

Anti-inflammatory effect of hydromethanolic extract from *Marrubium vulgare* Lamiaceae on leukocytes oxidative metabolism: An *in vitro* and *in vivo* studies

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Background: *Marrubium vulgare* Lamiaceae, known as white horehound, is frequently used in folk medicine to treat many illnesses from ancient times. **Aims:** The present study is conducted to evaluate the anti-inflammatory activity of hydromethanolic extract of the aerial part of *M. vulgare*. **Materials and Methods:** The oxygen consumption and the production of superoxide anions, by rat pleural polymorphonuclear leukocytes stimulated by opsonized zymosan, were performed. PMNs were collected after induction of an acute inflammatory reaction by injection, in the rat pleural cavity, of suspension of calcium pyrophosphate crystals. Plant extract was tested at a concentration range 10-100 mg/ml and 100-400 mg/kg/day for *in vitro* and *in vivo* assays respectively. **Statistical analysis:** All values are expressed as mean \pm standard error. The significance of differences between control and treated samples *in vitro* was analysed using the Student's *t*-test. For *in vivo* studies, data were analysed using one-way analysis of variance. Differences with $P \leq 0.05$ were considered to be statistically significant. **Results:** Hydromethanolic extract of *M. vulgare* showed, *in vitro* a significant anti-inflammatory effect in a dose-dependent manner. *In vivo*, only the high amounts of the plant extract (300 and 400 mg/kg/day) exhibited a significant anti-inflammatory effect. The plant extract contains a high amount of polyphenols. **Conclusion:** Hydromethanolic extract of *M. vulgare* had an anti-inflammatory effect, which corroborates the usefulness of this plant in the treatment of inflammatory disorders.

Key words: Anti-inflammatory effect, *Marrubium vulgare*, oxygen consumption, polymorphonuclear leukocytes, polyphenols, superoxide anions generation

INTRODUCTION

Polymorphonuclear leukocytes (PMNs), the most numerous of neutrophils in human, play an important function of resistance against invading microorganisms. Upon stimulation, there is a marked increase in oxygen (O_2) consumption, which is converted into various reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide, hydroxyl radicals or similar species.^[1] ROS generated by phagocytes play a crucial role in the inflammatory response, which is required for immune defence and regeneration after injury.^[2] However, when acute inflammation is not resolved, chronic inflammation occurs, which has a detrimental effect in various

disorders (atherosclerosis, cancer etc.).^[3,4] Thus, the objective of this study is to search for some natural compounds capable to modulate ROS metabolism of PMNs.

Marrubium vulgare Lamiaceae, commonly named white horehound, is a perennial aromatic herb of the Lamiaceae family. In Moroccan traditional medicine, decoction and/or infusion of aerial parts of *M. vulgare*, locally named 'merriwut' or 'merriwa', are used as expectorant, hypoglycaemic, anti-thyphoid, antipyretic, anti-diarrheic, anti-icteric, diuretic, choleric and tonic, in bilious stimulation and for menstrual pains.^[5]

Among its various use in folk medicine, the supposed anti-inflammatory activity has received our attention. Therefore, the current study was designed to investigate the *in vitro* and *in vivo* effect of hydromethanolic extract obtained from *M. vulgare* on the oxidative metabolism of pleural rat PMNs assessed by measurement of two metabolic parameters: O_2 consumption and $O_2^{\cdot-}$ production.

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MATERIALS AND METHODS

Plant Material

M. vulgare was collected during spring 2011 from the eastern region (Taza) of Morocco. Plant material was authenticated by Pr. R. Tellal (laboratory of plant ecology, Faculty of Sciences, El Jadida, Morocco). A voucher specimen (reference 15 L/11) is kept on file in our laboratory.

Preparation of Extract

Fresh aerial parts were washed twice, air-dried, powdered mechanically and sieved using a fine muslin cloth. Plant extract was prepared by macerating 150 g of the fine powder in a hermetically-closed glass vessel for 24 h at room temperature (25°C) under occasional shaking with 1500 ml of mixture distilled water-methanol (3 V/2 V).

The crude preparation was clarified by centrifugation at 5000 g for 45 min (Sigma 2K15). The supernatant was concentrated, in a rotary evaporator, under reduced pressure at 25°C. The extract was sterilized by filtration through nitrocellulose membranes (0.22 µm). Sterile extract (40.45 g) was stored at -20°C until use.

Animals

Male Sprague-Dawley rats (weighing 180-200 g) were used for all experiments. Each group consisted of 5 animals. The rats were maintained under standard laboratory conditions with food and water *ad libitum*.

All protocols performed in this study were conducted in accordance with internationally accepted principles for use and care of laboratory animals.

Preparation of Opsonized Zymosan

OZ was prepared by incubating 10 mg of zymosan A (Sigma) in 800 µl of phosphate buffer saline (PBS) and 200 µl of freshly prepared normal rat serum for 40 min at 37°C. After centrifuging this at 1700 g for 15 min, the OZ was resuspended at a concentration of 10 mg/ml in PBS and stored at -80°C before use.^[6]

Isolation of PMNs

PMNs were collected from the rat pleural cavity 4 h after injection of 1 ml of a 1% suspension of calcium pyrophosphate (CaPP) crystals in sterile PBS according to the modified technique described by Willoughby *et al.*^[7] Cells (95% PMNs) were washed twice in Hank's balanced salt solution, resuspended in PBS and stored at +4°C for O₂⁻ production and O₂ consumption assessment. Viability of the cells was determined by trypan blue exclusion test.

For *in vitro* study, rat pleural PMNs (2 × 10⁷ cells/ml) were incubated with various concentrations of *M. vulgare*

extract (10, 20, 40, 60, 80 and 100 mg of crude extract/ml of 0.9% NaCl) at 37°C for 30 min with gentle shaking. Control cells were incubated in medium vehicle alone (0.9% NaCl). The incubation was stopped by the addition of 4 ml of ice-cold PBS and the mixture was centrifuged at 250 g for 5 min at +4°C. The cell pellet was then resuspended in 1 ml of PBS and stored at +4°C (less than 30 min) before O₂ consumption and O₂⁻ production assessment.

For *in vivo* study, 35 rats were used. Treated group (*n* = 25) was subdivided into five lots of five animals each. Four lots received *M. vulgare* extract orally by an intragastric tube daily for 7 days at a dose of 100, 200, 300 or 400 mg/kg body weight/day while the 5th lot receives any treatment (control). Untreated group (*n* = 10), subdivided into five lots of two animals each, received or not (control) in the same conditions vehicle solution (0.9% NaCl) at a dose of 100, 200, 300 or 400 mg/kg body weight/day. At 8th day, pleural PMNs from treated and untreated groups were collected according to the method described above.

Cell Viability Assay

The potential toxicity of the studied extract towards PMNs was measured by the release of the cytosolic enzyme lactate dehydrogenase (LDH) as previously described by Paul *et al.*^[8] The presence of LDH activity in the extracellular medium represents cellular damage. 400 µl of PMNs suspension (5 × 10⁶ PMN/ml) was incubated at 37°C for 30 min in the presence of *M. vulgare* extract at different concentrations (10-100 mg/ml). After centrifugation, LDH activity was determined by the rate of reduced NADH (nicotinamide adenine dinucleotide) converted to NAD⁺ at 340 nm in the presence of pyruvate. The results were expressed as the percentage of LDH activity.

Determination of O₂⁻ Generation

O₂⁻ generation was measured by the reduction of ferricytochrome c (horse heart type III; Sigma) as described by Johnston *et al.*^[9]

For *in vitro* studies, pre-treated PMNs (2.10⁶ cells/ml), with or without (control) different concentrations of plant extract, in presence of 0.4 mM ferricytochrome c, were incubated during 5 min at 37°C and then stimulated with OZ (1.5 mg/ml) for 10 min in a shaking water bath at 37°C. The final volume of the reaction mixture was adjusted to 1.0 ml with PBS. Incubation was stopped by placing the tubes in an ice-water bath (5 min) and then centrifuged at 700 g for 10 min at +4°C. The absorbance optical density (OD) of the supernatants was read at 550 nm in a spectrophotometer (Cecil). The amount of O₂⁻ produced was calculated from the difference in absorbance of the sample before and after incubation using an extinction coefficient of 28 mM⁻¹ cm⁻¹ at 550 nm.

For *in vivo* studies, $O_2^{\cdot-}$ generation of PMNs, exuded from treated and untreated rats, was performed in the same conditions as above.

The results are expressed in nmol of released $O_2^{\cdot-}/10^6$ cells. The specificity of the reaction was verified by adding bovine erythrocyte superoxide dismutase, (Sigma) dissolved in PBS at a final concentration of 25 $\mu\text{g}/\text{ml}$ to test whether the reduction of cytochrome c was mediated by $O_2^{\cdot-}$.

Quantification of Oxygen Consumption

The parameter was quantified with a Clark oxygen electrode (Strathkelvin instrument oximeter 949) according to the method of Kvarstein and Halvorsen.^[10]

In vitro pre-treated PMNs or pleural PMNs from *in vivo* treated rats (5.10^6 cells/ml), were injected into a thermostated ($37.0 \pm 0.1^\circ\text{C}$) 2 ml measuring cell with no head space. After 5 min at 37°C , in order to initiate oxygen consumption, OZ (1.5 mg/ml) was added at a final concentration of 1.5 mg/ml. The rate of oxygen consumption was graphically determined from the initial slope promoted by the addition of the OZ to the incubation medium. The results are expressed in nmol of O_2 consumed/ 10^6 cells.

Standardization of oximeter was achieved at 0% with bisulfite sodium solution (3.85 M) and at 100% with air saturated distilled-water solution.

Total Polyphenols Content

The colorimetric method of Singleton *et al.*, was used.^[11] 0.5 mg of the extract was added to a mixture of 7 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent. After 3 min, 2 ml of 20% Na_2CO_3 was added before heating at 100°C for 1 min in a water bath. After cooling, the absorbance (OD) was measured at 685 nm in comparison with a standard solution of Gallic acid. Colorimetric results were expressed in mg of standard substances/100 g of extract.

Statistical Analysis

Data are expressed as mean \pm standard error. The significance of differences between control and treated samples *in vitro* was analysed using Student's *t*-test.

For *in vivo* studies, data were analysed using the one-way analysis of variance. If the overall F-value was found statistically significant ($P < 0.05$), further comparisons among groups means were compared using Duncan's multiple range test. Differences with $P \leq 0.05$ were considered to be statistically significant.

RESULTS

In order to appreciate the relative quiescent state, the dose-related effect and the maximal effect, we first

quantified the $O_2^{\cdot-}$ production by PMNs after stimulation with various concentrations of OZ. Figure 1 shows the stimulatory effect produced by increasing concentrations of OZ on $O_2^{\cdot-}$ production of PMNs, the maximal extent is reached at 1.5 mg/ml of OZ.

In a second stage, the *in vitro* activity of the plant extract was evaluated. For that PMNs were incubated, during 30 min at 37°C , with increasing concentrations of *M. vulgare* extract (10, 20, 40, 60, 80 and 100 mg/ml).

Table 1 summarizes, the effect produced by plant extract on the oxidative metabolism of rat PMNs stimulated with OZ (1.5 mg/ml) assessed by O_2 consumption and $O_2^{\cdot-}$ production.

The inhibition was calculated by the following equation:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Where, A_c is the absorbance of control and A_s the absorbance of the treated simple.

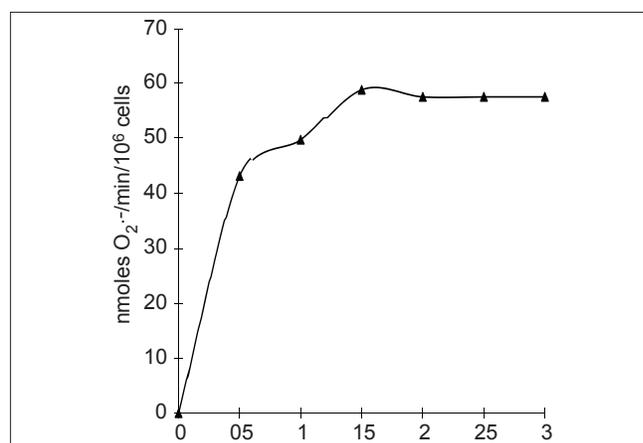


Figure 1: Effect of various concentrations of opsonized zymosan on superoxide anions production by pleural rat polymorphonuclear leukocytes. *Each value represents the mean \pm SD of five replicates of pool cells. **SD \leq 5%

Table 1: *In vitro* effect of *Marrubium vulgare* hydromethanolic extract on the opsonized zymosan (1.5 mg/ml)-induced oxidative metabolism of pleural rat polymorphonuclear leukocytes

Plant extract	Oxygen consumption (nmol $O_2/10^6$ cells)	Superoxide anions generation (nmol $O_2^{\cdot-}/10^6$ cells)
Control	73.63 \pm 4.23	42.33 \pm 3.07
10 mg/ml	72.01 \pm 4.46*	41.81 \pm 3.08*
20 mg/ml	64.35 \pm 4.94**	37.63 \pm 3.22**
40 mg/ml	55.65 \pm 4.12**	32.07 \pm 3.45**
60 mg/ml	44.79 \pm 3.66**	26.87 \pm 3.93**
80 mg/ml	34.23 \pm 3.27**	23.29 \pm 3.17**
100 mg/ml	34.51 \pm 3.05**	23.33 \pm 3.01**

Each value represents the mean \pm SE of five replicates experiences. *Not significantly different from the values of the controls at $P < 0.05$; **Significantly different from the values of the controls at $P < 0.05$

As can be observed in Figure 2, the extract induced a significant and concentration related inhibitory effect on both quantified metabolic parameters. The maximal extents of inhibition levels, obtained at 80 mg/ml, were about 54% ($P < 0.05$) and 45% ($P < 0.05$), for O_2 consumption and $O_2^{\cdot-}$ production respectively.

In the same conditions as shown in Table 2, the effect produced *in vivo* by plant extract on PMNs oxidative metabolism depend on the dose used. The low doses (100 and 200 mg/kg/day) were inefficient while the high doses (300 and 400 mg/kg/day) induced a similar and significant inhibitory effect. For both parameters, the maximal inhibitory effect was about 23% [Figure 3].

Both studies showed a good correlation ($r = 0.99$) between O_2 consumption and $O_2^{\cdot-}$ production confirming the validity of the methods used.

Otherwise, the hydromethanolic extract of *M. vulgare* contains a high amount of polyphenols (25.76 mg/100 g of extract).

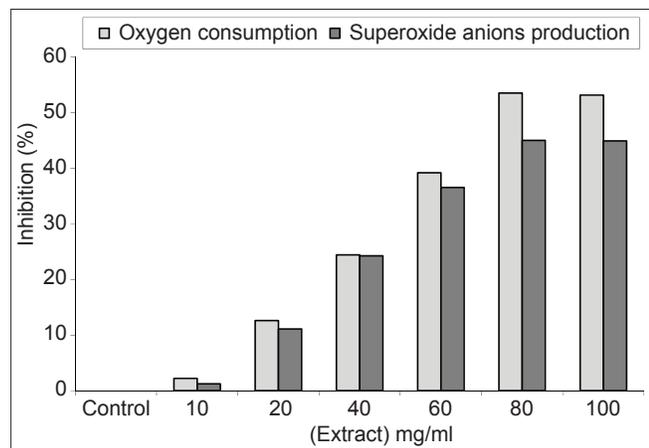


Figure 2: *In vitro* inhibitory effect of *Marrubium vulgare* hydromethanolic extract on the opsonized zymosan (1.5 mg/ml) -induced oxidative metabolism of pleural rat polymorphonuclear leukocytes

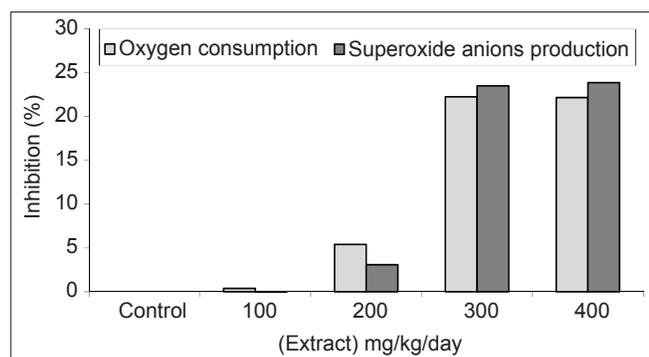


Figure 3: *In vivo* inhibitory effect of *Marrubium vulgare* hydromethanolic extract on the opsonized zymosan (1.5 mg/ml) -induced oxidative metabolism of pleural rat polymorphonuclear leukocytes

To evaluate the potential cytotoxicity, PMNs were incubated (30 min at 37°C) with different concentrations of the plant extract (10-100 mg/ml). The viability of pleural rat PMNs, evaluated by the extracellular LDH activity, was not significantly modified. The cells mortality did not exceed 10-15% for control and plant extract (data not shown). It could be concluded that the plant extract did not exhibit noticeable cytotoxicity for PMNs.

DISCUSSION

Many people take medicinal plant to maintain good health as much as to treat illnesses. The genus *marrubium*, comprising approximately 30 species, is considered one of the most popular herbal remedies used in the folk medicine throughout the world.

To screen new anti-inflammatory agents from medicinal plants, we have selected *M. vulgare*, based on its wide use in folk medicine. Thus, the aim of this study was to investigate the effect produced, *in vitro* and *in vivo*, by hydromethanolic extract of this plant on O_2 consumption and $O_2^{\cdot-}$ generation by rat pleural PMNs stimulated with OZ.

It is important to note that although, these cells were obtained by exudation, 4 h after induction of a non-specific inflammatory reaction by injection of suspension of CaPP in rat pleural cavity, their basal oxidative metabolism remains weak at resting state, which allows them to be used for such study.

Table 2: *In vivo* effect of *Marrubium vulgare* hydromethanolic extract on the opsonized zymosan (1.5 mg/ml)-induced oxidative metabolism of pleural rat polymorphonuclear leukocytes

Dose	O_2 consumption (nmol $O_2/10^6$ cells)	$O_2^{\cdot-}$ generation (nmol $O_2^{\cdot-}/10^6$ cells)
Untreated group (0.9% NaCl)		
Control	72.65±5.05	42.15±4.25
100 mg/ml	71.70±5.75*	41.85±3.95*
200 mg/ml	71.05±6.25*	40.75±3.75*
300 mg/ml	70.50±5.75*	41.05±3.50*
400 mg/ml	71.75±5.45*	41.25±4.15*
Treated group (plant extract)		
Control	72.05±4.63	42.90±3.40
100 mg/kg body weight/day	72.33±2.99*	42.89±3.09*
200 mg/kg body weight/day	68.15±4.05*	41.57±3.24*
300 mg/kg body weight/day	56.01±3.55**	32.81±3.15**
400 mg/kg body weight/day	56.07±4.12**	32.65±2.89**

Each value represents the mean±SE of 5 replicates experiences. *Not significantly different from the values of the controls at $P < 0.05$, **Significantly different from the values of the controls at $P < 0.05$

Our results clearly showed the ability of plant extract to modify the PMNs oxidative metabolism assessed by two parameters (O_2 consumption and $O_2^{\bullet-}$ production) after OZ stimulation. *In vitro*, the plant extract exerted a significant ($P < 0.05$) and dose-dependent inhibitory effect on both parameters used. *In vivo*, comparing the results of the treated group with those of the reference group, we found that only high doses (300 and 400 mg/Kg/day) exerted the same and significant ($P < 0.05$) inhibitory effect. These results are in agreement with those found by Kanyonga *et al.*,^[12] who demonstrated the anti-inflammatory, antinociceptive and antimicrobial potential of *M. vulgare* methanolic extract.

Otherwise, previous phytochemical investigations performed with *M. vulgare* have led to the characterisation of different metabolites. A lot of them belongs to the class of polyphenols, particularly flavonoids (luteolin, apigenin and quercetin), phenolic acids (caffeic and coumaric acids) and labdane diterpenoids (marrubiin as the main component).^[13-18]

The anti-inflammatory effect exerted by *M. vulgare* extract on pleural rat PMNs could be linked to one or more of these polyphenolic compounds. Several studies have demonstrated that plant polyphenols profoundly impair the production of reactive oxygen intermediates produced by neutrophils and other phagocytic cells.^[19-21] For example, experiments performed with quercetin showed an inhibitory effect on $O_2^{\bullet-}$ production by neutrophils stimulated with OZ.^[22] or with N-formyl-Methionyl-Leucyl-Phenylalanine peptide,^[23] while, marrubiin showed an antioedematogenic effect.^[24]

The mechanism by which these polyphenols impair the PMNs oxidative metabolism remains unclear. However as reported by various studies, the mechanisms of action could probably linked to their ability to inhibit leukocytes nicotinamide adenine dinucleotide phosphate oxidase.^[25] and/or their capacity to scavenge ROS.^[26]

CONCLUSION

The anti-inflammatory effect of *M. vulgare* hydromethanolic extract on oxidative metabolism of rat pleural PMNs stimulated with OZ was demonstrated by highly significant diminution of both oxygen consumption and $O_2^{\bullet-}$ formation. These results corroborate the usefulness of this plant in the treatment of inflammatory disorders. However, further investigations are needed to identify the active compound (s).

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