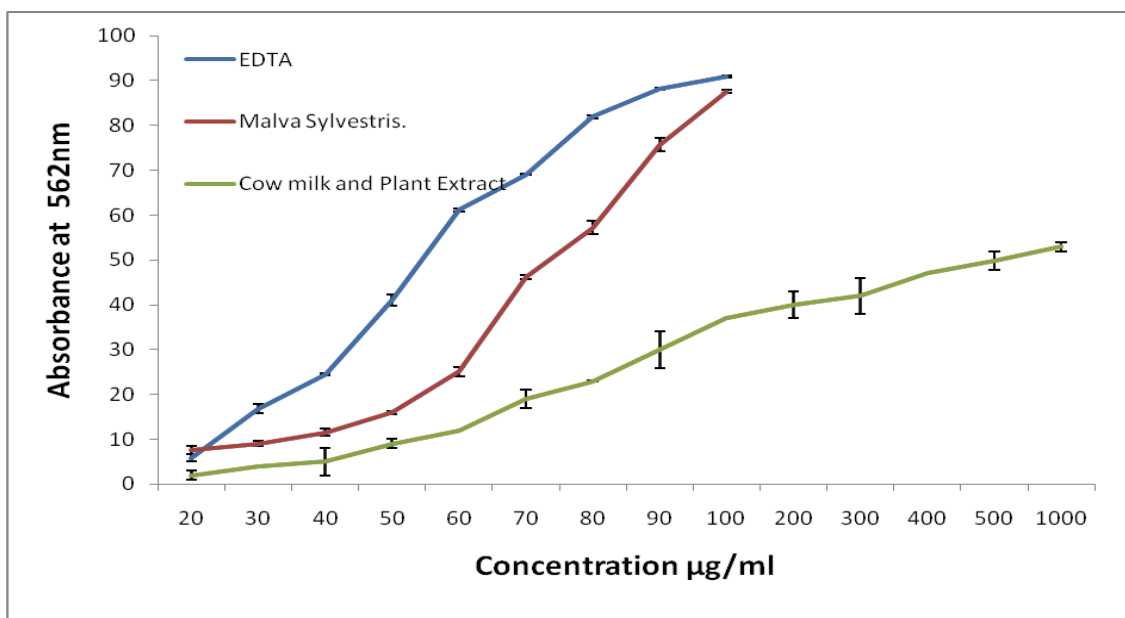


Figure 4. Ferrous ion chelating activity of EDTA and aqueous extract of *Malva Sylvestris* alone and combined with cow milk.

Ferric Reducing Power

Extract showed concentration-dependent reducing power. However, its reducing power (IC₅₀=46.7 ± 0.85 µg/ml) was lower than that of ascorbic acid (IC₅₀=88.17±1.39 µg/ml) (IC₅₀=211.02 ± 35.27 µg/ml).

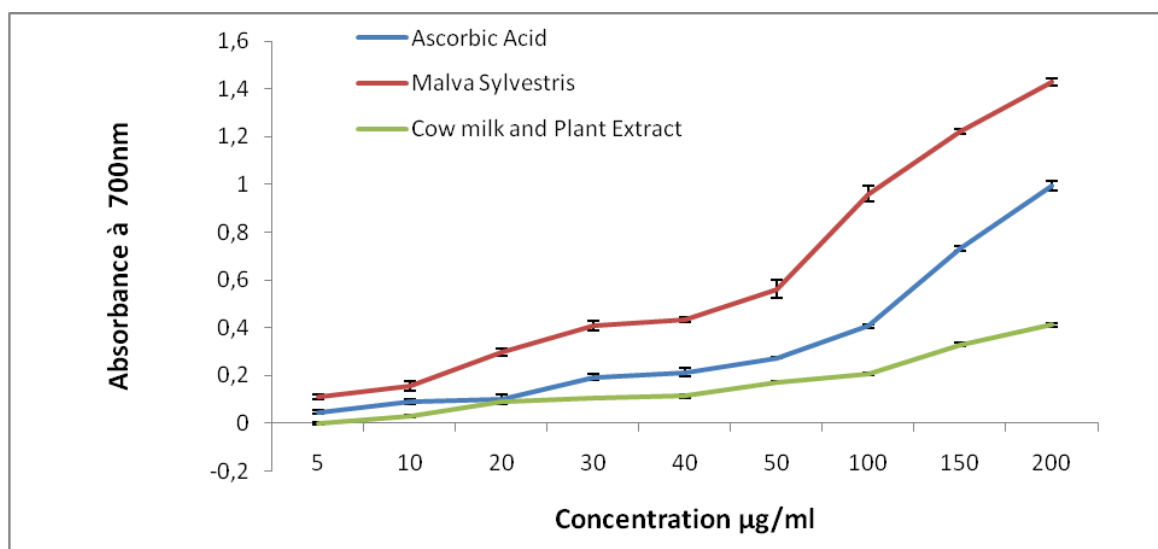


Figure 5. Ferric reducing power of ascorbic acid and aqueous extract of *Malva Sylvestris* alone and combined with cow milk.

Total Antioxidant Capacity

The basic principle of this test is based on the formation of a green phosphate / Mo (V) complex resulting from the reduction of Mo (VI) to Mo (V) by the acidic pH extract. Results showed antioxidant activity of extract and ascorbic acid in dose dependent manner at concentration 100 to 500 µg/ml. The IC₅₀ value of antioxidant capacity for the ascorbic acid (292 ± 7.54 µg/ml) was greater than extract IC₅₀ (348.357 ± 6.03 µg/ml).

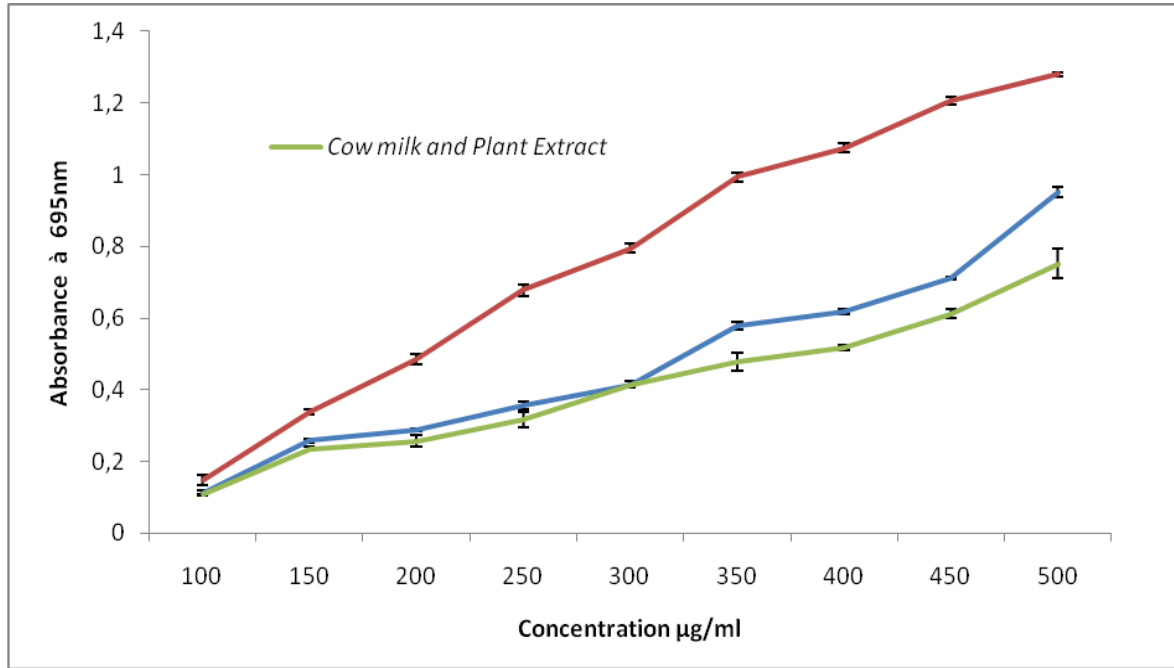


Figure 6. Total antioxidant capacity of ascorbic acid and aqueous extract of *Malva Sylvestris* alone and combined with cow milk.

Heat Induced Hemolysis

As shown in Figures 7, the extract prevented the erythrocyte membrane against lysis induced by heat compared with aspirin. The maximum protection recorded at 1200 µg/ml is 62.97 ± 2.1% for aspirine followed by the two samples studied for which no significant difference in their percentage of protection was found and around 90% for both

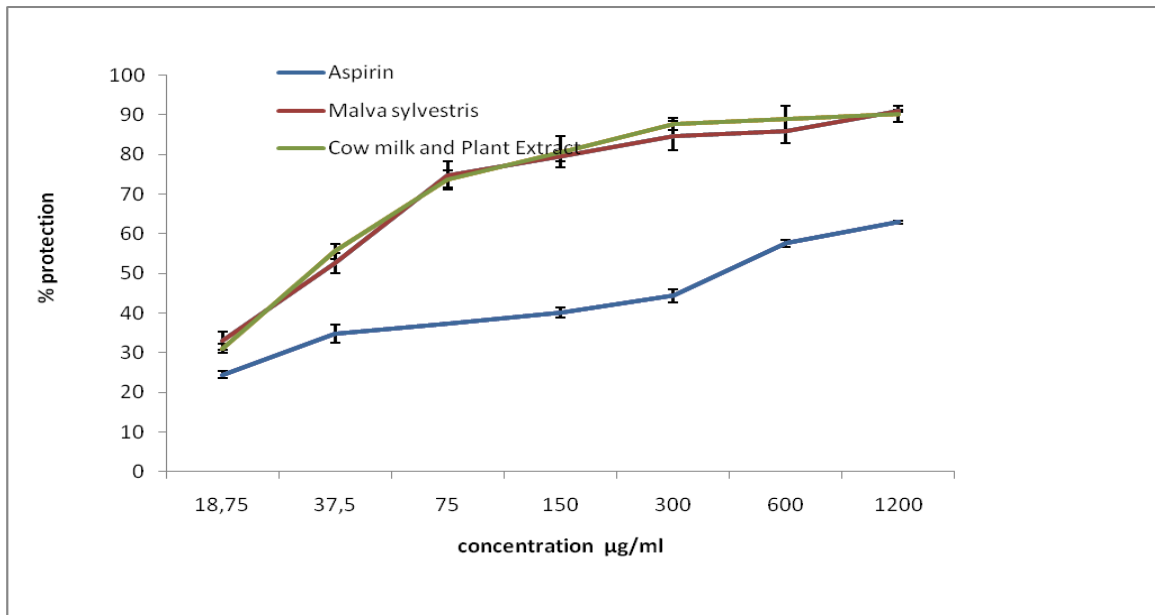


Figure 7. Effect of ascorbic acid and aqueous extract of *Malva Sylvestris* alone and combined with cow milk. on heat-induced hemolysis.

Hypotonic Solution Induced Hemolysis

In favor of the results indicated in Figure 8, it clearly appears that there are few differences between the two samples studied, namely the native extract of *Malva sylvestris* and that with added cow's milk, taking into account their protective power of red blood cells against -with respect to osmotic stress. Thus we respectively record a maximum of protection at the concentration 0.3% NaCl of the order of 79.57% and 80.48% and at the concentration of 0.7% NaCl 60.3% 66.12%.

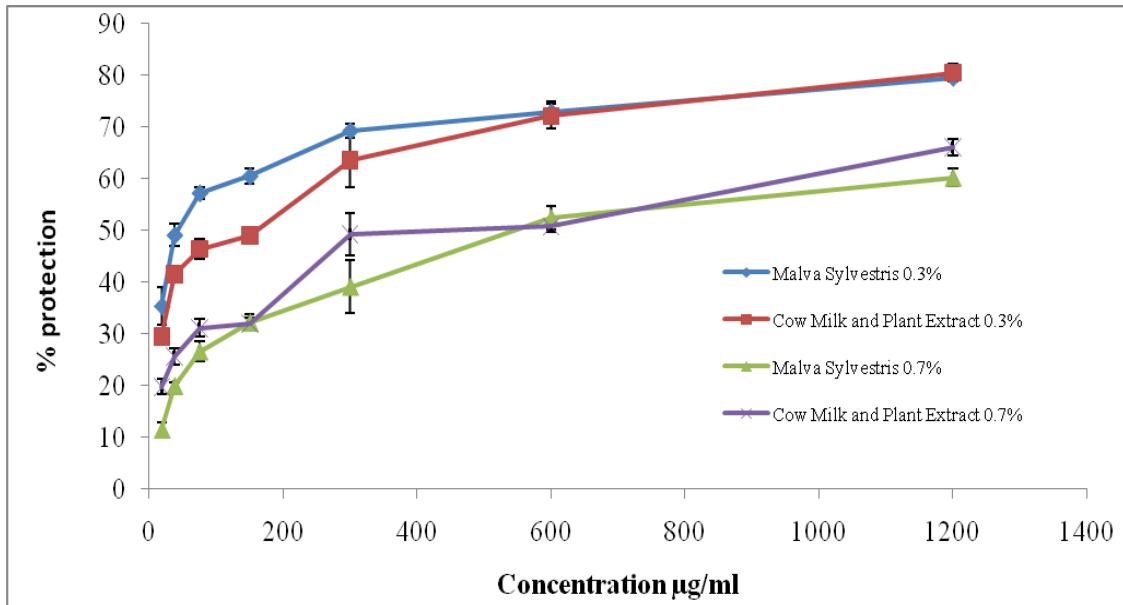


Figure 8. Effect of ascorbic acid and aqueous extract of *Malva Sylvestris* alone and combined with cow milk. on hypotonicity-induced hemolysis.

Oxidant Induced Hemolysis

The results that we recorded taking into account oxidative stress indicate in Figure 9 that the extract added with cow's milk displays a protection percentage of 80.32%, which remains relatively higher than the native extract where we find a protection rate of 63.10%.

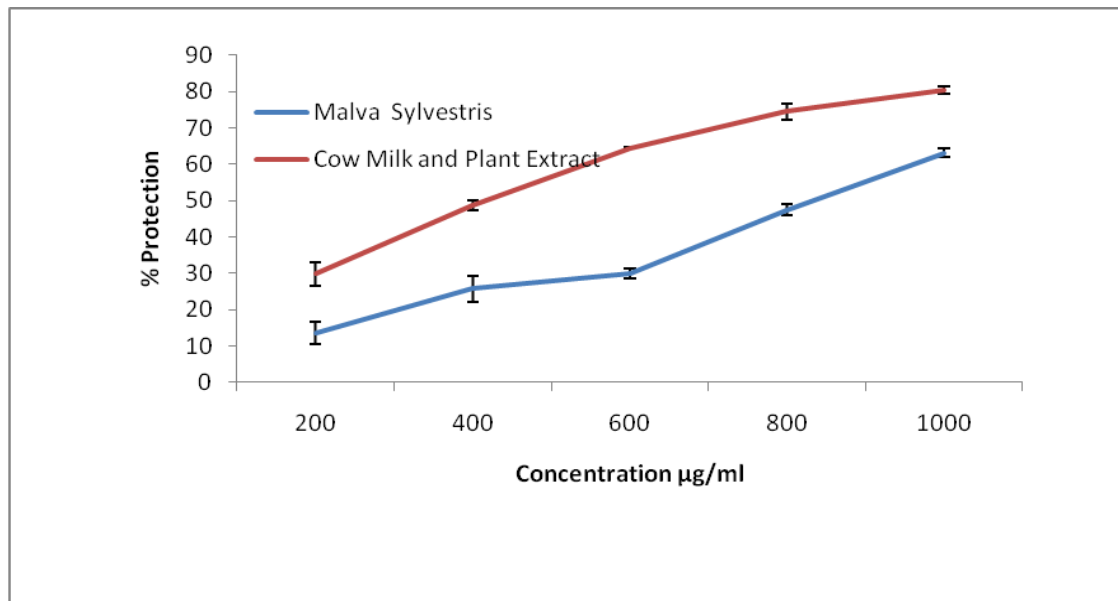


Figure 9. Effect of ascorbic acid and aqueous extract of *Malva Sylvestris* alone and combined with cow milk. on HOCl induced hemolysis.

As little data is available in the literature regarding the anti-inflammatory and antioxidant potential of the combination of the aqueous extract of *Malva Sylvestris* and cow's milk, it is difficult to compare our results. Compared to the data we collected on the aqueous extract of *Malva sylvestris* leaves in the Tizi-Ouzou region, the sample from the M'sila region presents relatively superior antioxidant and anti-inflammatory properties (Moualek et al., 2020). As for the potential of the activities expressed by the combination of the aqueous extract of *Malva sylvestris* leaves with cow's milk, the results recorded mark three levels of interactions. Thus, we note the existence of a synergy between cow's milk and the plant extract for radical inhibition (DPPH[•] and OH[•]) as well as the protection of biological membranes against an oxidant (HOCl). Considered as the most reactive free radical, hydroxyl radical is most often implicated in the pathology of free radical because of its ability to interact with intracellular targets such as DNA, thus causing significant damage. In a second level of interaction and for the chelation tests, reduction of iron and molybdate, we find a negative effect of the association which gives rise to a loss of the original potential of the plant extract. Knowing that the chelation potential of a biomolecule indicated a significant protective activity of the extract against oxidative damage by sequestering iron (II) ions that may turn into catalyst for Fenton-type reactions or participate in metal-catalyzed hydroperoxide decomposition reactions (Adefegha & Oboh, 2011). The absence of repercussions of the association between the plant extract and cow's milk was recorded for the trapping of H₂O₂ as well as the protection of erythrocyte cells against osmotic and thermal stress. Knowing that Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it can give rise to hydroxyl radical in the cells (Halliwell, 1991; Kumar et al., 2012). Thus, the removal of H₂O₂ is very important for antioxidant defense in cell systems or food (Turkoglu et al., 2010). Knowing that the erythrocyte membrane resembles lysosomal membrane and as such, the effect of extracts on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (Omale & Okafor, 2008). The anti-inflammatory activity can also be explained by the inhibition of release of lysosomal content at the site of inflammatory (Govindappa et al., 2011). This study demonstrated in vitro antioxidant and anti-inflammatory activities of, through scavenging, chelating and reducing activities indicated in the performed tests, showed a good antioxidant activity. Furthermore, the protection of RBCs indicated a membrane stabilizing effect of the extract. Conclusion It emerges from this preliminary study that the mixture studied presents a significant anti-radical potential as well as strong protection of erythrocyte membranes with respect to HOCl. The sum of the data collected indicates a disparity of results taking into account the uniformity of the impact of the combination of plant extract and cow's milk on the antioxidant and anti-inflammatory potential of the mixture. As we are faced with two matrices (plant and animal) rich in compounds of diverse and varied natures, it would be interesting to deepen the investigations and to highlight the type of interactions existing between the constituents of the mixture. It would also be interesting to verify the impact of *Malva Sylvestris* supplementation on the composition of cow's milk as well as on its antioxidant and anti-inflammatory potential, in vitro and in vivo.

Scientific Ethics Declaration

The authors declare that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the authors.

Acknowledgements

* This article was presented as an oral presentation at International Conference on Veterinary, Agriculture and Life Sciences (www.icvals.net) held in Antalya/Turkey on November 16-19, 2023.

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To cite this article:

Moualek, I., Benarab, K., Belounis, Y., & Houali, K. (2023). Anti-inflammatory and antioxidant activities of cow's milk supplemented with aqueous extract of *malva sylvestris*. *The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)*, 11, 1-11.