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Evaluation of the Anti-inflammatory and Anti-hemolytic Potential of Polyphenolic Components of Common Mallow (*Malva sylvestris*)

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ABSTRACT

Background: This work aims at the assessement of anti-inflammatory and anti-hemolytic effect of *Malva sylvestris*.

Methods: The anti-inflammatory potential was evaluated by the inhibition of protein denaturation method. It was followed by the study of anti-hemolytic potential, based on two methods (haemolysis by hydrogen peroxide (H_2O_2) and by hypotonic haemolysis).

Result: The macerated aqueous extract of *M. sylvestris* (250 µg/ml) exhibited the highest inhibition percentage of BSA denaturation compared to other extracts but it appeared to be slightly lower than the drug diclofenac sodium (80.97±1.23%). On the other hand, the macerated aqueous extract showed more protective power against haemolysis (93.42±3.45%). While it was almost similar to the percentage recorded for ascorbic acid (93.68±3.21%). For the second method, it was observed that the decocted acetone extract of *M. sylvestris* showed a rate of haemolysis inhibition which was the highest (98.09±1.26%) but that it remained slightly lower than aspirin (98.77±0.44%). All of these results showed that *M. sylvestris* extracts have interesting anti-inflammatory and anti-haemolytic potential and therefore have considerable interest as an alternative treatment against inflammatory mechanisms.

Key words: Anti-hemolytic, Anti-inflammatory, *Malva sylvestris*, Polyphenols.

INTRODUCTION

Due to the availability of thousands of bioactive natural substances, plants served as the foundation for the pharmacopeia and therapies of ancient civilizations (Hussain *et al*., 2014). Among these notably phenolic chemicals give medicinal efficacy and their studies are becoming more crucial due to their health benefits as they have been effective against cancer, atherosclerosis, diabetes, neurodegenerative diseases and arthritis, which are linked to oxidative stress and inflammatory pathways (Hui *et al*., 2017).

A complicated biological reaction of vascular tissues to damaging stimuli is often referred to as inflammation. It involves increased membrane damage, protein denaturation and vascular permeability (Ferrero-Millani *et al*., 2007). The body's response mechanism is sped up by the chemical mediators from wounded or migrating tissues and cells (Chandra *et al*., 2012). Non-steroidal anti-inflammatory treatments that treat diseases can be used to treat inflammation. The use of more natural alternatives, such as medicinal herbs, is being encouraged by patients' rising mistrust of allopathic medications and their side effects (Yougbaré-Ziébrou *et al*., 2016).

Malva sylvestris L., commonly referred as mallow, having flavonoids and other antioxidant polyphenolic chemicals, their leaves have demonstrated highly potent antioxidant effects. Extracts of *M. sylvestris* were used as a legume and to treat problems with the liver (Jaradat *et al*., 2015). The present investigation was planned to evaluate the anti-inflammatory *in vitro* and anti-hemolytic potential of *M. sylvestris* extracts.

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MATERIALS AND METHODS

The experiment was conducted during 10-2020 and 06-2021 at the institute of nature and life sciences in Mustapha Stambouli University, Mascara, Algeria.

Plant material

Common mallow (*Malva sylvestris* L.) were harvested from Tighennif region, Mascara (Algeria) (35° 25′ 00″ north, 0° 19′ 59″ east) in March 2021. The identification and confirmation of the species was carried out by botanist of the biology department in Mustapha Stambouli University, Mascara. After harvest, *M. sylvestris* was cleaned and then prepared for the extraction.

Preparation of extracts

For the maceration, 10 g of *M. sylvestris* were macerated in 100 ml of the solvent (acetone or distilled water) with magnetic stirring for 48 hoursat room temperature. For the decoction, 10 g of *M. sylvestris* were combined with 100 ml of the solvent (acetone or distilled water) in a reflux apparatus. In order to create a dry powder that could be stored in sterile bottles, the mixture was filtered and then concentrated in a rotavapor (Romani *et al*., 2006).

In vitro anti-inflammatory assay

Protein denaturation inhibition

Using the protein denaturation inhibition method described by Lavanya *et al.* (2010), the *in vitro* anti-inflammatory capacity of *M. sylvestris* extracts was assessed. Briefly, 0.05 ml of extract (500 µg/ml) or Diclofenac sodium as a reference anti-inflammatory with the same concentration was added to 0.45 ml of bovine serum albumin (BSA) 5%. The control included 0.05 ml of distilled water and 0.45 ml of BSA 5%. After bringing the pH of each of the listed solutions to 6.3, the combination was incubated for 20 minat 37°C. The temperature was then raised to 57°C for 3 minutes before being lowered. The solutions received 2.5 ml of the PBS (pH 6.3) phosphate buffered saline solution. Absorbance (A) was determined at 420 nm and protein denaturation inhibition was estimated using the formula:

$$\text{Inhibition percentage (\%)} = \frac{100 - (A_{control} - A_{sample})}{(A_{control})} \times 100$$

Anti-hemolytic activity

Hemolysis by hydrogen peroxide (H_2O_2)

The test was performed using the Girish *et al.* (2012) method to assess the preventive impact of *M. sylvestris* extract against oxidative damage caused by free radicals on human erythrocytes. The reaction mixture was added to 200 µl of 10% (v/v) red blood cell suspension, 50 µl of extracts (3.12-50 mg of the extract prepared in PBS) and 100 l of H_2O_2 200 µM. The mixture was then incubated for 30 minutes at 37°C before being centrifuged at 2000 rpm for 10 minutes. 800 µl of PBS were added to 200 µl of the supernatant and the absorbance at 410 nm was calculated. The control was made by completely hemolyzing the erythrocyte suspension incubated with H_2O_2 directly and the absorbance of the supernatant was determined as previously indicated. The standard antioxidant employed was ascorbic acid and the hemolysis percentage was computed by assuming that the hemolysis with H_2O_2 200 µM was 100%.

$$\text{Protection percentage (\%)} = \frac{A_{control} - A_{Sample}}{A_{control}} \times 100$$

Membrane stabilization assay

The method described by Debnath *et al.* (2013) was performed to study the efficacy of *M. sylvestris* extracts to prevent hemolysis brought on by the hypotonic solution. In brief, 1 ml of extract at gradual concentrations (125, 250, 500 and 1000 µg/ml) or 1 ml of aspirin (0.1 mg/ml), which was used as a reference, were added to 100 µl of the erythrocyte's suspension diluted to 10% in an isotonic solution. Erythrocyte suspension in a hypotonic solution made up the control that was thought to have undergone 100% hemolysis. Centrifugation was performed at 3000 rpm for 10 minutes following an incubation period at room temperature for10 minutes. At 450 nm, the supernatant's absorbance was determined. The following formula was used to determine the percentage of hemolysis inhibition:

$$\text{Hemolysis percentage (\%)} = \frac{A_{Sample}}{A_{Control}} \times 100$$

The membrane stabilization percentage was determine dusing the formula:

$$\text{Protection percentage (\%)} = \frac{100 - A_{Sample}}{A_{Control}} \times 100$$

Statistical analysis

Data were presented as means±SD. Statistical analysis was assessed by one-way of variance ANOVA analysis. The difference was considered significant at $P<0.05$.

RESULTS AND DISCUSSION

In vitro anti-inflammatory assay

Protein denaturation inhibition

Fig 1 showed the percentage of inhibited BSA denaturation that *M. sylvestris* extracts presented compared to diclofenac sodium. When denatured, the majority of biological proteins ceased to function biologically. The modification of electrostatic, hydrogen, hydrophobic and disulfide linkages in proteins during protein denaturation may be the cause of the generation of autoantigens in inflammatory disorders (Kar, 2012).

It was shown that the macerated aqueous plant extract (250 µg/ml) exhibited the highest percentage inhibition of BSA denaturation (79.7±1.02%) compared to the decocted aqueous, decocted acetonic and macerated acetonic extract which showed an inhibition equal to 75.9±2.3, 67.1±2.5 and 18.47±1.11%, respectively. The comparison with diclofenac sodium showed that the latter presented the inhibition (%) higher than all the extracts (80.97±1.23%). There was evidence that nonsteroidal anti-inflammatory medications inhibited the synthesis of pro-inflammatory prostaglandins and protein denaturation (Sivaraj, 2017).

The inhibitory activity of *M. sylvestris* extracts could be due to the interaction of components with two sites rich in tyrosine, threonine and lysine. Duganath *et al.* (2010) reported that plant components used in traditionally exerted their pharmaceutical effects through their ability to bind to plasma proteins. Shallangwa *et al.* (2013) confirmed that the inhibition of albumin denaturation was attributed to the action of flavonoids. Many studies have supported that the compound malvidin 3-glucoside appeared to be primarily

responsible for this effect. Thus, the leaves of *M. sylvestris* possessed topical anti-inflammatory properties (Benso *et al*., 2015; Mousavi *et al*., 2021). It was shown that the extracts of *M. sylvestris* have a pharmacological capacity, in particular through anti-inflammatory and anticancer effects (Paul, 2016; Anuradha *et al*., 2018).

Martins *et al.* (2014) measured the pro-inflammatory mediators PGE2 in U937 cells to assess the anti-inflammatory effects of alcoholic extracts of *M. sylvestris*. They hypothesized that the modification of these mediators may be connected to the anti-inflammatory effects induced by *M. sylvestris*. Several terpenoids, including blumenol A, linalool, malvone, linalool-1-oic acid and dehydrovomifoliol have been found in the extract of fresh *M. sylvestris* leaves. Some of these ingredients have been noted to have anti-inflammatory and antioxidant properties (Ghosh and Gaba, 2013; Hamedi *et al*., 2015).

Anti-hemolytic activity

Hemolysis by hydrogen peroxide (H_2O_2)

Table1 presented the percentage protection of *M. sylvestris* extracts against hemolysis by hydrogen peroxide (H_2O_2) compared to ascorbic acid. The protection percentage against hemolysis induced by hydrogen peroxide (H_2O_2) showed a proportional relation with the concentration of *M. sylvestris* extract. For example: for the macerated aqueous extract, increasing the concentration from 3.12 to 50 µg/ml gave a percentage of 38.17±2.31 to 93.42 ±3.45%. While the other extracts presented percentages of protection slightly lower than this extract (86.35±2.12%, 85.48±0.99% and 79.69±2.7%) for the decocted aqueous, macerated acetonic and decocted acetonic respectively, for a concentration of 50 µg/ml. On the other hand, it was noted that ascorbic acid recorded the highest protection (93.68±3.21%) for a concentration of 50 µg/ml. Compared to the protective effect reported by Ghaffar and El-Elaimy (2012) (48.52±3.03% at 500 µg/ml), the extracts showed better protective activity for 1/10 of this concentration (50 µg/ml).

Inhibition of H_2O_2 was very important step for antioxidant defense in cellular systems (Turkoglu *et al*., 2010). This was because even though H_2O_2 itself was inactive, it can be toxic to cells as it can give rise to a hydroxyl radical (Kumar *et al*., 2012). When red blood cells were treated with H_2O_2 (toxic), the percentage of hemolysis was found to be increased because of the oxidative nature of hydrogen peroxide which allowed to cell membrane degradation and release of hemoglobin from the cell (Devjani and Barkha, 2011). Hydrogen peroxide also caused the mobilization of iron by calcium *via* a Fenton reduction which stimulated the production of hydroxyl radicals (Anirban *et al*., 2013). All these combined factors caused destabilization of the cell membrane, which was probably the key in cell lysis (Devjani and Barkha, 2011).

The protective activity of *M. sylvestris* extracts against hemolysis by hydrogen peroxide was attributed to the action of polyphenols to scavenge and inhibit free radicals. Polyphenols were known to cause scavenging activity due to their ability to lose protons, form chelators, dismutate radicals and shed hydrogen atoms from their hydroxyl groups with radicals (Aksoy *et al*., 2013). The quantitative

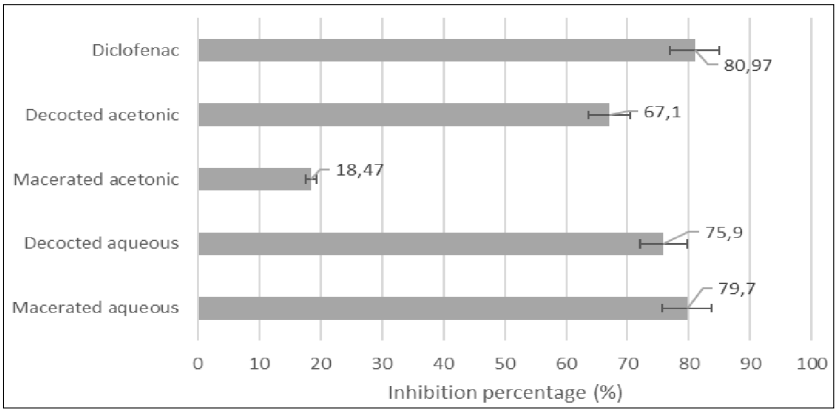

Fig 1: Percentage of BSA denaturation inhibition by *M. sylvestris* extracts.

Table 1: The percentage of protection of *M. sylvestris* extracts against hemolysis by hydrogen peroxide (H_2O_2).

Extraits	Concentrations (µg/ml)				
	3.12	6.25	12.5	25	50
Macerated aqueous	38.17±2.31	57.31±1.44	68.64±0.5	79.70±2.11	93.42±3.45
Decocted aqueous	31.39±0.56	43.71±3.55	58.59±4.55	75.88±1.23	86.35±2.12
Macerated acetonic	30.84±0.66	45.10±0.87	57.26±1.95	65.68±0.56	85.48±0.99
Decocted acetonic	29.14±1.34	35.87±1.9	53.11±2.76	67.07±1.76	79.69±2.7
Ascorbic acid	41.31±2.55	56.97±1.9	79.76±2.89	90.12±3.11	93.68±3.21

analysis carried out on these extracts showed us the richness of the studied plant in biomolecules. Polyphenols possessed numerous biological effects, mainly attributed to their antioxidant activities in scavenging free radicals, inhibiting peroxidation and chelating transition metals (Omale and Idris, 2014). Polyphenols were the main components for the scavenging ability of *M. sylvestris* extracts (Dellagreca *et al*., 2009). Due to the scavenging effect of these bioactive elements, *M. sylvestris*was able to scavenge free radicals, leading to the defense against biological molecules oxidation.

According to Bonarska-Kujawa *et al.* (2011), phenolic chemicals were integrated into the outer hydrophilic layer and have no impact on the fluidity of the hydrophobic portion. The incorporation of the extract's phenolic components into the hydrophilic portion of the membrane appeared to operate as a barrier between the cell and harmful external elements like free radicals (Louerred *et al*., 2016).

Membrane stabilization assay

The anti-hemolytic effect of *M. sylvestris* extracts was evaluated against hypotonic hemolysis to study the stabilization of the red blood cell membrane against osmotic stress. For comparative purposes, aspirin was used. Table 2 and 3 presented the hemolysis% and the protection% of the extracts of *M. sylvestris* against hypotonic hemolysis. It was observed to be inversely proportional to the concentration of *M. sylvestris* extracts. This showed a proportional relation between the protection against hemolysis and the concentrations of the extracts. For the concentration of 1000 µg/ml, a hemolysis rate of 8.02±0.33%, 16.23±0.54%, 21.64±1.67% and 1.91±0.78% was recorded for the macerated aqueous extract, decocted aqueous extract, macerated acetonic and decocted acetonic, respectively. While aspirin showed the lowest hemolysis rate (1.22±0.09%). On the other hand, it was noted that the decocted acetonic extract of *M. sylvestris* showed a hemolysis inhibition rate which was 98.09 ± 1.26%. It was followed by macerated aqueous extract, decocted aqueous extract and macerated acetonic by an average of 91.97±2.87%, 83.76±2.56% and 78.35±0.96%, respectively. It was also found that aspirin recorded the highest percentage of protection (98.77±0.44%) for a concentration of 1000 µg/ml.

A hypotonic medium was a medium oflower osmotic pressure than the intracellular pressure; this imbalance induced a diffusion of water towards the interior of the cell (hypertonic medium) through the membrane. The massive influx of water into the red blood cell caused it to swell then burst and released its cytoplasmic content, in particular hemoglobin. This was the phenomenon of hemolysis which was observed at NaCl concentrations below 0.9% (Vadivu and Lakshmi, 2008).

By lipid peroxidation caused by the free radicals, cell membranes damages will further increase the cell's susceptibility to several harm (Umapathy *et al*., 2010). By regulating the passage of sodium and potassium ions, membrane proteins allow for the regulation of cell volume and water content. Damage to the membrane will impact this function. Inhibiting or delaying the lysis of these cells and the subsequent release of their cytoplasmic contents can reduce tissue damage and, consequently, the inflammatory response (Arora, 2019).

The cell membrane stabilizing potential of *M. sylvestris* extracts could be attributed to the presence of oleanolic acid4, a triterpenoid with proven anti-hemolytic and anti-inflammatory properties (Lais *et al*., 2015; De La Cruz *et al*., 2016). Studies have shown that this protective action can be explained by the ability of the extract to modify the influx of calcium into erythrocytes (Chopade *et al*., 2012). So, the stabilization of erythrocytes by extracts could be extrapolated to the stabilization of the lysosomal membrane (Govindappa *et al*., 2011).

Table 2: The percentage of hypotonic hemolysis.

Extraits	Concentrations (µg/ml)			
	125	250	500	1000
Macerated aqueous	51.83±1.99	25.47±1.8	13.78±2.11	8.02±0.33
Decocted aqueous	80.27±1.44	63.69±1.88	32.28±0.22	16.23±0.54
Macerated acetonic	98.08±2.01	95.98±2.43	57.76±2.66	21.64±1.67
Decocted acetonic	30.19±3.77	24.95±1.06	9.07±1.85	1.91±0.78
Aspirine	21.98±0.7	10.64±1.02	3.49±1.99	1.22±0.09

Table 3: The percentage of protection of *M. sylvestris* extracts against hypotonic hemolysis.

Extraits	Concentrations (µg/ml)			
	125	250	500	1000
Macerated aqueous	48.16±2.99	74.52±1.98	86.21±1.05	91.97±2.87
Decocted aqueous	19.72±1.87	36.3±1.56	67.71±2.22	83,76±2.56
Macerated acetonic	1.91±1.09	4.01±2.65	42.23±1.7	78.35±0.96
Decocted acetonic	69.8±2.23	75.04±2.9	90.92±1.43	98.09±1.26
Aspirine	78.01±0.44	89.35±0.76	96.5±1.12	98.77±0.44

It has been demonstrated that the incorporation of phenolic compounds, in particular flavonoids, into the membrane of erythrocytes improved the stability of the latter against hypotonic lysis. This property can be explained by the increase in the volume/surface ratio of the cells which could be obtained either by the expansion of the cell membrane. In addition, the deformability and cell volume of erythrocytes were closely related to the intracellular calcium content. Therefore, it found that the protective effect of the *M. sylvestris* extract would be due to its ability to modify the influx of calcium into erythrocytes (Chopade *et al*., 2012).

According to Vidhya and Shobana (2016), the anti-hemolytic effect of plant extracts was due to their inhibitory effect on the enzymes involved in the production of chemical mediators of hemolysis and inflammation as well as on the metabolism of arachidonic acid. Some polyphenols bind to membrane proteins inducing a change in their conformation. Others bind to PLA2 through hydrophobic interactions with three amino acids from the enzyme active site (Rana and Dahiya, 2019).

CONCLUSION

It can be inferred from the current study that *M. sylvestris* shown the ability to limit protein denaturation to reduce inflammation and to control hemolysis by using a mechanism to stabilize HRBC membrane and also to inhibit H_2O_2 hemolysis. Furthermore, it indicated that the bioactive fraction and its main constituent may be a promising lead for the development of new treatment for the prevention against chronic inflammatory diseases.

Conflict of interest: None.

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