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High inhibitory effect on lipid peroxidation by *Hammada elegans* phenolic extracts

Khaled Djokhdem[1], Amar Djeridane[1,2], Nourelhouda Renane[2], Mohamed Yousfi[1,2]

Correspondence to:
Amar Djeridane
amardjeridane@yahoo.fr; a.djeridane@mail.lagh-univ.dz

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Abstract

The antioxidant activity and phenolic content of extracts and solvent–solvent partition fractions from the aerial parts of three medicinal plants were evaluated. Aerial parts of *Hammada elegans*, P*lantago ciliata* and *Thymelaea microphylla* were extracted using the polarity gradients of different solvents. The samples were extracted by liquid–liquid partition with different organic solvents to obtain 16 fractions. Next, the phenolic contents of all fractions were analysed using the Folin-Ciocalteu method and their inhibition of the peroxidation of linoleic acid and sunflower oil was evaluated by the lipid peroxide ammonium thiocyanate method.

The total phenolic content of the different *Hammada elegans* fractions varied from 0.045 to 0.0714 mg/g dry weight, expressed as gallic acid equivalents (GAE). The percentage inhibition of peroxidation of linoleic acid and sunflower oil was found to be higher in methanolic fractions and was comparable to that of α-tocopherol, Trolox, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). In this study, a direct relationship between total phenolic content and inhibition of lipid peroxidation was observed. This indicates that phenolics are the main contributors to the observed antioxidant activities of the different plant extracts.

These results indicate that the level of antioxidant activity in these plants varies greatly. They also suggest that the phenolics in these plants provide a substantial amount of antioxidants. Additional research may reveal further benefits of these medicinal plants. The flora of Algeria appears to be a rich and interesting source of plants for ethnomedicinal and phytochemical studies.

Introduction

The beneficial effects of saturated versus unsaturated fatty acids have been debated among the world's leading nutritional experts. A diet containing unsaturated fats has been shown to help prevent atherosclerosis and coronary heart disease [1], while long-term diets containing monounsaturated fatty acids have been shown to reduce platelet aggregation and decrease plasma LDL-cholesterol levels [2].

Natural antioxidants, including polyphenolic compounds, are considered beneficial because of their potential role in preventing food spoilage arising from lipid peroxidation. Polyphenols also have protective roles in the pathogenesis of multi-

[1]Laboratory of Applied Chemical and Physical Sciences, ENS of Laghouat, BP4033 Rue Martyres, Laghouat, Algeria
[2]Laboratory of Fundamental Sciences, University of Amar Telidji, BP37G, Laghouat, Algeria

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ple diseases associated with oxidative stress. They can inhibit lipid peroxidation by acting as chain-breaking electron donors (by reducing ROO·), by chelating metal ions (as these ions help to initiate the reaction), or by acting as chain-breaking electron acceptors by oxidizing R· [3].

Traditional herbs with medicinal functions are widely used, but there is often no scientific evidence to support their beneficial effects. We have demonstrated that methanolic extracts from several medicinal plants including *Hammada elegans*, *Plantago ciliata* and *Thymelaea microphylla* possess 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity [4, 5]. In this study, in order to examine the protective effects of these plants against free radical-induced damage, we used the thiocyanate method to investigate inhibition by three medicinal plants (*Hammada elegans, Plantago ciliata* and *Thymelaea microphylla*) and their components of the peroxidation of linoleic acid and sunflower oil.

Materials and methods

Extraction and analysis of phenolic extracts

The medicinal plants were collected in September 2014 from Sidi Makhlouf (40 km north of Laghouat in the steppe region of Algeria). Information on the plants (local name, medicinal uses, parts of the plant used, methods of preparation and administration) were collected from local inhabitants knowledgeable about the plants' curative properties. Samples were identified at the National Institute of Agronomy of Algeria, and voucher specimens were deposited at the Laboratory of Fundamental Sciences, University of Laghouat. The plant parts were thoroughly washed with tap water, dried at room temperature, and ground to a fine powder. All chemicals were of the highest quality available and were purchased from Sigma-Aldrich (France).

A 25 g sample of the air-dried plant material was crushed and extracted at reflux temperatures using a Soxhlet apparatus with five solvents with different polarities: *n*-hexane (0.0), diethylether (2.8), dichloromethane (3.1), acetone (5.1) and methanol (5.1). The obtained extracts were filtered and evaporated under reduced pressure. The precipitate was dried, dissolved in absolute methanol and stored at 4°C.

Analytical thin layer chromatography (TLC) was performed on precoated plates (5×10 cm, silica gel 60 F_{254}, 230–400 mesh, 0.25 mm; Merck) using an elution system (diethylether/cyclohexane/formic acid 7/3/0.1, v/v/v). Spots were visualized by examination under ultraviolet light (254 and 366 nm).

To isolate the active products, the methanolic extract of *Hammada elegans* was fractionated using preparative plates in streaks. The chromatoplates were developed with a mixture of diethylether/cyclohexane/formic acid (8:2:0.1, v/v/v). Bands were detected under UV light. The components were separated out into eight defined zones. The different bands were scrapped off, extracted with methanol, dried over anhydrous sodium sulphate and evaporated to dryness to obtain the purified products.

The concentration of total phenolics in plant extracts was estimated by the Folin-Ciocalteu procedure [6], which is considered the best method for the determination of total phenolics (including tannins) [7]. The total phenolic content of the sample was expressed as gallic acid equivalents (GAE), which is the amount of gallic acid (mg) in 1 g of dry material.

Inhibition of lipid peroxidation assay

The inhibition of lipid peroxidation assay measures linoleic acid and sunflower oil oxidation by radicals generated by 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) added to the solution. The peroxides formed from linoleic acid were evaluated by the thiocyanate method. The experiment was performed according to the modified methodology of Foti *et al.* [8] and Haraguchi *et al.* [9]. Test samples were prepared by mixing 1 ml of linoleic acid or sunflower oil solution with 1 ml of 0.05 M Tris–HCl buffer, pH 7.4, containing 0.3% Tween-20 and 0.1 ml of phenolic extracts in different concentrations. All samples were incubated at 50°C for 10 min. Then 0.1 ml of 2% AAPH solution was added, and the samples were vortexed and kept in a water bath at 50°C with no light exposure for 20 min for linoleic acid solution and 50 min for sunflower oil solution. The per-

oxide value was determined using the thiocyanate method of Haraguchi *et al.* [9] by adding 0.1 ml of $FeCl_2$ (20 mM in 3.5% HCl) to each sample. After 1 min, 0.1 ml of 1% ammonium thiocyanate solution was then added. The solution was mixed and the solution absorbance was measured at 505 nm in a UV-visible spectrophotometer (Optizen, Poland). Triplicate measurements were carried out and activity was calculated as the percentage of inhibition of the radical reaction. The antiperoxidation activity obtained for each plant extract was compared with that of α-tocopherol, Trolox, BHT and BHA.

Statistical analysis

All treatments were performed in triplicates and each data point in the results is the mean of three replicates. All experiments were repeated at least once. The values are expressed as mean±SEM. Statistical significance was set at $p<0.05$.

Results and discussion

Previous screening of the inhibitory activity of peroxidation of linoleic acid of the different plant extracts showed that the crude extracts of *Hammada elegans* were the most effective (Table 1). Consequently, we choose this plant for investigation.

The yield of the plant extracts and the concentration of total phenolic content (mg/g dry weight) are shown in Table 2. Methanolic extract had the highest extraction yields (3.05%) and total phenolic content (47%) among the samples. The hexane fraction with the lowest percentage yield obtained as the non-polar solvent used was expected to collect fat soluble compounds, for instance lipids or chlorophylls. The work of Herode *et al.* [10] shows that the percentage of extraction yields

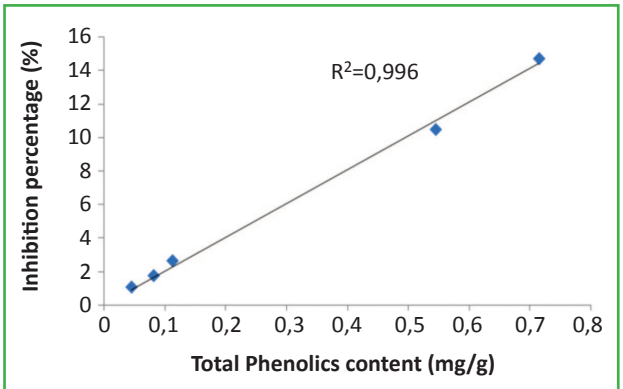

Figure 1 - Linear correlation of lipid peroxidation inhibition percentages with total phenolics content of five *Hammada elegans* extracts

increases with the particle size of the sample, extraction temperature, type of solvent and method of sample extraction. Analysis revealed that hexanic extract had the lowest total phenolic content. The correlation coefficient between the total antiperoxidative activity and total phenolic contents of five *Hammada elegans* fractions was $R^2=0.99$ (Fig. 1). This result suggests that 99% of the antioxidant capacity of these extracts is due to the contribution of phenolic compounds, either combined or individually. In addition, the synergism between the antioxidants in the mixture shows the antioxidant activity is dependent both on the concentration and on the structure of and interaction between the antioxidants. This explains why diethylether and dichloromethane extracts, with similar concentrations of total phenolics, may vary in their antioxidant activities. The results suggest that phenolic compounds contribute significantly to the antioxidant capacity of this medicinal plant. Several studies on total phenolic content have been published. The 18 plants studied by Djeridane *et al.* [4] had higher total phenolic contents than found in this study, with that of *Thymelaea microphylla* being around 10 mg GAE/dry matter. Different levels reported in other studies may

Solvent of plant extract	Inhibition percentage (%) at 0.1 g/l		
	Hammada elegans	*Plantago ciliata*	*Thymelaea microphylla*
n-Hexane	0	0	5.61±0.01
n-Butanol	2.34±0.02	0	0
Ethyl acetate	6.85±0.02	1.50±0.05	2.11±0.03
Methanol	7.91±0.03	0	0

Table 1 - Inhibition percentage of linoleic acid peroxidation of the different plants extracts

Plant extract	Yield of extracts (%)	Total phenolic content (mg of GAE/g dry mater)
Hexane	0.55	0.045±0.001
Diethylether	0.40	0.112±0.003
Dichloromethane	0.30	0.081±0.002
Acetone	0.45	0.544±0.003
Methanol	3.05	0.714±0.003

Table 2 - Extraction yield and total phenolic content of Hammada elegans extracts

be due to the use of different plants, procedures and standards to determine total phenolic contents. The use of Folin-Ciocalteu reagent also was measured based on the colour measurement which was non-specific on phenol. Perhaps other components that react with the reagent were present, such as ascorbic acid [11]. In addition, different phenolic compounds have different responses to this assay [6]. However, colour changes after 2 h of storage could indicate the presence of phenol in samples and may be due to the antioxidant properties of the plant extract that act as a reducing agent in redox reactions.

In addition, oxidative degradation produces lipid hydroperoxides, leading to complex changes that result in food rancidity and off-flavours. Therefore, the antioxidant activity of the different plants extracts has been evaluated in this study by the thiocyanate method, which measures the amount of peroxides formed in emulsion during incubation in the presence of linoleic acid and sunflower oil, the target of lipid peroxidation. The addition of different extracts of the medicinal plants to the linoleic acid or sunflower oil emulsion reduced peroxide formation. In particular, methanolic extract of *Hammada elegans* showed strong inhibitory activity (Fig. 2). In our previous study, extracts of these plants also exerted high DPPH radical-scavenging activity [4].

As the methanolic extract of *Hammada elegans* showed the best antioxidant activity compared with the other extracts, we sought to isolate its active compounds and investigate its antioxidant activity. However, fractioning of this extract by preparative plates indicated the presence of eight spots (Fig. 3).

The inhibitory effects of the compounds isolated from the crude methanolic extract on lipid peroxidation are presented in Fig. 4. Of the fractioned compounds, which were all tested at the same concentration, only two showed significant and dose-dependent inhibition of lipid peroxidation. This inhibitory activity increased as peroxide levels increased. Also, these two fractions had relatively high lipid peroxide inhibitory activity compared to other antioxidants, such as α-tocopherol, Trolox, BHT and BHA.

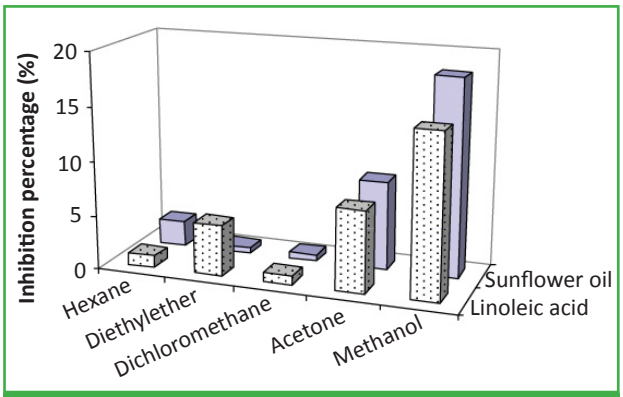

Figure 2 - Antioxidant activity of *Hammada elegans* extracts defined as inhibition percentage of linoleic acid and sunflower peroxidation assay

The concentration required to inhibit 50% of the lipid peroxidation of the isolated compound (fraction 6) was determined and compared to that of standard antioxidants. As seen from Table 3, the isolated compound had anti-free radical properties with an EC_{50} of 0.27 g/l. These data also showed that this natural compound had better free radical scavenging activity than reference antioxidants.

However, this active compound could not be characterized by spectrophotometric methods in the present study because there was only a very small amount (4 mg) and no plant matter was present. Therefore, re-isolation of this compound in larger amounts will be necessary so its characterization can be completed. Investigation of the other fractions is also being carried out using the bioassay to guide the isolation of further active components.

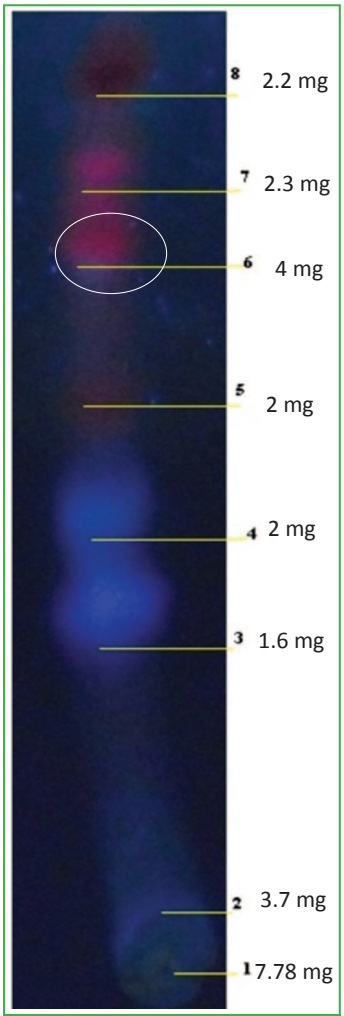

Figure 3 - Thin layer chromatography of *Hammada elegans* extract

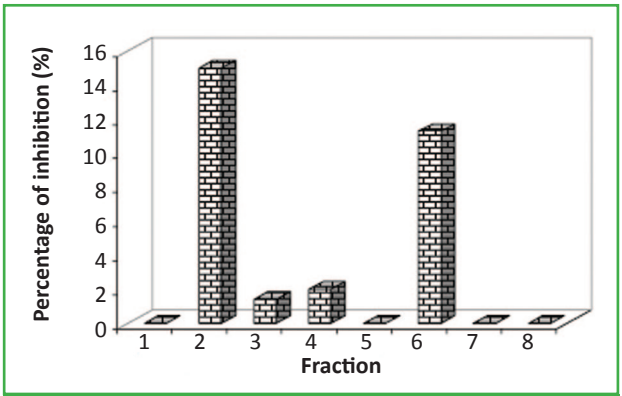

Figure 4 - Antioxidant activity of the eight fractions obtained from methanolic *Hammada elegans* extract defined as inhibition percentage of the sunflower peroxidation assay

	EC_{50} (g/l)	
	Linoleic acid	**Sunflower oil**
Isolated compound	0.10±0.01	0.27±0.01
Vitamin E	0.21±0.01	0.30±0.02
Trolox	0.10±0.002	0.32±0.01
BHA	2.30±0.003	19.37±0.09
BHT	3.52±0.005	09.96±0.08

Table 3 - Effect of the compound isolated from *Hammada elegans* extract and reference antioxidants on peroxidation generated by linoleic acid and sunflower oil

It has been reported that antioxidant compounds responsible for antioxidant activity could be isolated and then used as antioxidants for the prevention and treatment of free radical-related diseases [12]. Therefore, research to identify antioxidant compounds is required. Although it remains unclear which components of medicinal plants are the active compounds, our study suggests that *Hammada elegans* and its components have potential for the treatment of oxidative stress-related disorders.

Conclusion

In conclusion, our screening yielded one active extract from *Hammada elegans* leaves and initial fractionation yielded natural products with strong and interesting antioxidant activity. In vitro studies have shown that *Hammada elegans* has a protective effect on the polyunsaturated fatty acids found in cell membranes. These results suggest that it is safer to use natural antioxidants than artificial antioxidants such as BHT and BHA to preserve food products.

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