Citrus × Clementina (leaf): Phytochemical and antioxidant activity

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Abstract

The petitgrain *'Citrus*clementina'* is an aromatic plant classed as a green waste. Even though it has benefits, few researchers studied it. The aim of this study is to extract the essential oils from the leaves of *C. clementina* by using two methods, the conventional hydrodistillation and the steam distillation, and to evaluate the content of phenolic compounds and the antioxidant activities of their extracts.

The constituent of volatile compounds obtained by conventional hydrodistillation (Heh) and steam distillation (HEv) methods were analyzed by (GC-MS) and their results were compared.

The major compounds of their essential oils were sabinene, linalool, limonene, carina <δ-3-> and ocimene <(E)-β->. The chemical composition of essential oil from each method were compared and found to be similar.

Decoction and Soxhlet are the used methods for extract of phenolic compounds. The aqueous and hydro-ethanolic extracts from the Soxhlet method registered the highest phenolic contents.

The antioxidant activity of *C.clementina* extracts were performed by ferric reducing antioxidant power (FRAP) and total antioxidant capacity (CAT). The results indicate that the decocted and hydroethanolic extracts show a greater antioxidant capacity compared to the aqueous extract.

Thus, this result is a relevant source of bioactive compounds with antioxidant properties of interest for consumers and cooperatives as well as participating in socioeconomic development.

Key words: Antioxidant activity, *Citrus clementina* Hort., Phytochemistry, Total phenolic content

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Abbreviation

EO: Essential oils — *C. clementina: Citrus clementina* hort

Ed: Decocted extracts — GC-MS: Gas chromatography coupled with mass spectroscopy

HEv: steam distillation — HEh: hydrodistillation

Ee: Aqueous extract — Eet-e: Hydro-ethanolic

EC_{50}: Exhibitory concentration 50% of ferric reducing antioxidant power

I. Introduction

Aromatic and Medicinal Plants (AMP) have caught the interest of people working in different sectors and organizations. Citrus is one of those AMP that has emerged in world-renowned cultures, with a potential socio-economic impact (Dalia et al., 2016). More particularly, Morocco has become home to some of the most important genetic centers of citrus, thanks to promising production zones in the regions of Souss, Gharb, Moulouya, Tadla, Haouz, and Loukkos (Mohamed Afechtal et al., 2015). Indeed, citruses are dicotyledonous angiosperms belonging to the Rutaceae family (a subfamily of Aurantioidæ) (Audray, 2015)**.** This family includes 2000 species and 150 genera of plants. The group formed by the citruses includes six sexually compatible genera, namely Citrus, Fortunella, Poncirus, Microcitrus, Eremocitrus and Clymenia, the majority of which belong to the genus Citrus (Jamali et al., 2006)**.**

The genus *Citrus* displays a diverse basic taxon. Clementine (*Citrus clementina)* is among the species accounting for nearly 20% of the annual production of citrus fruits in the Mediterranean Basin countries (30% in Morocco). It ranks second behind oranges (55%). Additionally, most of the studies carried out on this topic confirm that this species results from the natural hybridization of the tangerine tree (*Citrus reticulata*Blanco) and the bitter orange tree *Citrus aurantium*L. (Barbier, 1964; Chahidi et al., 2008; Loizzo et al., 2018)**.***Citrus clementina hort.* is a two to three-meter-high tree, bearing highly fragrant leaves and flowers. The leaves are dark green, 3-4 cm long, glistening and lanceolate. As for the flowers, they are white and joined in clusters. Flattened at the poles, the fruit has a spherical shape as well as a thin, orange, and slightly adherent peel. The pulp is juicy, fragrant and practically seedless (Hamdani, 2018; Robin, 2011)**.**

In fact, the clementine fruit is widely consumed as a natural source of antioxidant compounds such as vitamin C, vitamin B, pectin, carotenoids, etc. (Ghasemi et al., 2009; Mahrouz et al., 2002) while the leaves are regarded as waste. Nevertheless, these wastes are a potential source of bioactive compounds, including natural antioxidants, particularly essential oils, flavonoids, and other phenolic compounds. Besides, numerous studies have demonstrated that these citrus leaves have

antimicrobial, antioxidant, anti-inflammatory, antidiabetic, and nutritional properties (Al-Gendy et al., 2017; Bissim et al., 2020; Butelli et al., 2019; Hamdani, 2018; Leporini et al., 2020; Menichini et al., 2011; Rao and Gajula, 2016; Singh et al., 2020).Furthermore, the use of antioxidant plant extracts has recently been suggested both as an alternative to food preservation technology and as a prophylactic agent for some human diseases(Khettal et al., 2017).This study aims at defining the chemical composition of essential oils and quantifying the contents of polyphenols, flavonoids, and tannins. An in vitro evaluation of the antioxidant activity of *C. clementina* hort. leaf extracts has been carried out, using the iron reduction method (FRAP) and that of the total antioxidant capacity. The results could contribute to the valorization of this species in the agri-food and traditional medicine fields.

II. Materials and Methods

1. Plant Material

C. Clementina hort. leaves were collected in December 2018 at a site in Beni Mellal (Laayayta) by staff members from AGRI LOTFI, a company engaged in the valorization of AMP. The dried leaves were pulverized and used to make the set of extracts.

2. Extraction and analysis

2.1. Humidity level

To measure the moisture content of plant material, three samples of 5 g, placed in a tare dish 2 mm thick, are dried in an oven set at 105 ± 2 ° C. The weight of the samples was evaluated following 24 hours of the drying process. Then, the samples were cooled to ambient temperature using a desiccator. The humidity level was measured according to the below formula:

$$TH\% = \frac{(m_0 - m)}{m_0} \times 100 \quad \text{(eq. 1)}$$

Where:

m: mass of sample after drying,

m_0: mass of sample before drying

TH%: percentage of humidity

2.2. Essential oil extraction

2.2.1. Hydrodistillation

The hydrodistillation method was employed in the extraction of dried *C. clementina* leaves. 100 g of the *C. clementina* leaves was placed in a 2L container along with 1L of water and kept in a Clevenger-type apparatus for three hours.

The yield in essential oil is the ratio between the weight of extracted essential oil and the weight of dried plant biomass. The yield is calculated from three extractions and it is expressed as a percentage (%) (Bousbia, 2011).

$$Y(\%) = \frac{M_{EO}}{M_{DP}} * 100 \text{ (eq. 2)}$$

Y (%): the yield in essential oil (%).

M_{EO}: weight of essential oil extracted (g).

M_{DP}: weight of dried plant material.

2.2. **Steam distillation**

The steam distillation method was adopted for the extraction of dried *C. clementina* leaves. The still used in the process has a separate boiler and an injected steam volume of 3000 liters. 500 kg of plant material was placed in a still, while 300 liters of water were being heated in the boiler. The water vapors are injected directly into the plant material in the still. Thus, the EO is extracted from the leaves and transferred via water vapor through a cooling system that makes it condensed. Then, it is recovered in an essencier and stored at 4° C.

2.3. Gas chromatography-mass spectrometry analysis (GC–MS)

The EO were analyzed using the method of gas chromatography coupled with mass spectrometry (GC / MS) in a lab (*Centre d'analyses*) at the Faculty of Sciences of Moulay Ismail University, Meknes. The EO chromatographic analysis was conducted using a Hewlett-Packard type gas chromatograph (6890 series) coupled with a mass spectrometer (HP 5973 series). Fragmentation was performed by electron impact intensity 70eV. The gas chromatograph is equipped with a Split injector, a fused silica column bonded on the inside with a stationary phase DB-5 (5% phenyl-methyl-siloxane). The carrier gas is nitrogen with a flow rate of 1 ml / min. The chromatographic separation took place on a DB-5 MS column (5% phenyl-methyl-siloxane) (30m x 0.25mm, film thickness: 0.25µm). The column temperature is set at 50 to 200° C at a rate of 4° C / min. The device is connected to a computer system managing a NIST 98 mass spectrum library.The identification of the constituents was made on the basis of the comparison between their calculated

Kovats indexes (according to the formula below) and those of the standard compounds contained in the available databases: Adams (Adams, 2007)andNational Institute of Standards and Technology (NIST) (http://webbook.nist.gov/chemistry/).

The Kovats index is calculated according to the following formula:

$$IK = \left[\frac{Tx-Tn}{Tn+1-Tn} + n\right] * 100 \qquad \text{(eq. 3)}$$

Where IK= KOVATS index.

Tx: Retention time of compound x to be identified.

Tn: Retention time of the hydrocarbon (with the number of carbon atoms being n) eluted before compound x.

Tn + 1: Retention time of the hydrocarbon (with the number of carbon atoms being n + 1) eluted after the compound x.

n: Number of carbon atoms of the hydrocarbon eluted before compound X.

2.4. Phytochemical screening of plant material

Phytochemical screening is a preliminary qualitative analysis based on color and/or precipitation reactions and aimed at highlighting the main chemical classes of secondary metabolites. This analysis was performed on various extracts obtained from the leaves of the plant that was analyzed in an aqueous medium through decoction and infusion; as well as in an organic medium by maceration of two solvents: ether and sulfuric acid. The tests were carried out according to the widely used phytochemical methods, described for the most part by (Dey PM and Harborne JB, 1989; GIBBS, 1974).

Various reagents were required for the phytochemical screening. The compounds belonging to the group of flavonoids were recognized based on the cyanidin reaction. Ferric chloride reaction led to the characterization of tannins. The differentiation between the two groups of tannins was exhibited by means of the Stiasny reaction. The characterization of alkaloids was carried out using two reagents, namely the Dragendorff reagent and the Mayer reagent. The detection of reducing compounds was performed using Fehling's solution. The detection of saponosides is indicated by the appearance of foams after stirring the aqueous solution. The search for sterols and terpenes was done by way of the Liebermann-Buchard reaction.

The results were evaluated as follows: +++: Strongly positive; ++: Moderately positive; +: Weakly positive; -: Negative.

2.5. Quantitative phytochemical analysis

2.5.1. Extraction of total phenolic compounds

Total phenolic compounds were extracted from the leaves of *C. clementina* based on two different methods, namely Soxhlet extraction; using two solvents, water and an ethanol-water mixture (70/30) (v/v); and extraction by "decoction," the method recommended in traditional medicine.

a. Decoction extraction

Decoction is the method of extraction recommended in traditional medicine. This extraction method was implemented, in accordance with the protocol described by (Djewe, 2012; Konkon et al., 2006) but with slight modifications. In our case, the decoction was performed based on the powder of dried and smashed *Citrus clementina* hort. leaves. 30 g of this plant's powder was placed in a customized container with 600 ml of distilled water. Then, the mixture was brought to the boil at a temperature of 80° C, with moderate stirring that lasted for 60 minutes. After filtration, a solution of about 200 ml was obtained and then evaporated using an all-stainless-steel automatic sterilizer (CE 0499) at a temperature of 60 ° C in order to make a dry extract (decocted Ed) which can be pulverized and stored **at room temperature until use.**

b. Soxhlet extraction

Soxhlet extraction is a conventional technique used for the extraction of thermolabile substances(Luque de Castro and García-Ayuso, 1998)**.** Indeed, 30 g of dry *C. clementina leaves* turned into powder are extracted using 600 ml of solvent. In our case, we used water and the ethanol-water mixture. The obtained aqueous (Ee) and hydro-ethanolic (Eet-e) extracts were dried in a ventilated drying oven at 60° C. The dried crude extracts of various solvents were collected in sterile vials and stored at ambient temperature until use.

c. Extraction yield

Extraction yield is defined as being the ratio between the mass of the obtained dry extract and the mass of the processed plant extract. This yield of extraction is calculated using the following equation:

Where $$Rt\% = \frac{M_e}{M_v} \times 100 \quad \text{(eq. 4)}$$

Rt (%): Extraction yield in %,

Me: Mass of the extract after evaporation of the solvent,

Mv: Mass of plant material used for extraction.

2.5.2. Determination of polyphenols, flavonoids and condensed tannins contents

a. Polyphenols contents

The determination of polyphenols from different extracts of the plant is carried out using the Folin-Ciocalteu reagent, based on a slightly modified version of the method described (Singleton and Rossi, 1965). A volume ($0 < V \leq 100$) µl of extract is mixed with 1.5 ml of the Folin Ciocalteu reagent (10%) and 1.5 ml of Na_2CO_3 (7.5% - m / v), with the mixture being stirred in a 50 ml volumetric flask. Then, the volume is adjusted with distilled water up to the filling mark. Following a 60-minute incubation in the dark and at ambient temperature, the absorbance is measured at 760 nm using a spectrophotometer (V-1200), against a blank containing only the reagents. The results are expressed in milligrams of gallic acid equivalents per gram of extract (mg EAG / g E).

b. Flavonoids contents

The total flavonoid content in the crude extracts was determined using the aluminum trichloride colorimetric method described by (Chang et al., 2002), with a slight modification.

The method requires placing, successively, a volume of extract ($0 < V \leq 100$) µl, 2 ml of distilled water, and 10 µl of aluminum chloride $AlCl_3$(10%) in a test tube. Pure methanol is added to make a 5 ml solution. The mixture is stirred and then incubated in the dark for 30 min. The absorbance is measured at 433 nm using a spectrophotometer (V-1200) against a blank that consists of a mixture of reagents, replacing the extract with pure methanol. The obtained results are expressed in mg equivalent of quercetin / per gram of extract (mg EQ / g E).

3. Condensed tannins contents

The content of condensed tannins was determined using the vanillin method, i.e. reaction with vanillin in an acidic medium (Sun et al., 1998)**.** The method requires placing, successively, a volume of extract ($0 < V \leq 10$) µl, 3 ml of the vanillin/methanol solution (4%), and 1.5 ml of concentrated hydrochloric acid in a test tube. The mixture is stirred and then incubated in the dark for 20 min. The absorbance is measured at 499 nm by a spectrophotometer (V-1200) against a blank consisting of a mixture of reagents. The results are expressed in mg equivalent of catechin per gram of extract (mg EC/g E)

4. Antioxidant activity assays

Two methods were used for the evaluation of the antioxidant activity of the set of extracts of *C. lementina* leaves (hydro-ethanolic, aqueous, and decocted extracts), namely the ferric reducing antioxidant power (FRAP) and the total antioxidant capacity.

4.1. Total antioxidant capacity

The total antioxidant capacity of *C. clementina*. leaf extracts was evaluated using the method of (Prieto et al., 1999)**.** This technique relies on the reduction of molybdenum (VI) into molybdenum (V) in an acidic medium using a plant extract. 1 ml of the phosphomolybdate reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) is added to 10 µl of extract. The tubes are incubated at 95°C for 90 min after cooling for 20 min at ambient temperature. The absorbance is measured at 695 nm. The results are expressed in mg equivalent of gallic acid / 100 g of extract (mg EAG / 100 g E).

4.2. Ferric reducing power assay (FRAP)

This test allows the measurement of the tested *C. clementina* hort. leaf extracts' capacity to reduce the ferric iron (Fe^{3+}) found in the $K_3Fe(CN)_6$ complex into ferrous iron (Fe^{2+}). This research followed the experimental protocol cited in(Oyaizu, 1986)**.** Samples of 0.5 mL taken from various concentrations of each extract of 0.5 to 5 mg / mL diluted in distilled water are mixed with 2.5 mL of the phosphate buffer solution (0.2 M - pH 6.6), and 2.5 mL of potassium ferricyanide $K_3Fe(CN)_6$ at 1%.The mixtures are incubated at 50 ° C for 20 minutes before being cooled. Then, 2.5 mL of the trichloroacetic acid (10%) is added. 2.5 mL of the supernatant from each concentration is mixed with 2.5 mL of distilled water and 0.5 mL of iron chloride ($FeCl_3$) at 0.1%. Ascorbic acid is used as the standard antioxidant in this experiment. The absorbances are read at 700 nm against a blank using a spectrophotometer (V-1200). An increase in absorbance is associated with the increase in the reducing power of the tested extracts and therefore the antioxidant capacity.

5. Statistical analysis

All tests were conducted in triplicates and the data were presented on an average ± SD. Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad Inc., San Diego, California). Data were analyzed using one-way ANOVA (Analysis of Variance). Differences (between groups) were considered as statistically significant at $p < 0.05$

III. Results and Discussion

1. Yields and Chemical composition of *C. clementina hort.* leaf *EO*

Extraction of C. *clementina leaf* EO was performed using two methods; hydrodistillation and steam distillation. Indeed, the average yield obtained through the hydrodistillated extract (HEh) was in the range of (0.90 ± 0.05) %, while the yield obtained using steam distillation (HEv) was in the range of 0.2 %. We noted that the yield of citrus EO obtained using the hydrodistillation method is significantly higher than that obtained by steam distillation.

The EO yield result obtained by hydrodistillation is almost equal to the result found by (Fleisher and Fleisher, 1990). On the other hand, the ones provided by (El-hawary et al., 2013; Leporini et al., 2020) are characterized by low yields in the range of (0.43-0.65) % and (0.39-0.43) %, respectively.

The chromatographic analysis (GC / MS) of petitgrain clementine EO enabled us to identify **34** constituents for both HEh and HEv oils, with respective rates in the range of **99.88%** and **98.81%** (Table 1).

The hydrogenated monoterpenes constitute the most abundant group of all the identified compounds, with rates measured respectively at **68.28%** and **64.7%**, for HEh and HEv. They consist mainly of: Sabinene (30.44% and 29.68%), Limonene (9.21% and 10.33%), Carene <δ-3-> (9.1% and 6.31%), Ocimene <(E) -β-> (7.98% and 6.85) and <β-> Pinene (3.37% and 2.87%). Moreover, Linalol (13.06% and 15.75%), Citronellal (4.38% and 3.21%) and Terpinen-4-ol (2.07% and 3.54%) constitute the greater part of oxygenated monoterpenes with a percentage of (**22.14% and 24.99%)** for HEh and HEv, respectively. As for the oxygenated sesquiterpenes, their rate was measured at **8.87%** for HEh and **8.58%** for HEv. HEh and HEv are mainly made up of: Sinensal<β-> (3.78% and 1.93%), Sinensal<α-> (3.5% and 0.97%) and Geranyl acetate (1,23% and 1.04%), stated in a respective order. While a total of **0.59%** of HEh Sesquiterpenes is represented by Farnesene<(E, E) -α-> (0.09%) and Bicyclogermacrene (0.08%), a total of **0.54 %** of HEv Sesquiterpenes is represented by the chemical compound (Caryophyllene <(E) ->). We observed a great similarity in the qualitative and quantitative chemical composition of *C. clementina* essential oils extracted using both methods, HEh and HEv. Similar results were obtained from *C. clementina EO* originating in Italy (Germanà et al., 2013; Leporini et al., 2020), France (Lota et al., 2001) and Egypt (El-hawary et al., 2013), with the main compounds consisting of sabinene (22.59-23.32%), (36.8%), (33.1-49.80%) and (9.94- 19.76%), linalol (10.41-16.83%), (21.2%), (19.4-23.5%) and (10.23-18.78%) and limonene (5.88-6.62%), (4.2%), (2.5-6.9%) and (11.54-21.13%), respectively.

Additionally,(Thi Nguyen et al., 2015) indicated that the EO extracted from the leaves of Vietnamese *C. clementina* has the same major compounds found in the above-mentioned studies. However, this EO is distinguished by an elevated rate of other constituents such as: β-Elemene (10.50%) and β-caryophyllene (4.04%).

The difference in the yield content and chemical composition of our EO, compared to those stated in the literature may depend on various factors, including the extraction method, geographical origin, harvest period, and drying conditions of the studied plant species.

We have observed that both adopted extraction methods (hydrodistillation and steam distillation) provide similar results in terms of the quality and quantity of the obtained EO chemical

constituents. This proves that the stainless-steel quality of the still as well as its method of extraction did not modify the chemical constituents of the plant's essential oil. Despite the fact that the Clevenger apparatus produces an EO yield significantly higher than that of the still, the latter is capable of extracting 1 liter of EO from a ton of citrus leaves. Citrus leaves are considered as waste from the clementine tree, which we were able to valorize in this research by demonstrating the richness of their EO in bioactive molecules such as monoterpenes (sabinene and linalol) and sesquiterpenes (Sinensal<β->, Sinensal<α- >.

Table 1: The chemical composition of EO of the leaves of *C. clementina hort*. extracted by the Clevenger apparatus and the industrial Alembic

Kovats index	**Compound**	**Molecular Formula**	**AR (%) of EO of *C. clementina* extracts by Apparatus**	
			Clevenger (Laboratory)	**Alembic (Industrial)**
930	α-Thujene	$C_{10}H_{16}$	0.33	0.24
939	α-Pinene	$C_{10}H_{16}$	1.44	1.19
975	Sabinene	$C_{10}H_{16}$	30.44	29.68
979	β-Pinene	$C_{10}H_{16}$	3.37	2.87
1002	α-Phellandrene	$C_{10}H_{16}$	0.72	0.6
1011	δ-3-Carene	$C_{10}H_{16}$	9.1	6.31
1017	α-Terpinene	$C_{10}H_{16}$	0.74	1.03
1026	p-Cymene	$C_{10}H_{14}$	0.21	0.34
1029	β -Phellandrene	$C_{10}H_{16}$	1.19	1.14
1029	Limonene	$C_{10}H_{16}$	9.21	10.33
1037	(Z)-β-Ocimene	$C_{10}H_{16}$	0.28	0.31
1050	(E)-β-Ocimene	$C_{10}H_{16}$	7.98	6.85
1059	γ-Terpinene	$C_{10}H_{16}$	1.29	1.87
1070	cis-Sabinene hydrate	$C_{10}H_{18}O$	0.49	0.47
1088	Terpinolene	$C_{10}H_{16}$	1.98	1.94
1096	Linalool	$C_{10}H_{18}O$	13.06	15.75
1098	trans-Sabinene hydrate	$C_{10}H_{18}O$	0.09	-
1121	cis-p-Menth-2-en-1-ol	$C_{10}H_{18}O$	0.1	-
1153	Citronellal	$C_{10}H_{18}O$	4.38	3.21
1177	Terpinen-4-ol	$C_{10}H_{18}O$	2.07	3.54
1188	α-Terpineol	$C_{10}H_{18}O$	0.5	0.56
1225	Citronellol	$C_{10}H_{20}O$	0.93	1.46
1237	pulegone	$C_{10}H_{16}O$	0.26	-

1267	Geranial	$C_{10}H_{16}O$	0.16	-
1324	Methyl geranate	$C_{11}H_{18}O_2$	0.1	-
1381	Geranyl acetate	$C_{12}H_{20}O_2$	1.23	1.04
1419	(E)-Caryophyllene	$C_{15}H_{24}$	0.42	0.54
1500	Bicyclogermacrene	$C_{15}H_{24}$	0.08	-
1505	(E.E)- α -Farnesene	$C_{15}H_{24}$	0.09	-
1563	(E)-Nerolidol	$C_{15}H_{26}O$	0.13	-
1583	Caryophyllene oxide	$C_{15}H_{24}O$	0.15	-
1699	β -Sinensal	$C_{15}H_{22}O$	3.78	1.93
1756	α-Sinensal	$C_{15}H_{22}O$	3.5	0.97
1767	Tetradecanoic acid	$C_{14}H_{28}O_2$	-	0.82
1876	cis-9-hexadecanal	$C_{16}H_{30}O$	-	0.29
1910	Hepta decanal	$C_{17}H_{34}O$	-	0.54
1943	Phytol	$C_{20}H_{40}O$	0.08	-
1970	n-hexadecanoic acid	$C_{16}H_{32}O_2$	-	1.15
2147	Octadecanoic acid	$C_{18}H_{36}O_2$	-	0.31
2175	Oleic acid	$C_{18}H_{34}O$	-	1.53
Hydrogenated Monoterpenes (%)			**68.28**	**64.7**
Oxygenated Monoterpenes (%)			**22.14**	**24.99**
Hydrogenated Sesquiterpenes (%)			**0.59**	**0.54**
Oxygenated Sesquiterpenes (%)			**8.87**	**8.58**
Total (%)			**99.88**	**98.81**

Phytochemical screening of *C. clementina* leaves

The phytochemical screening of secondary metabolites from Petitgrain of clementine reveals their richness into chemical families (Table 2).

The family were alkaloids, Gallic and Catechic Tannins, Flavonoids, Leucoanthocyanins, sterols and triterpenes, Mucilages, Reducing compounds and Oses and holosides. However, Saponosides were almost absent. These results were found to be similar to the results found by by (Rao and Gajula, 2016) for the genus citrus aurantium.

Table 2: Results of phytochemical screening of *C.clementina* leaves by colored reactions

Chemical group		**Observations**
Tannins	Gallic	+++
	Catechic	+++
Flavonoids	Flavone	++
	Leucoanthocyanins	+++
Saponins		-
Alkaloids		++
Reducing compounds		++
Oses and holosides		+++
Mucilages		++
Sterols and triterpenes		+++

2. Polyphenol extraction yields from C. clementina leaves

The yields of extraction of the polyphenols from the various extracts of Petitgrain (Eet-e, Ee and Ed) were reported in figure **1**.

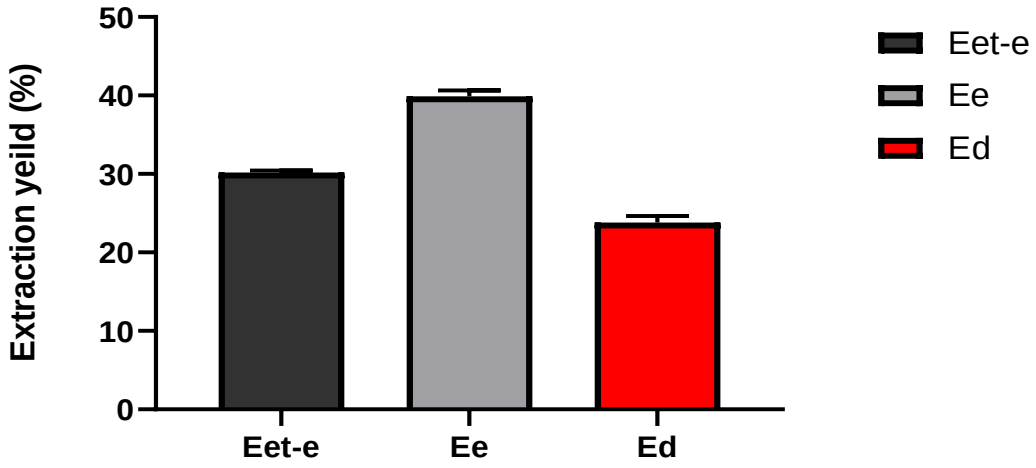
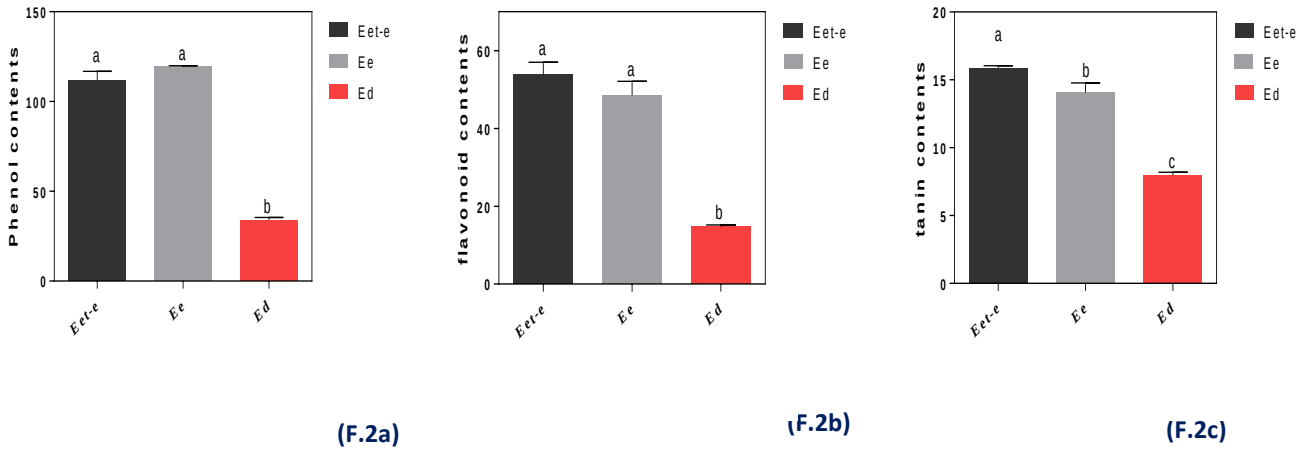


Figure 1: Extraction yields of polyphenols in (%) of *Citrus clementina* leaves

The results shown in Figure (**1**) illustrate that the hydroethanolic (Eet-e) and aqueous (Ee) extracts that were extracted by the Soxhlet technique gave the greatest yields respectively, (29.92 ± 0.07) % and (40.43 ± 0.76) % while the decocted extract (Ed) produced by the decoction process gave the smallest yield (23.20 ± 0.66) %. It can be appreciated that the yields of the extractions differed with the extraction technique used and the solvent applied.



(F.2a) (F.2b) (F.2c)

Figure 2: Phytochemicals content of *C.×clementina* leaves extracts

3. Determination of phenolic compounds of C. clementina leaves extracts

The contents of the Polyphenols, Flavonoids and Tannins of the leaves extracts of *C. clementina* are shown in Figure 2. The found results were calculated from calibration curves by using the references standards and the following equations are given below:

→ Polyphenols: $y = 0.0532x-0.0025$ $R^2 = 0.995$

→ Flavonoids: $y = 0.0509x + 0.0005$ $R^2 = 0.9948$

→ Tannins: $y = 0.0427x + 0.0036$ $R^2 = 0.9881$

3.1. Polyphenols contents

The total phenol content of *C. clementina* leaves extracts are represented as mg equivalent of Gallic acid by gram of extract, Figure **2a**. The contents of the extracts Ee, E(et-e) and Ed are respectively (119.256 ± 0.723) mg EAG/g E, (111.738 ± 5.064) mg EAG/g E and (34.043 ± 1.447) mg EAG/g E.

It can be noted that the polyphenol contents of the extracts (Ee and Eet-e) obtained by Soxhlet are largely greater than the extract from decoction (Ed) and that the use of water shows to be a better solvent compared to the hydroethanolic solution.

Studies carried out by (Khettal et al., 2017; Leporini et al., 2020) the hydro-ethanolic extract of petitgrain leaves indicated a polyphenol content in the range of (45.54 ± 3.70) mg EAG / g MS and (125.28 ± 4.15) mg EAG / g MS respectively. Our results seem to be different from those obtained by researchers in the studies cited above. Moreover, the study of Hachemaoui (Hachemaoui and Oumbiche, 2013) confirms that the content of phenolic compounds in *C.*

reticulata is greater when obtained in an aqueous medium using the soxhlet method, compared to the method relying on the hydro-ethanolic mixture. This result is similar to the findings of our study.

3.2. Flavonoids contents

Flavonoid contents were expressed as mg quercetin equivalents per gram extract (mg QE/g).

In Figure **2b**. we note that the hydro-ethanolic extract (Eet-e) obtained using the soxhlet method has the greatest content of flavonoids. i.e. 53.897 ± 3.119 mg QE / gE. compared to the aqueous extract (Ee) with a value equal to 48.483 ± 3.612 mg QE / gE. while the decocted extract (Ed) reflected a value in the range of 14.735 ± 0.454 mg QE / gE. found to be the lowest.

According to (Khettal et al., 2017), the flavonoid content in the aqueous extract of *C. clementina* leaves is in the range of 46.25 ± 2.58 mg QE / g DW. This value is close to the findings of our study. As for the hydro-ethanolic extract. (Leporini et al., 2020) stated a value in the range of 29.16 ± 2.92 mg QE/g DW, which is considerably lower than our findings.

3.3. Condensed tannins contents

The results of condensed tannin content in the extracts of *C. clementina* leaves were expressed as mg catechin equivalents in gram extract (mg CE / gE) (Figure **2c**).

The average content of condensed tannins of *Citrus clementina* leaves is relatively higher in the case of the hydro-ethanolic extract (Eet-e), which is in the range of 15.888 ± 0.162 mgCE / gE. followed by the aqueous extract (Ee), which is equal to 14.061 ± 0.721, and decocted extract (Ed), with a value of 7.972 ± 0.215 mgCE / gE.

A study on the hydro-ethanolic extract of *Citrus clementina* leaves carried out by (Rahmouni and Yaiche. 2014) indicate a value of 41.03 ± 1.63 mg EAT / 100g DW. This value is considerably higher compared to the findings of our study. On the one hand, these results allowed us to conclude that the polyphenolic compounds extracted using the Soxhlet method produce higher contents compared to those obtained by decoction. On the other hand, based on the studied solvent, we noted that the hydro-ethanolic extracts possess high contents of polyphenolic compounds compared to the aqueous extracts.

Consequently, the obtained results confirm that the contents of polyphenolic compounds vary significantly depending on the used solvent and extraction method.

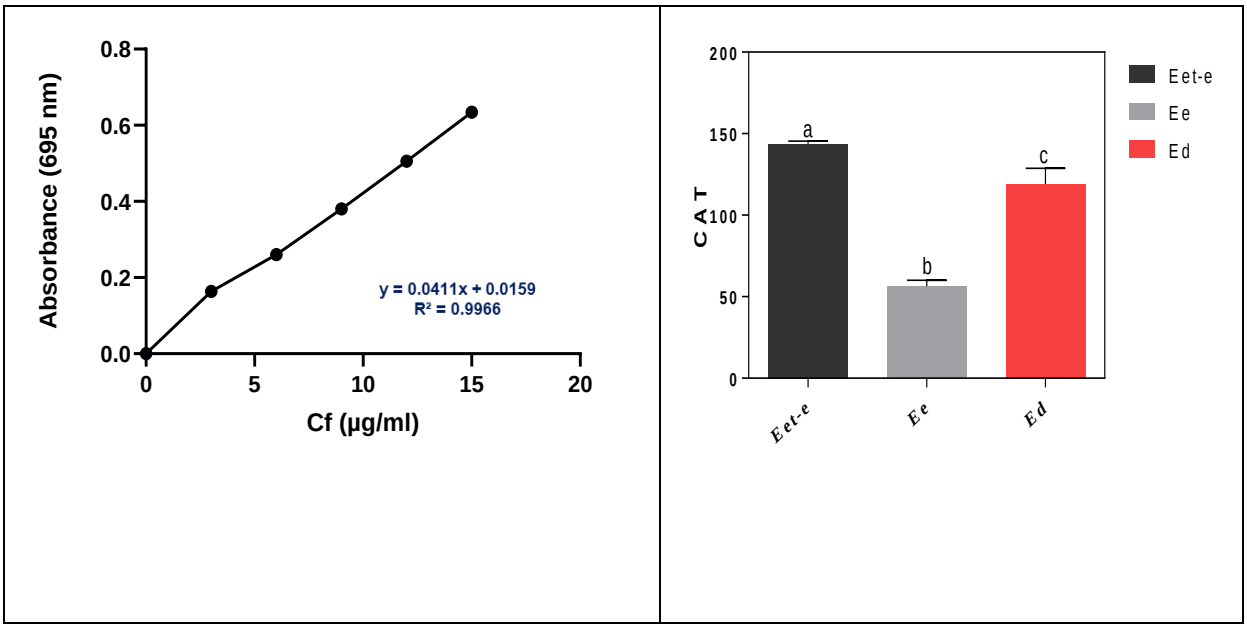


Figure 3: Evaluation of the reduction capacity of ferric iron by *C.×Clementina* leaves extracts

4. Antioxidant activity of C. clementina leaf extracts

4.1. Total antioxidant capacity

Determination of the total antioxidant capacity of the three extracts was performed using the phosphomolybdate method. This method is based on the extracts' capacity to reduce the molybdenum Mo (VI). present in the form of molybdate ions MoO_4^{2-}, into molybdenum Mo (V)MoO_4^{2+}, and the subsequent formation of a green phosphate / Mo (V) complex at acidic PH. The total antioxidant capacity of the analysed extracts were expressed as the number of equivalent of ascorbic acid in a gram of dry extract (mg EAA / 1 g E) (Figure. 3b). Using the calibration curve (y = 0.0411x + 0.0159. R^2 = 0.9966) (Figure 3a).

The antioxidant activity (TAC) results show a significant difference ($p < 0.05$) in the TAC of all extracts of *C. clementina* leaves. From these results, the hydroethanolic, aqueous and decocted extracts were found to be (143.722 ± 1.754) mgAA / gE, (119.165 ± 9.516 mgEAA / gE) and (56.380 ± 3.746) mg EAA / gE respectively (Figure **3**). It was found that the hydroethanolic extract of *C. clementina* leaves has a significant total antioxidant capacity compared to the decoction and aqueous extracts. However, for the same solvent used (water), the decoction extraction method reveals a significantly higher reduction activity than that of Soxhlet. It can be concluded that the type of solvent used and extraction technique that was adopted can influence the antioxidant capacity of an extract.

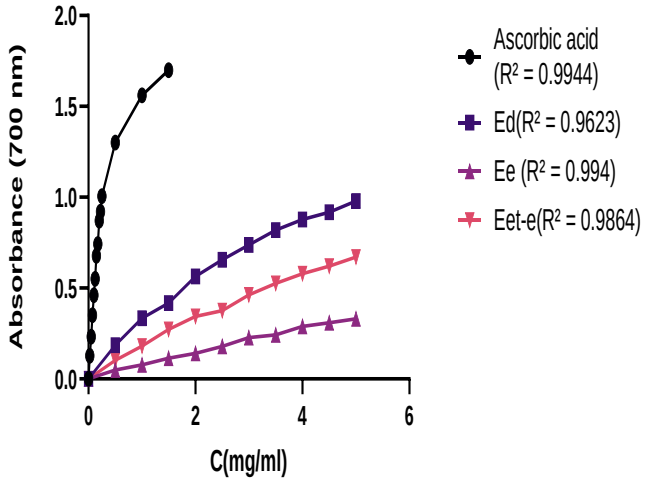


Figure 4a: Reducing power of ferric iron by *C.× clementina* leaves extracts

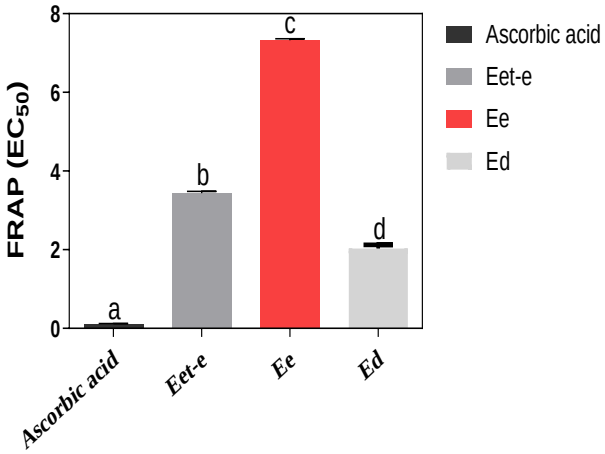


Figure 4b: values of the effective EC50 concentrations (mg/mL) of *C.× clementina* leaves

Figure 4: Evaluation of the reduction capacity of ferric iron by *C. × clementina* leaves extracts

4.2. Ferric Reducing power assay

The antioxidant activity assay by reduction of the iron ferric to iron ferrous is a widely used method in the evaluation of the most active extracts.

The measured results are shown in Figure 4. The powerful antioxidant used as standard is the ascorbic acid.

The results presented in **Figure4a.** reflect a remarkable increase in the reducing power of all *C. clementina* leaf extracts. with respect to the increase in concentration. The results were interpreted in relation to ascorbic acid for a concentration of 1 mg / ml. Indeed, the decocted extract (Ed) exhibited a greater iron reducing activity, with a value of optical density equal to 0.334% for a concentration of 1 mg / ml.As for the soxhlet extraction method, the hydroethanolic extract (Eet-e) reflects a significant iron reduction capacity of around 0.181%. compared to that of the aqueous extract which is equal to 0.078% for the same concentration of 1 mg / ml. By comparing the obtained results to the standard antioxidant (ascorbic acid, 1.56%) for the same concentration of 1 mg / ml, we find that the decocted extract (Ed) has a strong reducing capacity (turning ferric iron into ferrous iron) in comparison with the remaining extracts. And since the antioxidant capacity of the set of extracts is expressed using the determination of the effective concentration EC_{50}. Figure (4b) combines the obtained results.

Indeed, the decocted Ed. hydro-ethanolic E (et-e), and aqueous Ee extracts of *C. clementina* leaves trigger a reduction of the Fe^{3+} found in the potassium ferricyanide complex into ferrous

iron (Fe^{2+}). Their effective concentrations (EC_{50}) reducing 50% of Fe^{3+} ions are respectively in the range of (2.03 ± 0.094 mg / ml), (3.44 ± 0.047 mg / ml) and 7.3 ± 0.04 mg / ml.

By comparing the EC50s of the different tested extracts of our plant to that of the standard ascorbic acid (0.12± 0.001 mg / ml).

We find that there was a statistically significant difference ($p < 0.05$) observed between the ascorbic acid standard and all extracts of *C. clementina* leaves, and that the decocted extract (Ed) is generally classified as the most active compared to the other extracts.

The results obtained in this study proves that there is no correlation between the content of phenolic compounds. Flavonoids, tannins, and antioxidant power. Indeed, the decocted extract which proved to be less rich in these phenolic compounds has an antioxidant power greater than the other extracts obtained using the Soxhlet method. Water's polarity and the operating conditions of decoction help extract and preserve phenolic compounds with stronger antioxidant capacity.

IV. Conclusion

In the present research, we performed a phytochemical study of *C. clementina* leaves extracts, determined the total phenolic compounds and the antioxidant activities by FRAP and TAC assays. Also determined the chemical composition of their essential oils.

The results obtained in this study prove that the use of different solvents and methods in extraction had a big influence significantly ($p <0.05$) on the total polyphenolic contents, total antioxidant capacity, and antioxidant activity FRAP of obtained extracts.

We may conclude that the petitgrain *C. clementina* is a highly rich source of bioactive compounds. Including essential oil, polyphenols, flavonoids and condensed tannins. Moreover, the antioxidant properties of their extracts are highly sought after in the therapeutic and dietary fields. Thus, EO from the petitgrain clementine leaves extracts could become a natural alternative to certain synthetic additives. More researches are planned for the best valorisation of this vegetable biomass. Notably, the studies on the antimicrobial and the antidiabetic activity.

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