It is well known that reactive oxygen species (ROS) are involved in many pathological disorders such as atherosclerosis and related cardiovascular diseases, diabetes, and cancer. Reactive oxygen species, generated in vivo mainly by neutrophils, macrophages, and xanthine–oxidase system, appear to be responsible in these illnesses by inducing lipid peroxidation via a chain reaction process.

Most living species have protective systems against oxidative stress and toxic effects of ROS. Several studies have demonstrated that the antioxidant properties of plant compounds could be correlated with oxidative stress defense. Thus, antioxidant compounds can be used to counteract oxidative damage by reacting with free radicals, chelating free catalytic metals, and also by acting as oxygen scavengers.

The genus *Globularia* is commonly used in Moroccan folk medicine. Ethnomedicinal investigations have demonstrated that *Globularia alypum*, locally named ‘Ain Larneb,’ is one of the most traditional plant remedies. Its leaves are reported to be used in the treatment of diabetes and in renal and cardiovascular diseases. They are also used as laxative, cholangue, stomachic, purgative, sudorific, antihypertensive, and hypoglycemic. The involvement of ROS in most of these disorders prompted us to investigate the antioxidant properties of *G. alypum*, which has not been explored until now.

## Materials and Methods

### Plant material

Fresh *G. alypum* L. was collected during spring 2003 from the region of Taza, Morocco. Taxonomic identification was performed by Dr. R. Tellal (Laboratoire d’Écologie Végétale, Faculté des Sciences, El Jadida, Morocco). A voucher specimen (KS) is kept in the herbarium of the biology department.

### Preparation of plant extract

Fresh aerial parts (stems and leaves) were washed and air-dried in shade at room temperature. They were then mechanically powdered and sieved. One hundred grams of the obtained fine powder was macerated in a hermetically closed glass vessel for 48 h, at room temperature (25°C), under occasional shaking with 500 ml of a mixture of distilled water–methanol (3/2 v/v). The obtained crude preparation was concentrated under reduced pressure to obtain the hydromethanolic extract.

### ABSTRACT

Objective: To investigate the *in vitro* antioxidant activity of the hydromethanolic extract of aerial parts (leaves and stems) of *Globularia alypum* L. toward linoleic acid emulsion and human low-density lipoproteins (LDL) peroxidation.

Materials and Methods: Lipid peroxidation was carried out in the presence of *G. alypum* hydromethanolic extract (10 and 100 µg of extract/ml). CuSO₄ (10 µM) was used as the oxidation initiator. Conjugated dienes (CD) formation and oxygen consumption were assessed for monitoring the antioxidant properties of the plant extract. Butylated hydroxytoluene at 50 µg/ml was used as standard antioxidant. Quantification of total polyphenolic compounds was carried out according to the Folin–Ciocalteu method.

Results: The hydromethanolic extract of *G. alypum* exhibited significant antioxidant effect. There was a significant inhibition of CD formation in copper ions-mediated linoleic acid emulsion as well as human LDL peroxidation. Analysis of the plant extract revealed a high amount of polyphenols, suggesting a possible role of these compounds in the antioxidant properties.

Conclusion: The obtained results suggested that *G. alypum* could be a potential source of antioxidants. Further investigations are in progress to determine the active constituent(s).

KEY WORDS: Conjugated dienes, linoleic acid, lipid peroxidation.
centrifuged at 5000 g for 45 min (Sigma 2K15). After filtration, the supernatant filtrate was concentrated under reduced pressure at 25°C and the crude extract (10.75 g) stored at 4°C until use.

**Chemicals**

Linoleic acid (99%), ethylenediaminetetra-acetic acid (EDTA), Tween 20 (polyoxyethyleneborisorbitan monolaureate), butylated hydroxytoluene (BHT) and Folin–Ciocalteu reagents were purchased from Sigma. All other unlabelled chemicals and reagents were of analytical grade.

**Isolation of low-density lipoproteins**

Human low-density lipoproteins (LDL) (1.019<d<1.063) were isolated according to the method of Sattler,[10] using a Beckman Optima TLX ultracentrifuge equipped with a TLA 100.4 rotor, in the presence of EDTA (0.4 g/l). After separation, LDL was dialyzed overnight at 4°C with 1/100.4 rotor, in the presence of EDTA (0.4 g/l). After separation, LDL was dialyzed overnight at 4°C with 1/10 M sodium phosphate buffer (pH 7.0). For oxidation experiments, the LDL dialyzed solutions were adjusted by dilution to 100 µg/ml, and proteins were measured by commercial assay (Pierce method, Rockford III, USA).

**Measurement of oxygen consumption**

_Globularia alypum_ (Ga) extract (10 and 100 µg/ml) was added to 1.5 ml of a 7.5 mM linoleic acid emulsion in 10 mM aqueous phosphate buffer (pH 7.0) and 0.1% of Tween 20 (v/v) as emulsifier. The emulsion was air saturated. A freshly prepared solution of CuSO₄ was added, at a final concentration of 10 µM, to initiate the oxidation process. Measurement of the oxygen consumption, according to the modified technique of Genot[11] using Strathkelvin Instruments Oxymeter 949 was started by injection of the sample into a thermostated (25.0±0.1°C) 2 ml measuring cell with no headspace. Oxygen consumption was measured with a Clark electrode and reagents were of analytical grade.

**Measurement of conjugated dienes**

As shown in Figure 1, Ga hydromethanolic extract exhibited a significant inhibition of linoleic acid oxidation as assessed by CD formation. The inhibition extents were 24% (P <0.01) and 64% (P <0.001), respectively, at 10 and 100 µg/ml, while BHT, used as standard antioxidant at 50 µg/ml, gave 76% (P <0.001).

**RESULTS**

**Linoleic acid oxidation**

Linoleic acid oxidation

**Determination of total polyphenolic compounds (PPC)**

The amount of total PPC was measured by the method described by Taga[14] using the Folin–Ciocalteu reagent. Briefly, samples and standards were prepared in acidified (0.3% HCl) methanol–water solution (60/40 v/v). One hundred microliter of this preparation was added to 2 ml of 0.2% Na₂CO₃. After 2 min, 100 µl of Folin–Ciocalteu/methanol (v/v) reagent was added to start reaction at room temperature (25°C) during 30 min. The control mixture consisted of all reagents and solvent without extract. The phenolic concentrations were expressed as phenol equivalent by comparison with a standard calibration curve using phenol solution (0.01–1 mg/ml).

**Statistical analysis**

The results are presented as the mean±SD of five replicates. Data were analyzed using one-way analysis of variance (ANOVA) and group means were compared using Duncan’s multiple range test. P values <0.05 were considered significant.

**RESULTS**

Linoleic acid oxidation

As shown in Figure 1, Ga hydromethanolic extract exhibited a significant inhibition of linoleic acid oxidation as assessed by CD formation. The inhibition extents were 24% (P <0.01) and 64% (P <0.001), respectively, at 10 and 100 µg/ml, while BHT, used as standard antioxidant at 50 µg/ml, gave 76% (P <0.001).

Figure 1. Effects of Globularia alypum (Ga) hydromethanolic extract on Cu-induced linoleic acid oxidation monitored by conjugated dienes formation. Each point represents the mean of five replicates.
The kinetic parameters of oxidation [Table 1] showed that at a low concentration (10 µg/ml), the plant extract did not influence the lag time and the maximal propagation rate of linoleic acid oxidation, but induced a low and significant reduction (24%) (\(P < 0.05\)) in the maximal amount of CD formation. At a high concentration (100 µg/ml), the extract caused a mean increase in lag time of 52 ± 2.5 (\(P < 0.001\)) and inhibited both propagation rate (35%, \(P < 0.001\)) and maximal amount of CD formation (64%, \(P < 0.001\)).

The antioxidant properties of *G. alypum* were also demonstrated by the oxygen consumption method where the plant extract induced significant effect [Figure 2]. Thus, the Ga extract (10 and 100 µg/ml) reduced the rate of oxygen consumption by 63% (\(P < 0.001\)) and 84% (\(P < 0.001\)), respectively. It must be noted that the effect observed with a concentration of 100 µg/ml was more efficient than that obtained with BHT at 50 µg/ml (77%, \(P < 0.001\)) [Table 2].

### Human LDL peroxidation

In order to confirm the protective action of Ga extract on Cu-induced linoleic acid peroxidation, the human low-density lipoproteins (LDL) were used as a model system. The results showed that the Ga extract (10 and 100 µg/ml) reduced the rate of oxygen consumption by 61.2 ± 2.6 (\(P < 0.001\)) and 83.1 ± 2.9 (\(P < 0.001\)), respectively. It must be noted that the effect observed with a concentration of 100 µg/ml was more efficient than that obtained with BHT at 50 µg/ml (76.9 ± 1.2% \(P < 0.001\)) [Table 2].

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lag phase (min)</th>
<th>Propagation rate (nM/min)</th>
<th>([CD]_{max}) (mM)</th>
<th>% ([CD]_{max}) inhibition</th>
</tr>
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<tbody>
<tr>
<td>Linoleic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0a</td>
<td>90.1 ± 3.7a</td>
<td>29.0 ± 1.4a</td>
<td>0a</td>
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<tr>
<td>Ga 10 µg/ml</td>
<td>0a</td>
<td>89.3 ± 10.4a</td>
<td>22.1 ± 1.9b</td>
<td>23.9 ± 4.8b</td>
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<tr>
<td>Ga 100 µg/ml</td>
<td>52 ± 2.5b</td>
<td>58.8 ± 2.0b</td>
<td>10.4 ± 0.3c</td>
<td>63.9 ± 0.7c</td>
</tr>
<tr>
<td>BHT 50 µg/ml</td>
<td>60 ± 7.4b</td>
<td>48.6 ± 2.4b</td>
<td>7.0 ± 0.6d</td>
<td>75.8 ± 1.2d</td>
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<tr>
<td>One-way</td>
<td>F</td>
<td>140</td>
<td>27</td>
<td>136</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P</td>
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</table>

### Table 2

<table>
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<tr>
<th>Treatment</th>
<th>% oxygen consumption inhibition</th>
<th>Oxygen consumption rate (nM/l/s)</th>
<th>(I_{oxygen})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0a</td>
<td>172.5 ± 0.8a</td>
<td>1a</td>
</tr>
<tr>
<td>Ga 10 µg/ml</td>
<td>63.4 ± 1.4b</td>
<td>63.3 ± 2.9b</td>
<td>0.36 ± 0.02b</td>
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<tr>
<td>Ga 100 µg/ml</td>
<td>84.4 ± 1.4c</td>
<td>26.9 ± 2.0c</td>
<td>0.15 ± 0.02c</td>
</tr>
<tr>
<td>BHT 50 µg/ml</td>
<td>76.6 ± 1.25d</td>
<td>40.3 ± 2.4d</td>
<td>0.23 ± 0.02d</td>
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<tr>
<td>One-way</td>
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<td>1493</td>
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<tr>
<td>ANOVA</td>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
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</table>

Each value represents mean ± SD of five replicates. df = 3,16. Values with different superscripts (a, b, c, and d) differ significantly with each other at \(P < 0.05\) (Duncan’s multiple range test).
linoleic acid oxidation, we tested its effect on human LDL oxidation by quantifying the CD formation.

The obtained results [Figure 3] showed that copper-induced LDL peroxidation was significantly inhibited by Ga extract. The extent of inhibition of CD formation was 61%, (P < 0.001), 83% (P < 0.001) and 76% (P < 0.001), respectively, at 10 and 100 µg/ml of plant extract and BHT at 50 µg/ml.

The effects on kinetic parameters showed that the plant extract prolonged the lag time, diminished the propagation rate, and inhibited the maximal amount of CD formation [Table 1]. Standard antioxidant BHT (50 µg/ml) did not affect the lag time but inhibited to a lesser extent the propagation rate and the maximal amount of CD formation.

**Discussion**

The present investigation carried out on the antioxidant properties of hydromethanolic extract of *G. alypum* clearly showed the protective activity on lipid peroxidation. However, as previously described,[15][16] the efficiency of antioxidant activity was dependent on the used lipid system and the method of assessment.

Globally, in the presence of linoleic acid (simple lipid system), the antioxidant effect produced by the extract was lesser or higher than BHT, respectively, when assessed by CD formation or oxygen consumption. According to the kinetic parameters of linoleic acid oxidation, the plant extract (100 µg/ml) extended the lag time probably by increasing the initiation stage of the chain reaction. The plant extract also inhibited both the propagation rate and the maximal amount of CD formation.

After the CD formation, one of the first events of lipid peroxidation was the uptake of oxygen and formation of lipid peroxides.[17] In the presence of plant extract, linoleic acid oxidation showed significant decrease in the rate of oxygen uptake, which was higher than that induced by BHT. Probably, the plant extract reacts with peroxyl radicals inducing an inhibition of the lipid peroxidation chain propagation.[16]

The antioxidant effect exerted by the plant extract seems to be more efficient in protecting human LDL (complex lipid system) against peroxidation than linoleic acid. The extract increased the lag time and decreased the propagation rate and the maximal amount of CD formation, whereas BHT did not affect the lag phase and weakly decreased the propagation rate. Similar results were obtained with probucol[18] and with a synthesized antioxidant 4GBE43.[19]

Otherwise, the high amount of total phenolic content (120 mg of phenol equivalent per gram of extract) led us to suggest that these substances could be responsible of the antioxidant properties of the extract. Polyphenols were reported to have an important role in stabilizing lipid peroxidation[20] and are associated with a wide range of biological activities including antioxidant properties[21][22] due to their redox properties, as reducing agent or hydrogen atom donors.[21]

The implication of the polyphenolic compounds in the antioxidant activity of *G. alypum* is supported by previous results obtained with other Globularia species. Thus phenylethanoid glycosides extracted from *Globularia Trichosantha*,[23] *Globularia davissiana*,[24] and Globaritol obtained from *Globularia orientalis*[25] have been reported to possess scavenging properties.

In conclusion, the protective effect of Ga hydromethanolic extract toward linoleic acid emulsion and human LDL peroxidation was demonstrated by highly significant diminution of both oxygen consumption and CD formation. Further investigations are in progress to characterize the active compounds and to evaluate the usefulness in the treatment of disorders that involve oxidative stress.

**References**