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A New Phytochemical and Anti-oxidant and Anti-inflammatory Activities of Different *Lactuca sativa* L. var. *crispa* Extracts

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The phytochemical composition of *Lactuca sativa* L. var. *crispa*, commonly known as crisphead lettuce, from Morocco has been re-investigated for the presence of oxyprenylated cinnamic acid and coumarin derivatives. In the mean time also the anti-oxidant and anti-inflammatory effects of extracts from leaves obtained by the use of a Soxhlet apparatus have been assessed. The extracts had a high content of polyphenols with remarkable differences ranging from 0.19 to 0.38 mg gallic acid eq/mg, while the content of flavonoids ranged from 3.75 to 9.64 mg catechin eq/g.The hydroalcoholic extract with a polarity index of 5.8 displayed the best anti-oxidant, radical scavenging, and anti-inflammatory activities. The same extracts were screened by reverse-phase HPLC-UV/V analysis for the presence of biologically active oxyprenylated phenylpropanoids. Only 4'-geranyloxyferulic acid was recorded in detectable amounts. Results described herein enforce the nutraceutical value and depict a potential chemopreventive dietary feeding role for crisphead lettuce.

Keywords: 4'-Geranyloxyferulic acid, Inflammation, *Lactuca sativa*, Oxyprenylated secondary metabolites, Polyphenols.

It is nowadays well known how several nutraceuticals possess a high content of anti-oxidants that may protect cells from the damage caused by highly reactive molecules like free radicals and cell metabolism-derived oxidative species like ROS. Plant derived anti-oxidants include vitamins, polyunsaturated fatty acids, some minerals, terpenoids, phenolics, and nitrogen compounds [1]. *Lactuca sativa*.L, a plant commonly known as lettuce, is a member of the Asteraceae family. It is an important leafy vegetable largely consumed as food in many parts of the world, that is also appreciated for its medicinal properties [2]. Lettuce contains several minerals (Ca, Mg, K, Fe, Mn, Cu, and Zn) that are considered essential for humans [3]. It is also a valuable source of phytonutrients that play a key role in human nutrition, such as fibers, flavonoids, chlorophylls, and carotenoids [4]. Although several articles have been reported in the literature about the food and nutraceutical properties of *L. sativa*, no data have been cited about the same properties of the title plant growing in a northern African country, like Morocco, to the best of our knowledge. To this aim in this manuscript we wish to report the overall phytochemical screening of five extracts obtained from dried leaves of *L. sativa* var. *crispa* (crisphead lettuce) after extraction with a Soxhlet apparatus, and their antioxidant and anti-inflammatory properties. The characterization of a novel secondary metabolite, namely 4'-geranyloxyferulic acid (GOFA), recently showed to exert promising and valuable pharmacological properties in terms of dietary feeding, neuroprotective, anti-inflammatory, and anti-bacterial properties [5, 6] will be also described. Such a secondary metabolite is reported herein for the first time as a component of the phytochemical pool of a *Lactuca* species.

Dried leaves of *L. sativa* var. *crispa* have been extracted with five solvent mixtures: a) H_2O, b) H_2O / EtOH 3:1, c) H_2O / EtOH 1:1, d) H_2O / EtOH 1:3, and e) EtOH, having the following PI values 10.2, 8.7, 7.3, 5.8, and 4.3 respectively. For each extract we measured the total polyphenol and total flavonoids contents expressed as mg GA eq/g of extract dry weight and mg Cat eq/g of extract dry weight, respectively . Results are reported in Figures 2 and 3.

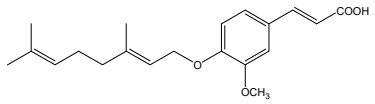

Figure 1: Structure of GOFA.

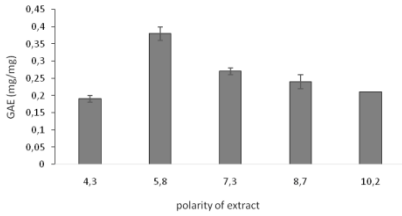

Figure 2: Total polyphenols content of extracts from *L. sativa* var. *crispa.*

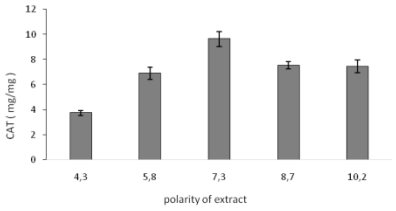

Figure 3: Total flavonoid content of extracts from *L. sativa* var. *crispa.*

The hydroalcoholic extract with a PI of 5.8 contained the highest quantity of phenolic substances. We subsequently assessed some

chemico-physical and biological parameters starting from the anti-oxidant capacities of the five extracts under investigation using the FRAP and DPPH scavenging assays using in both cases ascorbic acid as the reference. Results are reported in Figures 4 and 5.

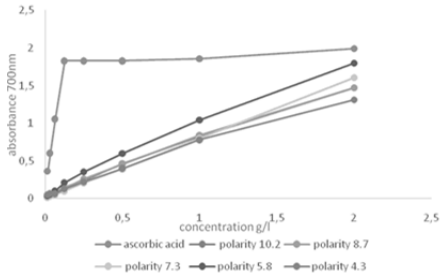

Figure 4: Results of FRAP assays.

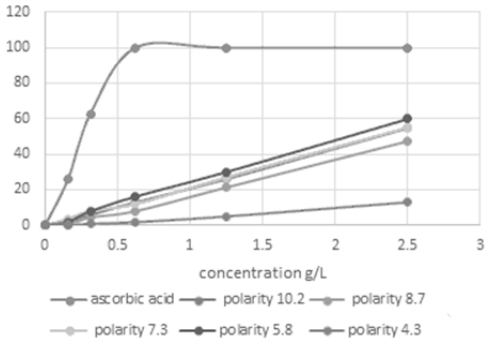

Figure 5: DPPH scavenging activity of *L. sativa* var. *crispa* extracts.

For what concerns both assays, the comparison between the five extracts under investigation shows that the hydroalcoholic extracts with a PI of 5.8 have a higher activity probably due to its higher content in polyphenols. Similar results have been obtained in the scavenging of ROS (e.g. H_2O_2) as reported in Figure 6.

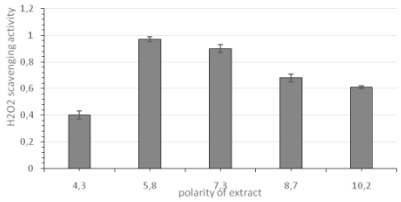

Figure 6: ROS scavenging effect of *L. sativa* var. *crispa* extracts.

Anti-oxidant capacities of a plant extract often well relates to its anti-inflammatory activity. To this aim we evaluated such an effect using the prevention of protein denaturation and, focusing only on the extract richest in polyphenols (e.g. PI = 5.8), the *in vivo* the inhibitory properties of the paw edema formation induced by formaldehyde after oral administration to animals. Results are reported in Figure 7 and Table 1 respectively.

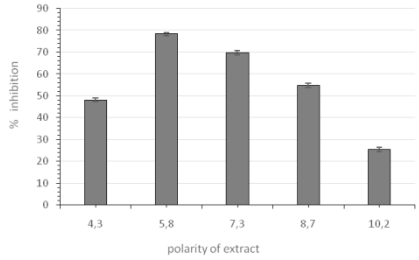

Figure 7: Inhibition of protein denaturation by *L. sativa* var. *crispa* extracts (results expressed as % of control = 100%).

Table 1: Inhibition of paw edema (%) in formaldehyde-induced chronic inflammation in rats for the *L. sativa* var. *crispa* extract with PI = 5.8. Doses are expressed as mg/kg of animal body weight.

Sample*	Dose (mg/Kg)	Paw volume (mL)**	% Reduction
Controls	--	1.60±0.09	--
Dexamethasone	10	0.81±0.08***	49.7
Extract PI 5.8	200	0.90±0.09***	43.8
	400	0.80±0.06***	50.1

* N = 6 animals for each group. ** Data are expressed as mean ± standard deviation. *** $p < 0.001$.

Data reported in Figure 7 show that the hydroalcoholic extract richest in polyphenols was able to prevent denaturation by nearly 80 %. For what concerns the anti-inflammatory activity the extract administered in the diet at the highest concentration recorded a value very close to that evoked by the treatment with the well known steroidal anti-inflammatory drug desamethasone. Very recently we demonstrated how some rare secondary metabolites, namely oxyprenylated pehnylpropanoids and polyketides, represent a class of natural products with a great therapeutic potential as anti-inflammatory and anti-cancer agents [6]. To this concern, we assayed the presence of such phytochemicals in the five extracts of *L. sativa* var. *crispa* herein under investigation by means of HPLC analysis with UV/Vis detection. The best results in terms of peak response (area and shape), chromatographic resolution, and overall run time were obtained with a GraceSmart RP18 (4.6 mm x 150 mm, 5 µm) thermostated at 10 ± 1 °C. Under these experimental conditions, recorded mean retention time for GOFA **1** was 7.55 ± 0.21 min.. The calculated capacity factors (k') was 3.94. The dead retention time, calculated with uracile, was 1.83 min. LOD was calculated by measuring S/N values obtained in the mobile phase spiked at 0.2 µg/mL level and extrapolation of the corresponding values to S/N = 3. The calibration curve showed a good linearity in the concentration range 0.5 - 25 µg/mL (r^2 = 0.9833). The back-calculated calibration standard points showed R.S.D. percentage values ranging from 1.7 % to 6.5 %. The differences in percentage between the standard concentration calculated from the calibration curves and the theoretical ones ranged from − 6.7 % to 3.4 %. LOQ was evaluated according to the guidance for industry on the validation of bioanalytical methods, as the lowest analyte concentration corresponding to a response at least 10 times higher than blank response and that can be determined with 80 – 120% accuracy and 20% precision. The back-calculated concentration value, obtained from calibration curves, allowed to assess 0.5 µg/mL as the validated LOQ. The accuracy and precision results for GOFA have been obtained analyzing QC samples prepared at three different concentration levels (1.0, 10 and 20 µg/mL). R.S.D. % values did not exceed 7.6 %. Bias values ranged from 3.05 % to 4.45 %. Under these experimental conditions the concentration of GOFA in the most active extract of *L. sativa* var. *crispa* was found to be 1.25 µg/g In the remaining four extracts the quantity of GOFA did not exceed in any case this value. In our study we investigated the polyphenols and flavonoids phytochemical profile of hydroalcoholic extracts of *L. sativa* var. *crispa* grown in Morocco and stated the presence for the first time in this genus of a biologically active ferulic acid derivative like GOFA. Lettuce is widely consumed as food all over the world, and it is nowadays well known how its consumption is associated with several beneficial effects for human welfare like a decreased risk of cardiovascular diseases [13], diabetes [14], liver syndromes [15], and several others [2]. Lettuce is cultivated in many geographical areas in the world across all the five continents [16]. The composition in total polyphenols and flavonoids of several cultivars of crisphead lettuce from different countries have been reported in the literature. Data in so far acquired indicate that the geographical area of origin as well as other factors like climate, soil, and fertilizers have a deep influence on the phytochemical composition of the title plant [17]. Limited information is available in the

literature about the phytochemical composition of lettuce plants grown in northern Africa countries featured by a hot climate like Morocco. Total polyphenols and flavonoid content of plants grown in such geographical area differ in an appreciable way with values recorded for plants grown in other areals, like north and Central America, East Asia, or the European side of the Mediterranean sea. In fact values reported in the literature indicate a mean content of about 0.25 mg/mg [18] of dry extract while the extract of *L. sativa* studied herein in which the total polyphenol concentration was higher reaching value of 0.37 mg/mg as indicated in Figure 2. Thus it is reasonable to hypothesize that the geographical origin (Morocco) and the hot climate may lead to an increase of this class of secondary metabolites. As a parallelism the extract having a PI value of 5.8 extert a powerful anti-oxidant, radical scavenging, and anti-inflammatory activity. In other recent studies we have demonstrated how several vegetable and food matrices like dill, anethum, wild celery [19], honey [20], propolis [21], and *Citrus* fruits [22] known to be rich in polyphenols contained also phytochemicals belonging to a rare class of secondary metabolites, namely the oxyprenylated phenylpropanoids, shown to exert valuable and promising *in vitro* and *in vivo* anti-inflammatory and anti-cancer effects. Such natural products may contribute by a synergism of action to the observed anti-inflammatory activity of extracts obtained form the above listed natural sources. To this aim, being lettuce a nutraceutical claimed to have marked anti-inflammatory effects [17], we investigated the presence of oxyprenylated compounds also in the extracts of the title plant. Differently from other Asteraceous plants, *L. sativa* var. *crispa* contained only one geranylated product, GOFA **1** in low concentration.

In the present study we put in evidence how crisphead lettuce plants grown in Morocco exhibited a high content of polyphenols and flavonoids. Results described herein may greatly contribute to enhance the consideration of lettuce as an effective epidemiological mean for a valuable prevention strategy of socially and economically severe syndromes like cancer and inflammatory-based diseases. This hypothesis is also enforced by the discovery for the first time in the genus *Lactuca* of an oxyprenylated secondary metabolite like GOFA, having a great therapeutic potential. Data reported herein may be also a stimulus for future studies aimed to correlate the presence of prenylated and unprenylated natural products with a potential dietary chemoprevention strategy based on feeding with vegetables commonly consumed as food all over the world. As witness that dietary feeding chemoprevention by botanicals and nutraceuticals is a field of current and growing interest, several reviews and articles on this topic appeared in the literature during the last fifteen years.

Experimental

Plant material: *L. sativa* var. *crispa* plants were collected from a farm located in Kenitra region (west Morocco) (GPS coordinates 34°28'58.32''N, 5°39'47.75''O). Dried leaves were crushed into a fine powder before extraction. A voucher specimen named as LSC-MOR-001 have been stored in the deposit of the laboratory of Phytochemistry and Chemistry of Natural Compounds at the Department of Pharmacy of the University "G. d'Annunzio" of Chieti-Pescara.

Preparation of extracts: Dried leaves were extracted with five solvent mixtures: a) H_2O, b) H_2O / EtOH 3:1, c) H_2O / EtOH 1:1, d) H_2O / EtOH 1:3, and e) EtOH using a 1:10 (w/v) ratio of herb to solvent, under a continuous reflux set-up in a Soxhlet extractor for 3 h. Extracts were then filtered and concentrated under reduced pressure. Each sample was kept in the dark and stored at 4 °C before performing analysis and biological assays. PI for each extracting solvent mixture was calculated according to literature methods [7].

Total phenol content: Total phenol content was calculated by a modification of the method reported by Chekroun and coworkers [8]. Briefly 100 μL of extract (1 mg/mL) were mixed with 3 mL of freshly prepared Na_2CO_3 2 % aqueous solution. After 5 min., 100 μL of the Folin Ciocalteu reagent 1 N were added to the mixture, the resulting solution left for 30 min. in the dark at r.t. and reading was performed against a blank at 765 nm. A calibration curve was drawn in parallel under the same operating conditions using GA as a positive control. The results are expressed as mg GA equivalent per mg of dry extract (mg GA eq/mg).

Total flavonoids content: The total flavonoid content was determined by a colorimetric method as described in the literature [8]. Results were expressed as catechin (Cat) equivalent per gram of dry extract (mg Cat eq/g).

Chemistry: GOFA **1** (Figure 1) was chemically synthesized as already reported and its purity (> 98 %) assessed by GC/MS and ^{1}H NMR [12]. Methanol (HPLC-grade) was purchased from Carlo Erba (Milan, Italy) and used without further purification. Double-distilled water was obtained by a Millipore Milli-Q Plus Waters treatment system (Millipore Bedford Corp., Bedford, MA, USA). HPLC analyses were accomplished using a Waters liquid chromatograph equipped with a model 600 solvent pump and a 2996 photodiode array detector. Empower v.2 Software (Waters Spa, Milford Massachusetts, USA) was used for data acquisition. A C18 reversed-phase packing column (GraceSmart RP18, 4.6 × 150 mm, 5 μm; Grace, Deerfield, IL, USA) was employed for the separation. The column was thermostatated at 10 °C ± 1°C using a Jetstream2 Plus column oven. The UV/Vis acquisition wavelength was set in the range of 210 – 600 nm. Analogue output channel A was set at wavelength 310 nm with a bandwidth of 9.6 nm. The qualitative analyses were achieved at a wavelength of 288 nm. The injection volume was 20 μL. The mobile phase was directly on-line degassed by using a Degassex, mod. DG-4400 apparatus (Phenomenex, Torrance, CA, USA). Mobile phase composition consisted in a mixture of double distilled water (solvent A) and methanol (solvent B) at a flow rate of 1.2 mL/min. following a gradient elution as the following: 0.01 – 3.00 min A/B 6:4, 3.01 – 8.61 min. A/B 1:9, 8.61 – 15.00 min. A/B 6:4. Column re-equilibration was achieved in 7.39 min. using the original composition of the mobile phase. All the sample solutions were centrifuged and the supernatant was directly injected into the HPLC-UV/Vis system. Stock solutions of GOFA **1** were independently prepared by dissolving the pure compound obtained by chemical synthesis in MeOH at r.t. to provide an initial concentration of 1 mg/mL and stored in aliquots at − 20 °C in amber glass tubes. Working standard solutions were then obtained by appropriately diluting the stock solutions to achieve concentration values in the range 0.5 – 50 μg/mL and then stored at − 20°C in amber glass tubes for not more than 4 weeks. Separate solutions were used to prepare calibration standards and QC samples.

Ferric-reducing antioxidant power assay (FRAP): The reducing power of different extracts was evaluated according to the method of Saumya and coworkers [9].

DPPH free radical scavenging activity: The different concentrations of plant crude extracts were prepared manually and poured in different test tubes at concentrations of 2.5, 1.25, 0.63,

0.31, 0.16 mg/ml. A volume of 50 µL of each sample was added to 2 mL of phosphate buffer 0.02 M, pH 6 and 1 mL of DPPH 0.2 mM. The mixture was shaken and left for 30 min. at r.t. and the absorbance was measured at 517 nm.

Hydrogen peroxide scavenging effect: A solution of H_2O_2 (20 mmol/L) was prepared in phosphate buffered saline (PBS, 0.1 mol/L, pH 7.4). 1 mL of sample or standards (1 mg/ml) was added to 0.6 mL of H_2O_2solution in PBS. The absorbance was measured at 230 nm, after 10 min. against a blank solution that contained extracts in PBS without H_2O_2 [9].

Anti-inflammatory activity: Inhibition of protein denaturation was evaluated by the method of Reshan and Arun [10]. Acetyl salicylic acid was used as positive control. The experiment was carried out in triplicates and % of inhibition for protein denaturation was calculated using the following equation:

% Inhibition=100 − ((A_1-A_2)/A_0)*100)
A_1: the absorbance of the sample
A_2: the absorbance of the product control
A_0: the absorbance of the positive control.

The anti-inflammatory effect of hydoalcoholic extracts was evaluated by formalin-induced paw edema. Acute inflammation of rat hind paws was induced according to the method of Al-Hejjaj and coworkers [11]. The ability of the anti-inflammatory drug to suppress paw inflammation was expressed as % of inhibition of paw edema using the following equation.

$$\% \text{ AUG} = \frac{paw\ volume\ at\ timeT - InitialVolume\ (V0)}{InitialVolume\ (V0)} * 100$$

Anti-inflammatory activity was evaluated by calculating the % of inhibition of the edema

$$\% \text{ INH} = \frac{\%\ AUGcontrol - \%AUGtreated}{\%\ AUGcontrol} * 100$$

Statistical analysis: All the data are reported as mean SD of three replicates of each sample and analyzed by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

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