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Essential Oil of *Citrus aurantium* L. Leaves: Composition, Antioxidant Activity, Elastase and Collagenase Inhibition

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Abstract: Sour orange *(Citrus aurantium* L.), which belongs to the Rutaceae family, is used around the Mediterranean Sea for ornamental and agronomic purposes as a rootstock for the *Citrus* species. Peels and flowers, the most-used parts of *Citrus aurantium* L., have constituted a largely promising area of research for their many medicinal properties. However, the leaves of sour orange have not yet been studied extensively. The present study aimed at investigating the essential oil composition of sour orange leaves grown in Algeria and determining their antioxidant and anti-inflammatory properties. Essential oil composition of leaves harvested before flowering was determined by GC-MS. Total phenol content, antioxidant activities (DPPH) and elastase and collagenase inhibition were assessed. Forty-three volatile compounds were detected in essential oil from leaves with a yield of 0.57%. The major compounds were linalool, linalyl acetate and α-Terpineol. Results show that the total phenol content and antioxidant activity of essential oil are low, 3.48 ± 0.10 mg/g (Gallic Acid Equivalent/EO) and IC_{50} > 10,000 mg·L^{-1}, respectively. In contrast, EO present an interesting level of elastase and collagenase inhibition. This result emphasizes the potential interest of the essential oil of sour orange mainly in relation to its anti-aging mechanism.

Keywords: sour orange; anti-elastase; anti-collagenase; linalool; linalyl acetate; α-terpineol

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1. Introduction

The growing interest in food safety is today gaining momentum for both consumers and the food industry as people are becoming more concerned about the multiple health and environmental impacts of foods. Natural sources of compounds used in cosmetics and pharmaceutics processing today play a prominent role; their multiple positive health and environmental impacts and benefits have made them the basic principles of what constitutes a healthy diet today and in the future [1,2].

Annual production of *Citrus* plants has reached more than 126 million tons [3], one fifth of which were produced in the Mediterranean region. Therefore, *Citrus* could be considered an economically important fruit tree crop. Appreciated for their fruits, *Citrus* species are rich in vitamins B9, C and E, antioxidants, essential oil (EO), coumarins and dietary fiber [3–6].

Citrus fruits are not only used as a dessert, but also for the preparation of functional foods and *Citrus* fruit-derived products such as jams and juices, as well as in the food industry around the world. The by-products of the industrial processing of products,

mainly peels, constitute an invaluable source for the production of EO. The latter are widely used in the cosmetic, fragrance, pharmaceutical and food industries [6,7].

The chemical composition of *Citrus* EO has been extensively studied and several compositional patterns owing to the species/cultivars, origin, climate, season, ripening stage, extraction and analytical methods have been published [8–10]. The chemical composition of EO of sour orange (*Citrus aurantium*) was assessed in different plant parts during different seasons. Many studies were focused on EO extract from *C. aurantium* peels and Limonene was found to be the major component [10–19]. In contrast, other studies carried out on EO extract from *C. aurantium* flowers showed that Linalool and Linalyl acetate are the main components [13,14,20,21]. However, the volatile oil constituents from leaves have not received much attention in the literature. Indeed, the few reported studies that focused on *C. aurantium* leaves [13,21,22] showed that linalool is the main component of EO. In addition, a large number of studies on *C. aurantium* were performed in Tunisia and Greece. To our knowledge, two studies were released in Algeria concerning *C. aurantium* peels and leaves and their antibacterial activities for foods applications [11] and antifungal properties [23]. Recently, Lin et al. [24] have explored the antioxidant and antibacterial activities of essential oils of several *Citrus* species. They have highlighted interesting antioxidant activities. Nevertheless, there are no reports concerning antioxidant and/or anti-elastase and anti-collagenase activities of EO of different organs of *C. aurantium.*

The main objective of this study is to investigate the EO composition of sour orange (*C. aurantium)* leaves from Algeria and to evaluate its antioxidant, anti-elastase and anticollagenase activities.

2. Materials and Methods

2.1. Plant Material

The samples of *Citrus aurantium* (L.) var *Amara* were collected during the phenological stage of the inflorescence buds swelling (51 of the BBCH scale) in March and April 2018, at the botanical garden of the Boufarik Regional Plant Protection Station, 36 km South of Algiers (36°34′00″ N and 2°55′00″ E) and at 63 m above sea level. Three samples were collected from three different trees within the same area (100 m^2).

The harvested plant material was sealed in zipped plastic bags for transport to the laboratory of the Department of Biotechnology, Faculty of Natural and Life Sciences (University of Blida 1, Algeria). The samples were dried for 48 h at 30 °C in a ventilated oven. Grinding was performed with a Retsch knife mill (model SM100, Retsch, Eragny sur Oise, France). After grinding, the samples were used immediately for the extraction of essential oils.

2.2. Essential Oil Extraction and Yield Estimation

An aliquot of 250 g of the ground leaves (of each harvested sample) was used for the extraction of EO by hydrodistillation using a Clevenger type apparatus. Pursuant to the directives of the European Pharmacopoeia, the extraction was performed during three hours. The EO yield was calculated as follows:

$$Oil\ yield\ (\%) = \frac{Weight\ of\ essential\ oil\ (g)}{Weight\ of\ dry\ sample\ (g)} \times 100$$

The oil was dried using anhydrous sodium sulfate, stored in a sealed vial and brought to 4 °C pending analysis.

2.3. Chemical Characterization of EO by Coupling Gas Chromatography/Mass Spectrometry

Analysis of the volatile part was carried out by using gas-chromatography (GC-2014 Shimazu Gas Chromatograph, Courtaboeuf, France), equipped with a flame ionization detector AOC-20i autosampler (Kyoto, Japan). An RTX-5MS (5% diphenyl/95% dimethyl polysiloxane) GC column of 30 m × 0.25 mm × 0.25 µm from Restek (Palo Alto, CA, USA) was used in this study. The sample of EO was prepared with hexane at the ratio of 1:10 (*v*/*v*). The injector operates in split mode with a ratio of 1/50. The temperature of the

injector was maintained at 250 °C and that of the detector at 270 °C. As to the temperature of the column, the following sequence was performed: an initial temperature of 50 °C was maintained for one minute, then raised from 50 to 175 °C at a rate of 5 °C per minute. The temperature of 175 °C was maintained for 10 min, then raised from 175 to 250 °C in one step at a rate of 15 °C per minute [25].

Identification of the individual components was based on the comparison of retention indices (RI) calculated, on the polar and apolar columns, with those of authentic compounds or data from the literature (National Institute of Standards and Technology, 2008) and commercial libraries [26,27], as well as on the analysis of each mass spectrum of the constituent compounds, or were identified by comparing their retention indices with those already described in the literature [28–32].

2.4. Assessement of Total Phenolic Content

Total phenolic content was assessed with Folin-Ciocalteu reagent, according to Salachna et al. [33], and measured by spectrophotometry. One hundred microliters of EO extract were added to 0.2 mL of Folin-Ciocalteu reagent, 1 mL of sodium carbonate (at 20%) and 2 mL of distillated water. The samples were kept at 20°C in darkness for 1 h. The absorbance of samples was measured at 760 nm. The standard curve was performed on the basis of gallic acid and results were expressed as mg gallic acid equivalents (GAE) per g of EO.

2.5. DPPH Radical Scavenging Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl, Carlo Erba, Val-de-Reuil, France) Radical Scavenging assay was used to evaluate antioxidant activity of sour orange EO according to Popovici et al. [34]. One hundred microliters of EO was mixed with 100 µL of a methanolic solution of DPPH at a concentration of 63.5 µM.

Following incubation for 30 min at 25 °C, absorbance was measured at 517 nm using a GQ-1300-UV-Vis spectrophotometer (UV—Vis-Cary 4000, Agilent, Les-Ulis, France). A blank test was also performed by applying the same procedure to a solution without the test solution and its absorbance was measured. The free radical scavenging activity of the EO was calculated as a percentage of inhibition pursuant to the following equation:

$$DPPH\ scavenging\ (\%) = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample)}{Absorbance\ of\ control} \times 100$$

The antioxidant activity of EO was determined by the calculation of the IC50 index, defined as the concentration of the test solution deemed necessary to reduce by 50% the initial concentration of DPPH. All measurements were made in triplicate.

2.6. ABTS [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] Radical Scavenging Activity

The method used for the determination of $ABTS^{\bullet+}$ free radical scavenging activity was the approach presented by Baragan Ferrer et al. [35]. A 70 mM $K_2S_2O_8$ solution was also prepared. The radical cation $ABTS^{\bullet+}$ was obtained by adding 50 mL of ABTS stock solution to 200 µL of $K_2S_2O_8$ solution. The mixture was kept in the dark at room temperature for 16 h. For the evaluation of essential oils, the $ABTS^{\bullet+}$ solution was diluted with PBS to give an absorbance of 0.800 ± 0.030 at 734 nm. Three milliliters of $ABTS^{\bullet+}$ solution was mixed with 200 µL of essential oil of *C. aurantium*. The mixture was shaken vigorously and then stored in the dark at 30 °C for 6 min. A PBS solution was used as a blank sample. All determinations were performed in triplicate. The results were presented as milligrams of Trolox equivalents per gram (mg TE·g^{-1}). $ABTS^{\bullet+}$ free radical scavenging activity (Inhibition = I%) was finally calculated according to the following equation:

$$I\% = \frac{AB - AA}{AB} \times 100$$

where I is the inhibition of $ABTS^{\bullet+}$, in %; AB is the absorbance of the blank; AA is the absorbance of EO (after 10 min).

2.7. Determination of Collagenase and Elastase Inhibition

The collagenase activity (*Clostridium histolyticum*, Fisher, Illkirch, France) was carried out on an N-[3-(2-*furyl*) *acryloyl*]-Leu-Gly-Pro-Ala substrate (VWR, Fontenay-sous-Bois, France) according to Wittenauer et al. [36] and Zemour et al. [37]. The reduction in absorbance was carried out at 335 nm for 20 min using a BioTek ELX800 microplate reader (Colmar, France). Collagenase activity was performed in triplicate. It is expressed as a percentage of inhibition relative to the control.

Porcine pancreatic elastase (Servilab, Le Mans, France) was used in this study on a substrate of N-*Succ*-Ala-Ala-Ala-p-nitroanilide (Servilab, Le Mans, France) [34,35]. The release of p-nitroaniline was measured at 410 nm with a BioTek ELX800 microplate reader (Colmar, France). Measurements were performed in triplicate and the activity was expressed as a percentage of inhibition relative to the control.

2.8. Statistical Analyses

For essential oil yield and composition, as well as for the measurement of all activities, mean values and standard deviations were calculated from at least three replicates using MS Excel 2003 (Microsoft-France, Issy-les Moulineaux, France). Statistical analysis was performed by using one-way analysis of the variance (ANOVA), followed by the Duncans' post hoc test to compare the means showing significant variation ($p < 0.05$).

3. Results and Discussion

3.1. Essential Oil Yield and Composition

Forty-three volatile compounds were detected in the EO of Algerian *C. aurantium* leaves whose major compounds, linalool, linalyl acetate and α-Terpineol, constitute more than 73% of the total components (Table 1). The chromatogram is provided in Figure S1.

Table 1. Essential oil composition of Algerian *Citrus aurantium* from leaves.

Pics	T_R (Min)	Constituants	%	$RI_{literature}$	RI_{exp}	Group
1	5.4	2-Ethyl furan	0.01 ± 0.00	689	692	Fur
2	6.9	1-Hexanol	0.02 ± 0.00	799	803	Alc
3	7	α-Pinene	0.20 ± 0.01	925	923	M
4	7.8	α-Thuyene	0.01 ± 0.00	926	925	M
5	8.9	Camphene	0.01 ± 0.00	943	945	M
6	9.2	β-Pinene	3.20 ± 0.02	945	947	M
7	10.1	Sabinene	0.40 ± 0.01	973	974	M
8	10.4	δ3−Χαρενε	0.01 ± 0.00	986	989	M
9	11.1	β-Myrcene	2.25 ± 0.01	989	992	M
10	11.8	α-Terpinene	0.03 ± 0.00	1008	1016	M
11	12.2	Limonene	0.71 ± 0.01	1023	1024	M
12	12.5	β-Phellandrene	0.05 ± 0.00	1023	1025	M
13	13	2-Hexanal	0.11 ± 0.01	1024	1028	A
14	13.7	Cis-β-Ocimene	0.81 ± 0.02	1027	1030	M
15	13.8	γ−Τερπινενε	0.05 ± 0.00	1028	1031	M
16	14.8	Trans-β-Ocimene	2.40 ± 0.02	1028	1032	M
17	15.4	p-Cymene	0.05 ± 0.00	1029	1033	M
18	18.3	Cis-Oxide linalool	0.10 ± 0.00	1059	1065	O M
19	23.5	Trans-Oxide linalool	0.05 ± 0.00	1072	1073	O M
20	25.1	Terpinolene	0.45 ± 0.01	1078	1079	M
21	29.8	Linalool	30.62 ± 0.04	1083	1084	O M
22	30.5	Terpinen-4-ol	0.15 ± 0.00	1124	1129	O M
23	32.9	α-Terpineol	9.57 ± 0.05	1173	1175	O M
24	33	Citronellol	0.05 ± 0.00	1212	1213	M
25	33.5	Nerol	2.01 ± 0.00	1214	1216	O M

Table 1. *Cont.*

Pics	T_R (Min)	Constituants	%	$RI_{literature}$	RI_{exp}	Group
26	36.5	Neral	0.02 ± 0.00	1226	1227	O M
27	36.7	Geraniol	5.53 ± 0.05	1234	1235	O M
28	37	Linalyl Acetate	33.01 ± 0.07	1239	1239	O M
29	37.6	Linalyl propionate	0.08 ± 0.00	1318	1319	O M
30	38.5	Terpenyl acetate	0.10 ± 0.00	1334	1338	O M
31	38.6	Citronellyl acetate	0.02 ± 0.00	1335	1336	O M
32	40.2	Neryl acetate	2.43 ± 0.02	1342	1345	O M
33	40.5	Geranyl acetate	4.51 ± 0.03	1359	1361	O M
34	40.8	β-Caryophyllene	0.40 ± 0.01	1417	1425	S
35	42	α-Humulene	0.05 ± 0.00	1437	1440	S
36	42.1	E-β-Farnesene	0.02 ± 0.00	1443	1444	S
37	44,3	B-Germacrene	0.10 ± 0.00	1475	1477	S
38	46.9	β-Bisabolene	0.02 ± 0.00	1496	1499	S
39	57.2	Nerolidol	0.15 ± 0.01	1547	1550	O S
40	57,8	Germacra-1,5-dien-4-ol	0.02 ± 0.00	1568	1569	S
41	61.5	Spathulenol	0.01 ± 0.00	1576	1573	O S
42	63.8	T-Cadinol	0.02 ± 0.00	1626	1624	S
43	66.8	α-Cadinol	0.05 ± 0.00	1652	1653	S
		Compound group (%)				
		Oxygenated Monoterpenes	86.02			
		Monoterpenes	7.6			
		Sesquiterpenes	0.47			
		Oxygenated Sesquiterpenes	0.25			
		Other	5.29			
		Total	99,63			

A: aldehyde; Alc: aliphatic alcohol; F: Furan; M: Monoterpene; O M: Oxygenated Monoterpene; S: sesquiterpene; O S: Oxygenated sesquiterpene.

The hydrodistillation of the ground leaves of *C. aurantium* resulted in a pale yellowish oil where the EO yield was 0.57%, which was quite similar to the results already reported for this species in Algeria [23]. This yield value was higher than those observed in Tunisian sour orange leaves (0.31–0.56%) collected from different regions and during different seasons [21,22]. This result corresponds to higher values reported in the study of Almeida et al. [38], who examined seven accessions of sour orange from Brazil. This disparity could be explained by environmental and/or genetic factors, particularly for this species, which presents glandular trichomes on the surface of the leaves [25,38–41].

Forty-three constituents make up the sour orange EO. The major components were linalyl acetate, linalool, α-Terpineol, geraniol and geranyl acetate (Table 1). β-Pinene, Neryl acetate, trans-β-Ocimene, β-Myrcene and Nerol were also detected, at least at 2% of total EO (Table 1). These results confirm that the EO of Algerian *C. aurantium* leaves belongs to the linalool/linalyl acetate chemotype, reported to be the most widespread one [21,38].

3.2. Phenolic and Antioxidant Activity

Total phenol content (TPC) from sour leaves of Algeria was 3.48 ± 0.10 mg/g (GAE/EO). This value is low compared to results reported in other studies where other organs of *C. aurantium* were analyzed [16,42,43].

It is well known that free radicals cause cell death and tissue damage leading to chronic diseases. Many studies have reported the potential value of using essential oils to eliminate these free radicals [14,15,24,44]. *Citrus* essential oils have been reported to present activities that allow them to fight against cellular damage caused by physiological oxidants. These activities limit the impact of physiological oxidants and free radicals on in vitro assays [19,24,42].

Few studies have evaluated the antioxidant activities of essential oils from *C. aurantium* leaves. We measured the antioxidant activity of essential oils with 2,2-diphenyl-1-

picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) tests, which are simple and widely used for antioxidant studies. These tests were chosen in order to compare our results with results reported in other *Citrus* species [12,16].

Sour orange EO presented a low antioxidant activity, determined by DPPH assays, with IC_{50} values > 10,000 mg·L^{-1}; meanwhile, ascorbic acid showed a IC_{50} value of 3.9 mg·L^{-1}. These values were typically low and concur with those reported in Tunisian and Serbian EO of *C. aurantium* [12,16]. In contrast, hydrosol and ethanol extracts from *C. auriatium* from Turkey have shown significant antioxidant activities [18]. Indeed, in this report [18], ethanol and hydrosol were used as solvents, while our study used EO as extracted by hydrodistillation. Moreover, many of the antioxidant activities observed in *C. aurantium* have been assessed in EO extracted from different organs or byproducts of *Citrus* transformation industries. There are no published reports on antioxidant activity of EO from leaves of sour orange. In our study, ABTS radical scavenging assay exhibited low antioxidant activity (38.6 mg Trolox equivalent·g^{-1}). Similar small antioxidant activities of essential oils of numerous plant species against ABTS radicals were detected [44–47]. These discrepancies are mainly due to the small content of phenols in the EO of sour orange [16]. Indeed, the portion of the fruit used for extraction notwithstanding, the major component of sour orange EO displayed no antioxidant effect [12,13,48,49]. This fact is also supported by the low TPC observed in our study. Nevertheless, Hsouna et al. [16] reported an interesting antioxidant activity of EO of sour orange peels. This difference could be due to the EO composition [6]. Phenolic compounds have been shown to serve as electron donors in free radical reactions and seem to be frequently associated with the EO antioxidant effects [18]. The results obtained for sour orange are different from those shown in four *Citrus* species essential oils of peels. Indeed, Lin et al. [24] have reported strong antioxidant activities measured both by DPPH and ABTS tests. Obviously, the chemical composition, environmental conditions as well as genetic background are among the factors which explain these differences.

3.3. Collagenase and Elastase Inhibition

Figure 1 shows the results of Collagenase and Elastase inhibition which were represented by anti-collagenase and anti-elastase activities of sour orange EO.

The EO of sour orange leaves is mainly composed of linalool, linalyl acetate and α-terpineol, which constitute more than 73% of the total components (Table 1). These components were reported to exhibit anti-aging properties [50]. Moreover, limonene, which was not present at elevated levels in the study's EO (Table 1), was reported to display higher anti-inflammatory properties [16,51]. In addition to their individual activities, these compounds could display a synergistic action [16,44]. Several Lamiaceae and Apiaceae EOs have been shown to exert anti-aging properties [48–50]. Zemour et al. [38] highlighted the same effect in safflower vegetable oil. All these reports emphasized that the inhibition of collagenase and elastase may spring from the inhibition of the production of pro-inflammatory mediators. Their results mirrored strong anti-aging activities. Even when evaluated using different methods, the unique available work [16] agrees with the findings of the current study. The exploration of skin protective formulations is linked to the use of plant constituents which have an antioxidant activity. This activity is correlated with the ability to protect the different layers of the skin (dermal and epidermal), mainly composed of elastin and collagen. Skin exposure to external aggressions (for example, UV, temperature, etc.) leads to the increase of the enzymes involved in the aging process, including collagenase and elastase. Their activity triggers the degradation of major components such as collagen and elastin. This in its turn accelerates the visible aging of the skin evidenced by age-related skin changes such as wrinkles and sagging skin depending on the exposure to primary risk factors [51,52].

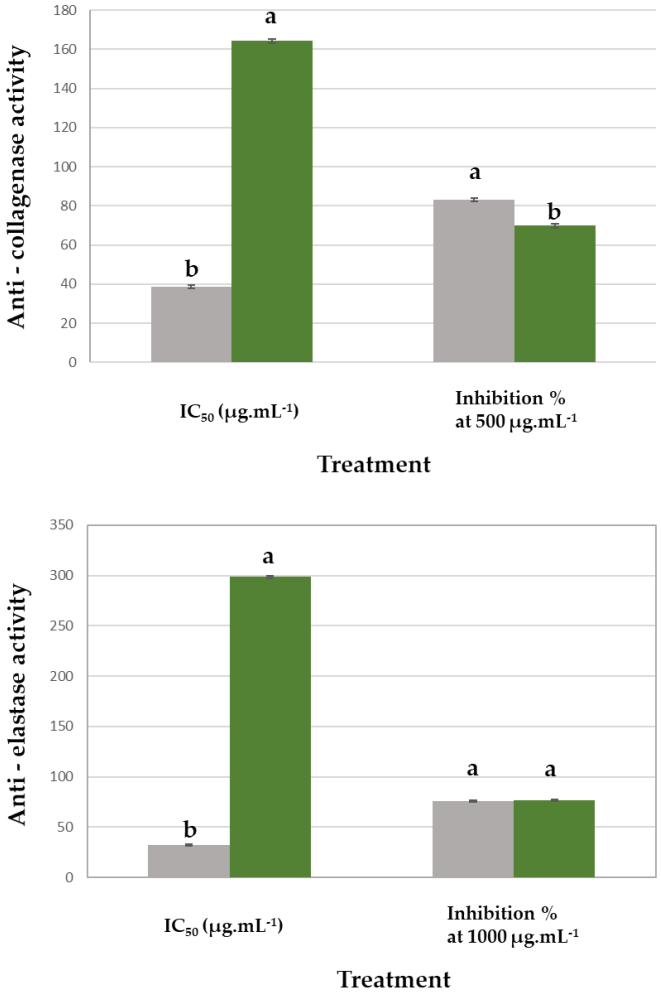

Figure 1. Collagenase and Elastase inhibition represented by anti-collagenase activity and anti-elastase activity of the EO of *Citrus aurantium* leaves. Means and standard deviation values are displayed. Grey columns represent control and green ones essential oil Similar letters for the same treatment indicate non-significant differences as indicated by Duncan's multiple range test, at $p < 0.01$.

These results highlight the interest of using the EO of *C. aurantium* leaves as a potential source of valuable components in the pharmaceutical, food and cosmetic industries [6,43]. This is also the case of the EO extracted as a by-product from peels and flowers. Indeed, the EO of sour orange leaves exhibited interesting insecticidal effects against saw-toothed grain beetle and rice weevil [47].

Fungicidal, bactericidal and insecticidal effects were also reported for the EO from leaves, flowers and peels of *C. aurantium* from North Africa and India [11,21,42,53]. In contrast, peel EO demonstrated a larvicidal activity against the Malaria Vector *Anopheles stephensi* [15,47,54]. Moreover, Costa et al. [55] reported an anxiolytic-like activity and LDL-cholesterol lowering effects of mature fruit EO. This effect was reported to be highly effective on patients diagnosed with chronic lymphocytic leukemia who have received EO of *C. aurantium* by inhalation [56]. It is well known that the EO of this species is used to treat gastric disorders [6,57]. Moraes et al. [58] emphasized that, after intraduodenal administration of a single dose of EO of bitter orange to rats, EO may contribute significantly to the development of a remedy for gastric damage prevention.

4. Conclusions

Natural compounds, their properties and their activities play a crucial role in maintaining human health and preserving the environment. Given their importance for perfumery, cosmetic and pharmaceutical uses, EO have attracted interest from researchers. EO from leaves of *C. aurantium* grown in Algeria was mostly constituted of linalool, linalyl acetate and α-Terpineol. EO presented a low phenol content and low antioxidant activity. In contrast, as a result of this composition, the inhibition of elastase and collagenase activity was very high. This finding constitutes a first report of anti-elastase and anti-collagenase activities reported in the EO of leaves from this species, which must be established by conducting more experiments using different methods. If confirmed, the anti-collagenase and anti-elastase activities of *Citrus aurantium* EO could increase its use in drug formulations and in pharmaceutical and cosmetic applications.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12061466/s1, Figure S1: Chromatogram of C. aurantium essential oil.

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