A comparative study of phytochemical investigation and antioxidative activities of six citrus peel species

A comparative study of phytochemical investigation and anti-oxidative activities of six Citrus peels species

Running Title: Citrus peel constituents and activities

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ABSTRACT

Over the past few decades, much effort has been devoted to the study of known food products for medicinal applications. Among these, Citrus fruits play a key role in providing a wide range of health beneficial effects but it generates a huge amount of waste products. In an attempt to recover those wastes, peel of six Citrus species (*C. aurantium*, *C. limetta,C. limon, C. reticulata*, *C. Sinensis* osbeck*, and C. Sinensis* thomson) was evaluated for yield, physicochemical properties, phenolic constituents, as well as antioxidant activities. LC-MS/MS analysis showed that the flavonoids neoreiocitrin, luteolin-7-*O*-neohesperidoside, scoparin and neohesperidin were chemical markers for *C. limetta*, whereas apigenin-6,8-di-*C*-glycoside was only detected in *C. Sinensis Osbeck*. PCA analysis revealed significant correlations between antioxidant activities and phenolic contents, highlighting a large interspecific variability.

These results suggest that *Citrus* peel by-products may be valuably recycled by industries due to their high yield and transformed into value-added products, with potential interest for the development of functional foods, cosmetics or preventive therapies for some diseases.

Keywords: Antioxidant activity; Citrus peel; Extraction; Flavones; LC-MS/MS identification.

Abbreviations: **BHT**, Butylated hydroxytoluene; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl; **NBT**, p-Nitro-tetrazolium blue; **CA**, *Citrus aurantium*; **CSO**; *Citrus sinensis osbeck*, **CLi,** *Citrus limetta*; **CL**, *Citrus limon*; **CST**, *Citrus sinensis thomson*; **RE**, *Citrus reticulata*; **TPC,** Total polyphenol content; **TFC**, Total flavonoid content; **TCT**, Total condensed tannins; **TAC,** Total antioxidant capacity; **PCA**, Principal component analysis.

1. INTRODUCTION

In the last decades, world production of Citrus fruit and fruit juice industry has increased continuously. Citrus tree is thus considered one of the world's major fruit crops[1]. It is distributed in the Euro-Mediterranean regions and Asia[2]. According to European Fruit Juice Association, fruit juice and nectar consumption was 38.5 billion litres in 2015 globally[3]. However, in that context, huge amounts of waste materials of Citrus are produced by the industries every year (40 megaT/annum) as peels, pomace and seeds[4]. These generated residues are either used as animal feed or directly discarded, without proper processing, thus causing environmental problems[5]. Therefore, reducing the amount of waste and promoting Citrus industry through waste processing remains a challenge[6]. Thus, many value-added compounds commercially important can be efficiently extracted from citrus peels, or can be reused in several ways.[4] Accordingly, it has been reported that Citrus peels in current medicine exhibit important pharmacological and nutraceutical properties[7,8]. These bioactivities are related to significant amounts of biologically active polyphenols, especially phenolic acids and flavonoids. Recently, special attention of many industries has been given to recover and recycle the added-value compounds from Citrus peels for exploitation during the production process. This attention is related essentially to the amount of citrus peel biomass and its availability throughout the year. The peels of Citrus fruits are a rich source of flavonoid glycosides, coumarins, sterols, glycosides and volatile oils[9]. Many polymethoxylated flavones are endowed with several important bioactivities, which are very rare in other plants[10]. The most abundant citrus flavonoids, generally known as the flavanones, include hesperidin, naringin, narirutin, and neohesperidin. These compounds have been found to provide health benefits such as antioxidative, anticancer, anti-inflammatory, and cardiovascular protective activities[11]. In this context, it was demonstrated that the consumption of naringin and hesperidin reduced cholesterol levels in hamsters by 32 to 40 %

[12]. In addition, it was reported that essential oil of orange peels are endowed with interesting antimicrobial activity [13].

Hence, the aim of the present investigation was to select the best fruit-peels from six Citrus species growing in Tunisia useful as a natural source of nutraceuticals. This selection was based on the identification of secondary metabolites present in the prepared extracts using HPLC-MS/MS analyze as well as the evaluation of their antioxidant activities by various tests.

2. MATERIALS AND METHODS

2.1 Plant material collection

Six Citrus species peels, *i.e. C. aurantium (CA)*, *C. limetta (CLi)*, *C. sinensis osbeck (CSO)*, *Citrus limon (CL)*, *Citrus sinensis thomson (CST)* and *Citrus reticulata (RE)* from the Rutaceae family, were harvested in February and March 2016 from a garden located in Sfax (Tunisia). Each plant was botanically identified by Z. Ennoumi, assistant professor at the Faculty of Sciences of Sfax. All the fruits were of eating quality, without harm or blemishes. Voucher specimens [C-19], [C-21], [C-22], [C-38], [C-39], [C-36] (in the same order as noted above) were submitted at the Laboratory of Biology & Physiology of Vegetation in Arid Environment Herbarium, Faculty of Sciences of Sfax. The fruits were peeled off carefully using a sharp razor blade to avoid any ravage of oil glands. All peel samples were rapidly washed with water, freeze-dried and then ground using a blender (Mettler AE 200).

2.2 Extraction procedure from citrus peel waste

Extraction of each fruit peels species was carried out by magnetic blend stirring of 30 g of dry powder in 300 mL of ethanol/water (4:1 v/v) with maceration for 5 h at 4°C. Ethanol/water mixture is considered as a green solvent affording no toxic extracts. Then, the aqueous

ethanol extract was filtered with N°1 Whatmann Millipore filter paper (0.45 µm, HAWP04700, Bedford, MA, USA), and concentrated using a rotary evaporator at 40±1°C under vacuum. Then, the residue was resuspended in ethanol and conserved at −27°C for analyses. Figure 1 shows a schematic diagram for the design of the experiment.

2.3 Total phenolic compounds

2.3.1. Total polyphenol content (TPC)

Polyphenols were measured at 760 nm according to **Dewantoet al.**[14] method and expressed as mg gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW).

2.3.2 Total flavonoid content (TFC)

Extracted flavonoids were determined according to **Dewantoet al.** [14] procedure. Absorbance was read at 510 nm and TFC were expressed as mg catechin equivalents per gram of dry weight (mg CE g^{-1} DW).

2.3.3 Total condensed tannins (TCT)

These compounds were quantified according the protocol of **Sun et al.**[15]. Absorbance was read at 500 nm and TCT were expressed as mg catechin equivalents per gram of dry weight (mg CE g^{-1} DW).

2.4 Citrus peel physicochemical properties

2.4.1 Water content

Samples of fresh peels were weighed before and afteroven a 3 h incubation at 105°C.

2.4.2 Ash contents

The ground dried citrus peel samples were reduced to ash by heating for 5 hours at 525°C and weighed[16].

2.4.3 Total sugar content

The method allows determination of reducing sugars using phenol and concentrated sulfuric acid[17]. The absorbance was determined at 490 nm.

2.5 Identification and qualification of flavones in citrus varieties by HPLC-PDA-ESI-MS

HPLC-PDA-ESI-MS analysis was performed employing a Surveyor LC system equipped with a diode array detector (Thermo Fisher, San Jose, CA, USA) and a Kinetex EVO C18 column (Phenomenex). The flow rate was 1 mL/min, UV detection was carried out at 254, 280 and 360 nm. The gradient program was as follows: solvent A, 0.01% FA; solvent B, CH_3CN; gradient, 5 min 10 % B; from 10 to 100 % B in 42 min; stay at 100 % B during 5 min; from 100 to 10 % B in 3 min; 5 min 10 % B. The injection volume was 4 μL. After flow splitting the LC system was coupled to an LXQ linear ion trap (Thermo Fisher). The experimental conditions for operation of the instrument in the (+)ESI mode were optimized. The optimal conditions were as follows: sheath gas flow, 50 arbitrary units; auxiliary gas flow, 8 arbitrary units; spray voltage, +4.0 kV; ion transfer tube temperature, 375°C; and capillary voltage, 35 V. Mass spectral data was recorded using full scanning in the mass range m/z 100–1800. For MSn experiments an isolation width of 2 Da was used and a normalized collision energy of 35 % was applied. All data were recorded and processed using Xcalibur software, version 2.0 (Thermo Fisher). Eluted metabolites were identified according to the comparison of their λ_{max}, retention times, and MS data with those reported in previous studies as indicated in the results and discussions section.

2.6 Antioxidant activities determination

Total antioxidant capacity, DPPH• radical-scavenging, β-carotene bleaching inhibition, superoxide anion radical scavenging activity and FRAP were successively measured to evaluate the antioxidant activity of *Citrus* peel extract.

2.6.1 Total antioxidant capacity

Total antioxidant capacity (TAC) of peel extracts was evaluated through the assay of a green phosphate/Mo^{5+} complex according to the method described by **Prieto et al.**[18] An aliquot (0.1 mL) of diluted samples was combined with 1 mL of reagent solution. Ethanol was used instead of sample for the blank. After being incubated in a boiling water bath for 90 min in the dark, the samples were cooled to room temperature and the absorbance was measured at 695 nm with a 160 UV-Visible spectrophotometer (Shimadzu). Antioxidant capacity was determined referring to the regression equation of a calibration curve (y = 0.0038x) established with gallic acid and expressed as mg gallic acid equivalents (GAE) per gram of dry weight (DW).

2.6.2 DPPH-scavenging activity

DPPH-scavenging activity was measured according to the method of **Chen et al.**[19] An aliquot of the reaction solution was mixed with 100 µL of 100 mM DPPH in 90 % methanol and 100 µL of peel extract diluted in ethanol at different concentrations. Mixtures were vigorously shaken and left for 30 min in the dark. Absorbance was measured at 517 nm using ethanol as a blank. The following equation was used to calculate quenching of DPPH radicals:

$$PI\ (\%) = 100 \times (A_0 - A_s)/A_0$$

Where A_0 is the absorbance of the control, and A_S is the absorbance of the tested sample. When an antioxidant scavenges free radicals by hydrogen donation, the DPPH assay solution becomes lighter in colour [20]. The quality of the antioxidants in the extracts was determined by the IC_{50} values, denoting the concentration of the sample required to scavenge 50% of the DPPH free radicals. Butyl hydroxytoluene (BHT) was used as a positive standard and all samples were analyzed in triplicate.

2.6.3 β-Carotene bleaching test

A slightly-modified method of that described by **Kolevaet al.**[21] was employed to estimate inhibition of β-carotene bleaching. β-carotene (2 mg) was dissolved in 20 mL of chloroform, and to 4 mL of this solution linoleic acid (40 mg) and Tween 40 (400 mg) were added. Chloroform was evaporated under vacuum at 40°C and 100 mL of oxygenated water was added, then the fresh emulsion was vigorously shaken. An aliquot (150 µL) of β-carotene/linoleic acid emulsion was distributed in 96-well microtiter plates (NUNC micro-plate, Fisher Bioblock) and methanol solutions of the test samples or authentic standards (10 µL) were added. Three replicates were prepared for each concentration. The absorbance of all wells was measured at 470 nm using a microtiter reader (Multiskan EAR 400, Labsystems), both immediately (t = 0 min) and after 120 min of incubation at 50°C. The antioxidant activity of the BHT standard and peel extracts was calculated as percentage of β-carotene bleaching inhibition as follows:

$$\% \text{ inhibition} = (S - C_{120} / C_0 - C_{120}) \times 100,$$

Where C_0 and C_{120} are the absorbances of the control at 0 and 120 min, respectively, and S is the sample absorbance at 120 min. Results were expressed as IC_{50} values (mg/mL).

2.6.4 Superoxide anion radical scavenging activity

Superoxide **($O_2^{\cdot-}$)** scavenging capacity was assessed according to **Pick.**[22] The reaction mixture contained 0.2 mL of peel extracts at different concentrations, 0.2 mL of 60 mM PMS, 0.2 mL of 677 mM NADH and 0.2 mL of 144 mM NBT, all in phosphate buffer (0.1 M, pH 7.4). After 5 min of incubation at room temperature, the absorbance was read at 560 nm against blank. The inhibition percentage of superoxide anion generation was calculated using the previous formula. As for the antiradical activity, the antioxidant activity in peel extracts was expressed as IC_{50} in mg. mL^{-1}. Samples were analyzed in triplicate.

2.6.5 FRAP assay

The capacity of plant extracts to transform Fe^{3+} to Fe^{2+} was determined according to the method of **Oyaizu**[23]. Samples at different concentrations were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1 % w/v). The tubes were incubated at 50°C for 20 min. Then, 2.5 mL of 10 % TCA were added in each tube and the mixture was centrifuged for 10 min at 1.000 *g*. An aliquot of the supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.5 mL of ferric chloride (0.1 % w/v), and the absorbance was read at 700 nm.

2.8. Statistical analyses

The XLSTAT-Pro 7.5.3 software was used to compare mean values. We used Duncan's multiple range tests with least significant difference (l.s.d.) ($P< 0.05$) for mean separation procedures. To compare the different extracts, a one-way analysis of variance (ANOVA) was used. To visualize the possible relationship between antioxidant assays and phenolic contents we performed a principle component analysis (PCA).

3. RESULTS AND DISCUSSION

3.1. Extraction yields of the studied Citrus peels

Extraction constitutes a detrimental experimentation for the recovery and segregation of natural compounds from plant materials prior to their biological activities investigation. The selection of the extraction solvents is of great importance for the type and the amount of bioactive phytochemicals in the extract [24]. After maceration of dry Citrus peels in ethanol (solvent with high polarity), the extraction yields were calculated as percentages of the initial quantity of sample of plant material. The obtained values are shown in **Table 1**. The highest extraction yield (12.28 %) was obtained for *Citrus aurantium* followed by those related to

211 *Citrus limon* (9.18 %), *Citrus reticulata* (6.13 %), *Citrus sinensis osbeck* (5.08 %), *Citrus*
212 *sinensis thomson* (4.79 %) and *Citrus limetta* (4.58 %).
213 The high extraction yields determined for the six varieties of citrus peels can be explained by
214 their richness in polar compounds such as carbohydrates, phenolic acids and flavonoids[25]. The
215 difference between the extraction yields can be due to the amount variation in polar
216 compounds present in the investigated Citrus peels. In this way, it was established that the
217 extraction yield of polyphenols from plants depends in various extraction parameters such as
218 polarity of solvent, extraction temperature and time as well as their affinity with the extraction
219 solvent [26].
220
221 **3.2. Phenolic assessment in Citrus peels extracts**
222 Three different classes of phenolic compounds (phenolics, flavonoids and condensed tannins)
223 were quantified against standards and presented in table 2. The latter showed the phenolics
224 richness of *C. limon* (6.65 mg/g DW), followed by *C. aurantium* and *C. reticulata*, compared
225 to the other *Citrus* species **(Table 2)**. These data corroborate with those of Belitz and Grosch
226 [27] who reported a high content of polyphenols in *C. limon*. The lowest content was registered
227 for *C. Sinensis thomson*. These variations might be due to genetic differences between
228 cultivars, to their origin, or growth conditions. In fact, high phenolic content of citrus peels
229 confirmed their nutrition value associated to bioactive compounds richness. Also, their
230 richness in phytochemical compounds makes Citrus peel a promisful source of phenolic
231 compounds for further utilization, including health care. It is suggested that polyphenolic
232 compounds part in various metabolism and have several pharmacological properties such as
233 antioxidant andanticancer activities[28]**.**
234 In addition, flavonoids content is reported in **table 2**. Our results showed that the *C.*
235 *limon* extract have the highest flavonoid content (5.11± 0.02 mg QE /g DW), followed by *C.*
236 *aurantium* and *C. reticulata*. Flavanones and flavones are two types of flavonoids that occur

in Citrus peel[29]. The main flavonoids exclusively found in fruit peel of Citrus are hesperidine, tangeretin, naringin and nobiletin[28]. The present findings reveal that the Citrus peel extracts may be considered as an attractive source of phytochemical compounds for further use. Accordingly, epidemiological studies indicated that Citrus flavonoids reduce the risk of coronary heart disease[30]. Also, flavonoids are attracting more attention as anticarcinogenic and anti-inflammatory agents[25].

The results showed that the fruit peel extract of *C. limon* contained the highest content of condensed tannins (1.52 ± 0.07 mg CE /g DW) **(Table 2)**. This high level of tannins in the extracts edible of *C. aurantium*, *C. Sinensis* osbeck, *C. sinensis* thomson, *C. limetta* and *C. reticulata* could be responsible for the astringency of their peels. Accordingly, it is reported that tannins have astringent properties [31], binding to salivary proteins, thus producing a taste that humans recognize as astringency [32].

3.3. Physicochemical properties of Citrus

It is well known that the low water content does not favour the proliferation of microbes and allows a good conservation of the plant material. Our results indicate that the peel water content in the different *Citrus* species ranged from 17.2 % to 42.9 % (**Table 3**). The highest water content was registered in *C. reticulata*, whereas the lowest activity was found in *C. Limon* extract. Besides, the ash content represents the total amount of minerals contained in the plant material. This parameter is essential to determine if the product is intended for animal or human nutrition. Our results given in **Table 3** evidenced the richness of the peel extracts of Citrus studied, in mineral elements. The ash content generally varies according to species, geographical locations and seasons [33]. Moreover, the sugar content, ranging from 0.161 to 0.332 mg/g DW (**Table 3**), varies according to the species of *Citrus*.

3.4. HPLC-PDA-ESI-MS analysis of Citrus peel extracts

263 Complementary analyses by LC–MS/MS were performed to confirm unequivocally the
264 identity of flavonoids extracted from Citrus peels (Figure 2). UV and MS spectra analysis, in
265 positive ion mode, led to the identification of thirteen compounds (Table 4): three
266 polymethoxyflavones (peaks 11, 12 and 13), three flavone *O*-diglycosides (peaks 2, 6 and 7),
267 five flavanone*O*-diglycosides (peaks 3, 5, 8, 9 and 10), one flavone di-*C*-glycoside (peak 1)
268 and one coumarin (peak 4). The less polar compounds (11, 12 and 13) were identified as
269 polymethoxylated flavones by UV and MS spectra **(Table 4).** Their protonated molecules ([M
270 + H]$^+$ at *m/z* 343, 373 and 403 for 11, 12 and 13, respectively) dissociated predominantly via
271 the loss of methyl radicals, producing the [M + H - Me]$^+$ ion (*m/z* 328, 358 and 388 for 11, 12
272 and 13, respectively) as a basic skeleton and other main fragments corresponding to the loss
273 of 30 amu ([M + H – 2Me]$^+$ at *m/z* 312, 343 and 373 for 11, 12 and 13), 61 amu ([M + H –
274 Me - CO - H_2O]$^+$ at *m/z* 282, 312 and 343 for 11, 12 and 13), and 48 amu ([M + H – 2Me -
275 H_2O]$^+$ at m/z 325 and 355 for 12 and 13). MS data indicated the presence of four, five and six
276 methoxyl groups in 11, 12 and 13, respectively, but did not allow their positions to be
277 established. However, according to the literature, compounds 11, 12 and 13 were tentatively
278 assigned to 5,6,7,4'-tetramethoxyflavone, tangeretin and nobiletin, respectively **(Figure 3 A;**
279 **B; C).** These compounds were previously identified in Citrus peels of tangerina Tanaka [34] and
280 of sweet orange, lemon, mandarin, and grape fruits [31].

281 Ion mass spectra obtained for the protonated molecules of the *O*-diglycosyl flavonoids
282 (peaks 2, 3, 5, 6, 7 , 8, 9 and 10, [M + H]$^+$ at *m/z* 597, 595, 611, 609, 579, 581, 723 and 755,
283 respectively) showed product ions with *m/z* 451, 449, 465, 463, 433, 435, 577 and 609 for
284 compounds 2, 3, 5, 6, 7, 8, 9 and 10, and *m/z* 289, 287, 303, 301, 271, 273, 415 and 447 for
285 compounds 2, 3, 5, 6, 7, 8, 9 and 10, due to the cleavage of two glycosidic linkages. The
286 losses of 146 and 308 amu generating the above ions indicated that the disaccharide sequence
287 is as follows: deoxyhexose-hexose-flavonoid. UV spectra of products 2, 6 and 7 provided two

maxima at 340 nm (band I) and 282 nm (band II), consistent with flavones, whereas compounds 3, 5, 8, 9 and 10 showed UV spectra (maxima at 285 nm and shoulder at 330-335 nm) characteristic of flavanones[35]. Compounds 2, 3, 5, 6, 7 and 8 could be identified as neoreiocitrin, luteolin-7-*O*-neohesperidoside, neohesperidine (**Figure 3 D; E; F**), neodiosmin, rhoifolin and naringin [36], respectively **(Table 1).** These compounds were previously identified in citrus peels of Aurantii fructus [37]. The positive product ion spectra ($[M + H]^+$ at m/z 723 and 755 for compound 9 and 10, respectively) showed $[M + H - CO_2 - H_2O]^+$ (m/z 661 and 691 for 9 and 10), $[M + H - C_4H_6O_3]^+$ (*m/z* 621 and 651 for 9 and 10), and $[M + H - C_6H_8O_4]^+$ (*m/z* 579 and 609 for 9 and 10) ions, which indicates the presence of a 3-hydroxy-3-methylglutaryl substituent[38]. The fragment ions at *m/z* 609 (10) and 579 (9), close to the protonated molecules of compounds 6 and 7, respectively, led to the assumption that 10 and 9 were conjugates of neodiosmin and rhoifolin, namely brutieridin and melitidin[38].

Compound 1 showed a UV spectrum suggesting the structure of a flavone di-*C*-glycoside derivative, and it yielded product ions typical of di-*C*-hexosyl flavones**.** MS data of compound 1 was superimposable to that of apigenin 6,8-di-*C*-glycoside, previously identified in *C. aurantinum* leaves[39]. Compound 4, with the characteristic UV spectrum of coumarins, was tentatively identified as scoparin, with a maximal UV absorbance at 330 nm[39]**.** The loss of an hexose moiety produced a fragment ion at *m/z* 301[28].

Overall, *C. limetta* exhibited the highest diversity of phenolics, with 11 compounds identified, whereas *C. reticulata* possessed only 4 of these phenolics. Moreover, *C. limetta* was the only Citrus species containing neoreiocitrin, luteolin-7-O-neohesperidoside, scoparin, and neohesperidin. Similarly, apigenin-6,8-di-*C*-glycoside appeared as a biochemical marker of *C. sinensis osbeck*. Conversely, *C. limetta* was the only Citrus species lacking melitidin. Finally, all the six Citrus taxa studied here contained 5,6,7,4'-Tetramethoxyflavone,

312 tangeretin, and nobiletin. This result is in agreement with the findings of Huijuan and co-
313 workers[40].

314

315 **3.5. Citrus peel extracts antioxidant activities**

316 **3.5.1. Total antioxidant capacity**

317 Total antioxidant capacity (TAC) of the different Citrus peel extracts is presented in **table 2**.
318 We note a significant variability among the Citrus extracts, ranging from 0.51±0.01 to
319 1.11±0.01 mg GAE/g MS. In fact, the highest antioxidant capacity was observed in *Citrus*
320 *limon*, whereas the lowest activity was found for the *C. reticulata* extract. Similar trends were
321 found in other Citrus tissues by Gorinsteinet al.[41] who confirmed that *Citrus limon* has an
322 important antioxidant activity. Many studies have reported antioxidant effects of Citrus and
323 edible oranges parts from different origins and varieties[42]. Accordingly, the Citrus extracts
324 were found to have a high antioxidant potential[41]. The plant antioxidant property is generally
325 associated to the presence of secondary metabolites including flavonoids, terpenoids,
326 coumarins and saponins[43,44]. In our case, the antioxidant activity measured suggested the
327 presence of natural antioxidants in the *Citrus* ethanolic extracts, including polyphenols.

328

329 **3.5.2. Radical scavenging activity (DPPH)**

330 In general, free radicals are attacked by antioxidants in order to fight various diseases. They
331 powered these radicals, by reactive oxygen species scavenge or by protecting the antioxidant
332 defence mechanisms protection. Therefore, the RSA of the methanol extracts of Citrus peel
333 was assessed by DPPH assay test used for scavenging free radicals. Our results showing the
334 antioxidant activities of ethanolic Citrus peel extracts were evaluated. Among the six Citrus
335 species, *C. limon* exhibited the highest antiradical activities followed by *C. aurantium* and *C.*
336 *reticulate* **(Table 2)**. Conversely, *C. Sinensis* thomson and *C. limetta* showed the lowest

activities. The decrease of DPPH absorbance is associated to the reaction between radical and antioxidant molecule resulting in hydrogen donation leading to radical scavenging[45].

3.5.3. $O_2^{\cdot-}$ scavenging activity

Superoxide is also produced endogenously by flavo-enzymes like xanthine oxidase, or by lipoxygenase and cyclo-oxygenase. In this sense, results from the superoxide-scavenging assay showed that the all extracts quenched superoxide anion to significantly different extents **(Table 2).** Moreover, the *C. limon* extract exhibited the highest antiradical potential (IC_{50} = 0.32 $mg.mL^{-1}$), representing a higher activity than the standard ascorbic acid (IC_{50} = 0.36 $mg.mL^{-1}$), followed by *C. aurantium* and *C. reticulata*. The other extracts showed a moderate activity.

3.5.4. β-Carotene bleaching inhibition

Likewise, β-carotene bleaching test has been used by many researchers to measure the antioxidant activities of the six Citrus peel extracts. In this method, fat-soluble antioxidants are measured more effectively than in aqueous system, thanks to tween 40 capable of keeping the components in emulsion. The β-carotene oxidation was inhibited effectively by the Citrus peel extracts, achieving the highest values for both *C. aurantium* and *C. reticulata* (IC_{50}= 0.028 $mg.mL^{-1}$), followed by *C. limon* **(Table 2)**. The antioxidant activity of the other three species was moderated in comparison with BHT bioassay. This moderation could be explained by the mechanism of β-carotene / linoleic acid bleaching. In fact, β-carotene is discoloured by free radicals generation from linoleic acid. The antioxidant presence can block the extent of β-carotene destruction following the neutralisation of the linoleate free radicals formed in the system[46].

3.5.6. Iron reducing power activity (FRAP)

363 Generally, compound reducing ability is related to the presence of reductants, which have an
364 antioxidant activity by donating a hydrogen atom and breaking the free radical chain[47].
365 Interestingly, the iron-reducing power of the six Citrus peel extracts is significantly different
366 **(Figure 4).** The *C. limon* extract exhibited the highest reducing capacity with a OD value of
367 0.315, followed by *C. aurantium* and *C. reticulata* (0.300 and 0.292 respectively). However,
368 the other three extracts have very limited reducing capacity, compared to the ascorbic acid
369 standard (0.399). Similar trends were previously recorded in other Citrus extracts, indicating
370 that their antioxidant compounds have reducing ability[48].
371
372 **3.6. Principal component analysis (PCA)**
373 In order to set out possible relationships between every studied parameter, data were
374 subjected to PCA. This chemometric tool reduces the dimensionality of the multivariate data
375 to two or three principal components (PCs), which can be visualized graphically, with more
376 information. For this reason, the data matrix was decomposed into matrices of scores
377 (different peels) and loadings (DPPH, TCA, β-carotene bleaching, TFC, TPC, and TCT),
378 providing information on samples and variables, respectively.
379 In the exploratory study, figure 5 revealed patterns and differences among citrus peel extracts
380 for the first time. As can be seen, the loading directions for samples of *C. limon*, *C. reticulata*
381 and *C. aurantium* were different from those of other Citrus (CLi, CSO and CST). The figure
382 also confirms important correlations between CAT, FRAP, phenolic compounds and the first
383 three types of Citrus mentioned above. Several authors support that antioxidant capacities
384 were strongly related to the phenolic profiles. In the same context, other previous studies
385 confirmed that phenolics, as an important source of substantial secondary metabolites,
386 especially within Citrus species undergoing in their native biotopes, hard environmental
387 factors[44].
388

4. CONCLUSION

This study reports simultaneously the phenolic compositions and antioxidant activities as well as the physicochemical proprieties of six varieties of *Citrus*. These species markedly differ in peel phenolic composition, antioxidant activities and physicochemical properties. Overall, our results highlight the strong potential of *Citrus limon* as a source of antioxidant activity. In the same context, the ethanol extract bioassay-guided fractionation of *Citrus limon* is under experimentation to elucidate identification of bioactive compounds, making this species an important source of ingredients for cosmetic, food and pharmaceutical applications.

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Disclosure statement

The authors declare that there is no conflict of interests.

Data Availability Statement

Data supporting the results of this study are available from the authors on reasonable request.

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Tables

Table 1. Extraction yields of the six citrus peels

	Ethanol		
	Initial mass (g)	**Mass (g)**	**Yield (%)**
(CA)	300	36.84	12.28
(CSO)	300	15.26	5.08
(CLi)	300	13.75	4.58
(CL)	300	27.55	9.18
(CST)	300	14.38	4.79
(RE)	300	18.40	6.13

Citrus aurantium ***(CA),*** *Citrus sinensis osbeck* ***(CSO),*** *Citrus limetta* ***(CLi),*** *Citrus limon* ***(CL),*** *Citrus sinensis thomson* ***(CST),*** *Citrus reticulata* ***(RE).***

Table 2. Antioxidant activities, Phenolic contents and Antidiabetic-activities of citrus peels extracts (50 % of ethanol).

	Antioxidant activities				Phenolic contents		
	DPPH[a]	**TAC[b]**	**$O_2^{\cdot-}$[c]**	**β-carotene[d]**	**TP[e]**	**TF[f]**	**CT[g]**
CA	0.340±0.003 [c]	0.340±0.005 [b]	0.970±0.004 [b]	0.228±0.002 [a]	4.80 ± 0.07 [b]	3.56 ± 0.01 [b]	1.27 ±0.07 [b]
CLi	0.460±0.005[g]	0.460±0.009 [g]	0.750±0.003 [g]	0.240±0.004 [c]	2.08 ± 0.05 [f]	1.08 ± 0.02 [f]	0.09 ±0.01 [f]
CST	0.540±0.006 [f]	0.410±0.008 [f]	0.810±0.001 [f]	0.243±0.003 [d]	2.76 ± 0.05 [e]	1.41 ± 0.01 [e]	1.02 ±0.07 [e]
CL	0.260±0.001 [b]	0.320±0.003 [a]	1.110±0.009 [a]	0.230±0.002 [b]	6.65 ± 0.04 [a]	5.11± 0.02 [a]	1.52 ±0.07 [a]
CSO	0.490±0.005 [e]	0.480±0.009 [e]	0.840±0.002 [e]	0.245±0.004 [e]	3.67 ± 0.07 [d]	2.19 ± 0.03 [d]	1.17 ±0.07 [d]
RE	0.430±0.004 [d]	0.370±0.005 [d]	0.510±0.004 [d]	0.228±0.002 [a]	4.50 ± 0.08 [c]	3.15 ± 0.03 [c]	0.90 ± 0.01 [c]
BHT	0.230±0.001 [a]	0.360±0.005 [c]	-	0.230±0.002 [b]	-	-	-
Ascorbate	-	-	0.360±0.005 [c]	-	-	-	-

Citrus aurantium **(CA),** *Citrus limetta* **(CLi),** *Citrus sinensis thomson* **(CST),** *Citrus limon (CL)*, *Citrus sinensis osbeck* **(CSO),** C*itrus reticulata* **(RE).**
[a]DPPH scavenging activity (mg mL^{-1}) , **[b]Total antioxidant capacity** (mg AAE g^{-1}DW) , **[c] Superoxide anion radical-scavenging** (mg mL^{-1}), **[d]ß-carotene bleaching test** (µg mL^{-1}), **[e]Total polyphenol content** (mg GAE g^{-1}DW) , **[f] Total flavonoid content** (mg EC g^{-1}DW), **[g]Condensed tannin content** (mg EC g^{-1}DW).
Values represent the means of three replicates ± SE of 6 samples.

Table 3. Physicochemical properties of citrus peels extracts (50% ethanol).

	Physicochemical properties		
	WC[a]	**AC[b]**	**TSC[c]**
CA	20.0 [c]	4.58 [c]	0.180 ± 0.003 [d]
CLi	19.8 [d]	2.50 [e]	0.160 ± 0.003 [e]
CST	23.9 [b]	7.52 [b]	0.200 ± 0.005 [c]
CL	17.2 [f]	2.62 [d]	0.330 ± 0.008 [a]
CSO	19.0 [e]	2.00 [f]	0.180 ± 0.001 [d]
RE	42.9 [a]	2.67 [a]	0.240 ± 0.003 [b]

*Citrus aurantium***(CA),** *Citrus limetta***(CLi),** *Citrus sinensis Thomson***(CST),** *Citrus limon* ***(CL)*****,** *Citrus sinensisOsbeck***(CSO),** C*itrus reticulata***(RE).**

[a] :**Water content** (%), **[b]** : **Ash content** (%), **[c]**: **Total sugar content** (mg mL^{-1})

Values represent the means of three replicates ± SE of 6 samples.

Table 4. Retention times, UV and ESI-MS data of compounds detected in Citrus peel extracts

Peaks	Compounds	RT (min)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	Other ions (m/z)	UV λ_{max} (nm)	CST	CL	CLi	CA	RE	CSO
1	**Apigenin-6,8-di-C-glucoside**	14.26	595	-	577, 559, 499, 475, 457, 439, 355, 325, 295	346.23	-	-	-	-	-	+
2	**Neoreiocitrin**	16.20	597	-	579, 451, 435, 418, 355, 289	340.29	-	+	-	-	-	-
3	**luteolin-7-*O*-neohesperidoside**	16.80	595	-	499, 287	331.28	-	+	-	-	-	-
4	**Scoparin**	18,09	463	-	391, 301, 283	330.24	-	+	-	-	-	-
5	**Neohesperidin**	18.15	611	633	593, 539, 465, 445, 303	330.28	-	+	-	-	-	-
6	**Neodiosmin**	18.34	609	633	463, 301	345. 29	-	+	-	+	-	+
7	**Rhoifolin**	19.91	579	-	433, 271, 243, 229	349. 28	-	+	-	-	-	+
8	**Naringin**	21.14	581	-	435, 283, 273	333. 28	-	+	-	-	-	+
9	**Melitidin**	22.8	723	-	722, 661, 621, 577, 579, 415	330.28	+	-	-	+	+	+
10	**Brutieridin**	22.87	755	-	713, 691, 651, 609,447 389	331.28	+	+	-	-	+	-
11	**5,6,7,4'-Tetramethoxyflavone**	27.67	343	365	328, 312, 282	340. 28	+	+	+	+	+	+
12	**Tangeretin**	28.91	373	-	358, 343, 328, 325, 312	340.28	+	+	+	+	+	+
13	**Nobiletin**	29.63	403	-	388, 373, 355, 343	349. 29	+	+	+	+	+	+

Citrus sinensis thomson **(CST),** *Citrus limon* ***(CL)*****,** *Citrus limetta* **(CLi),** *Citrus aurantium* **(CA),** *Citrus reticulata* **(RE),** *Citrus sinensis osbeck* **(CSO).**

Figures captions

Fig. 1. Schematic overview of experimental procedures.

Fig. 2. HPLC-PDA-ESI-MS of *Citrus* peel extracts.

Fig. 3. Mass spectra and chemical structures of the polymethoxyflavones [(A) 5,6,7,4'-Tetramethoxyflavone (11); (B) Tangeretin (12) and (C) Nobiletin (13)] and the glycoside flavonones [(D) Neoreiocitrin (2); (E) Neohesperidine (5) and (F) Luteolin-7-*O*-neohesperidoside (3), (Nh(Neohesperidose), Glu(glucose))].

Fig. 4. Iron reducing power (FRAP) of Citrus peel extracts.

Fig. 5. Principal components analysis (PCA) of six Citrus peel extracts (*C. aurantium (CA)*, *C. limetta (CLi)*, *C. Sinensis obseck (CSO)*, *Citrus lemon (CL)*, *Citrus sinenesis thomson (CST)* and *Citrus reticulata (RE))* under radical scavenging activities.

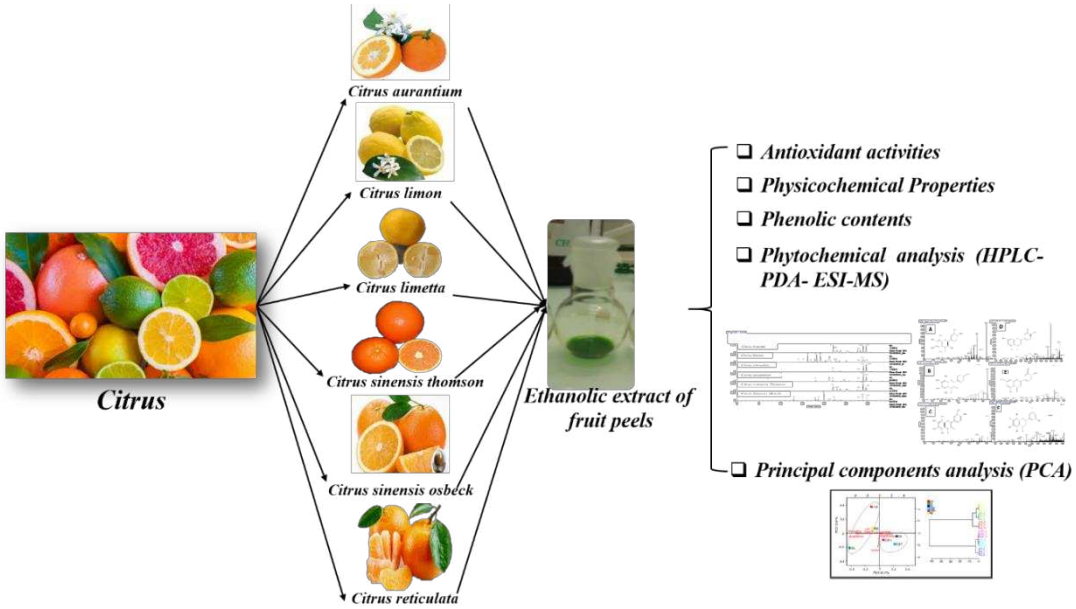
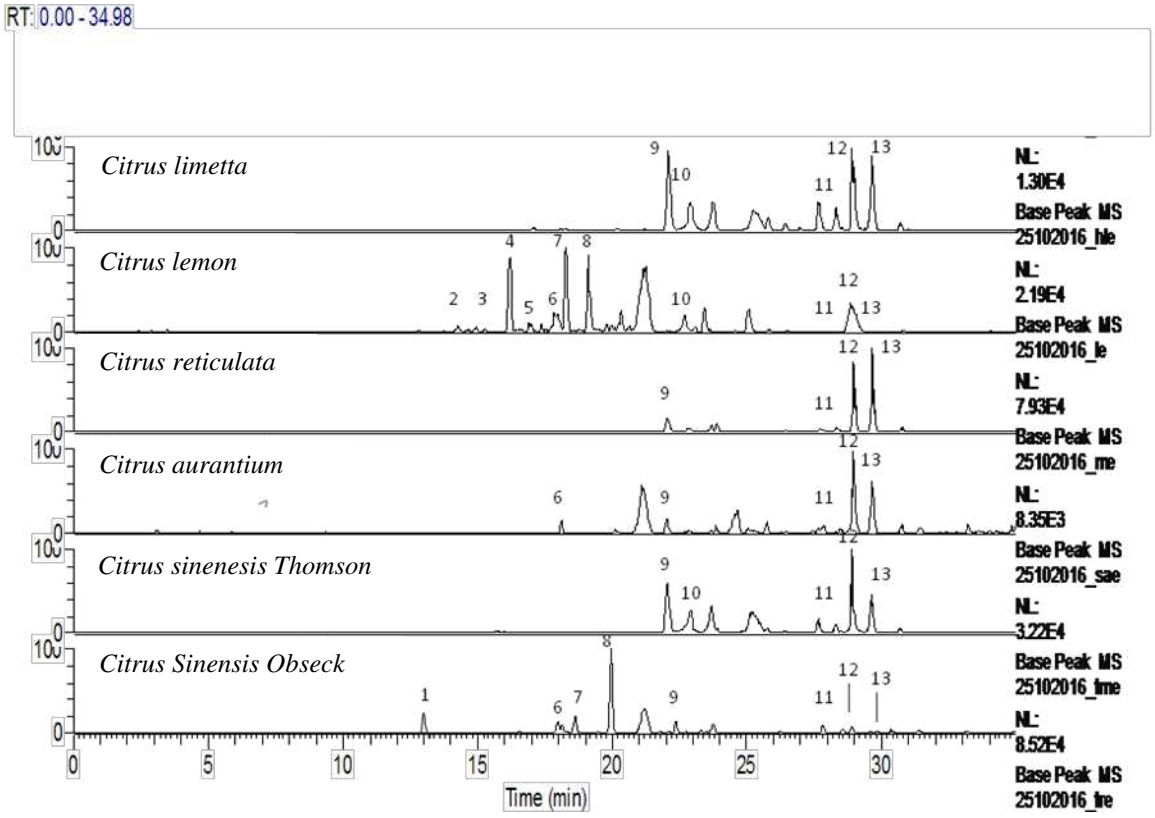

Citrus aurantium
Citrus limon
Citrus limetta
Citrus sinensis thomson
Citrus sinensis osbeck
Citrus reticulata
Citrus
Ethanolic extract of fruit peels
Antioxidant activities
Physicochemical Properties
Phenolic contents
Phytochemical analysis (HPLC-PDA- ESI-MS)
Principal components analysis (PCA)

Figure 1.


RT: 0.00 - 34.98
Citrus limetta
Citrus lemon
Citrus reticulata
Citrus aurantium
Citrus sinenesis Thomson
Citrus Sinensis Obseck
NL: 1.30E4 Base Peak MS 25102016_hle
NL: 2.19E4 Base Peak MS 25102016_le
NL: 7.93E4 Base Peak MS 25102016_re
NL: 8.35E3 Base Peak MS 25102016_me
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NL: 8.52E4 Base Peak MS 25102016_fre
Time (min)

Figure 2.

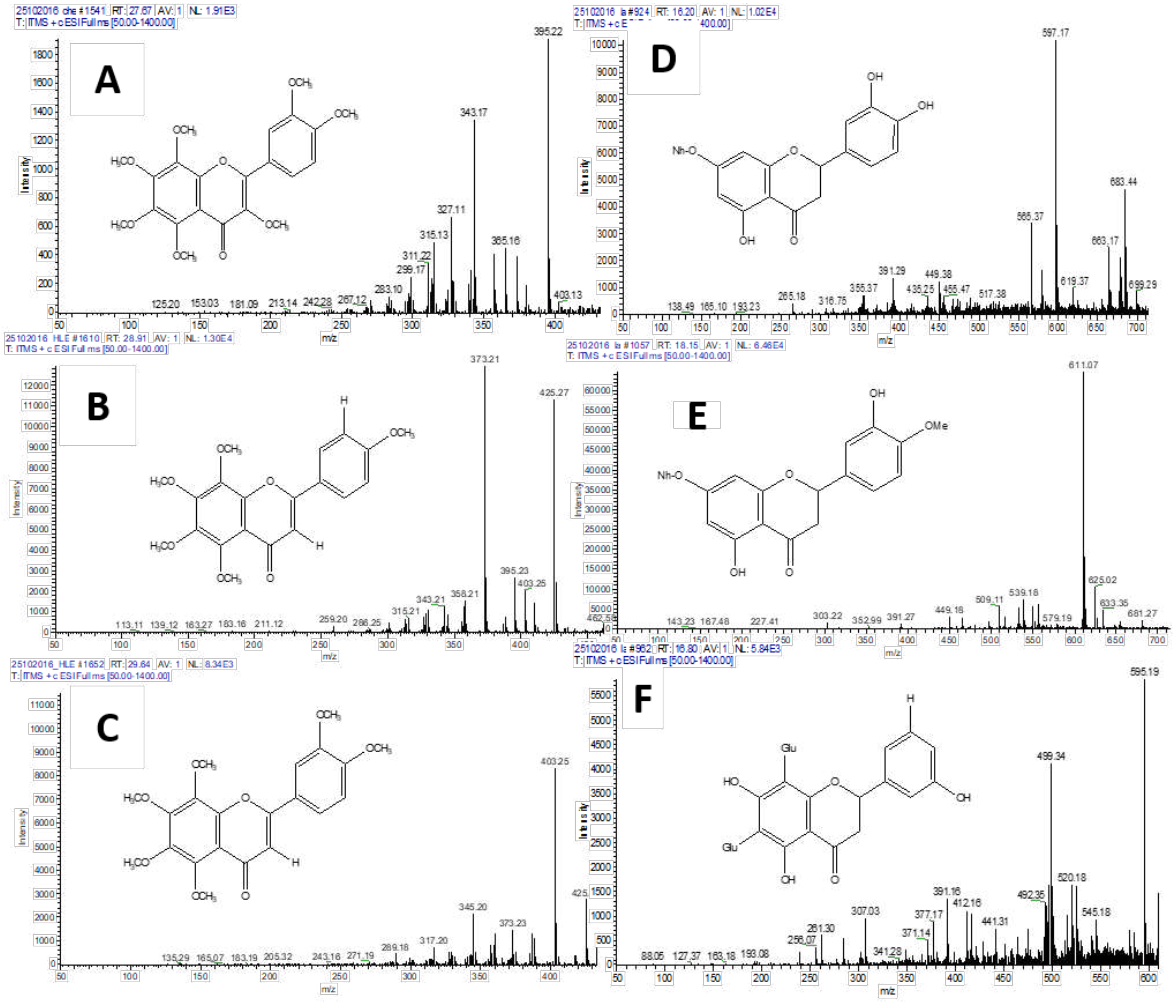
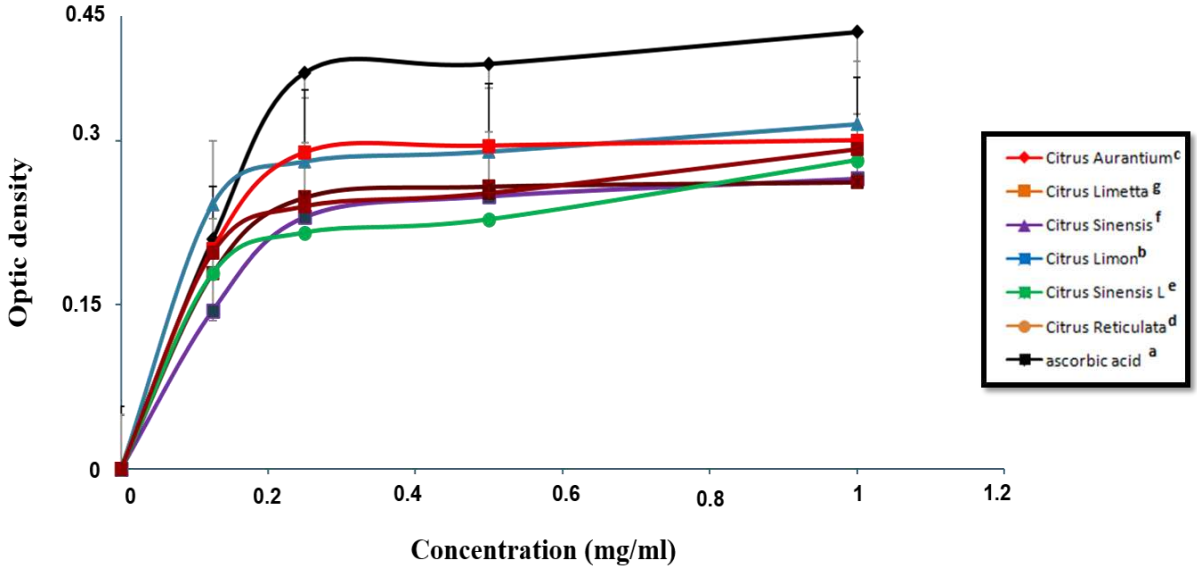


Figure 3.


0.45
0.3
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Optic density
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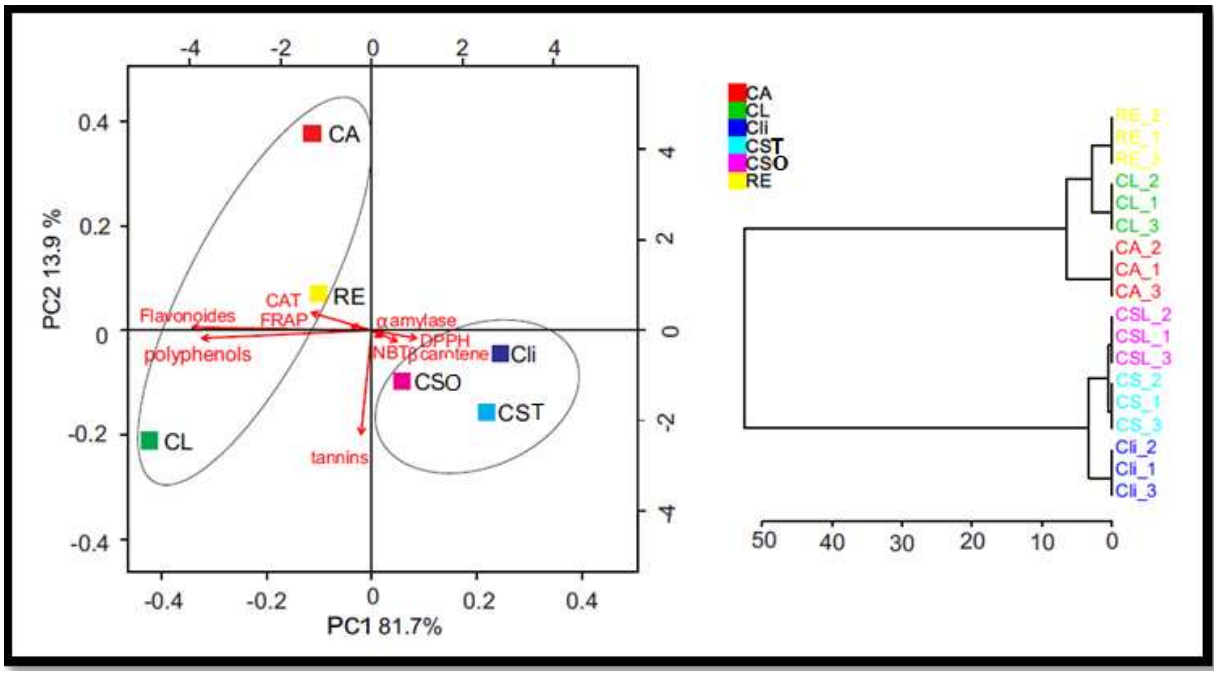
1
1.2
Concentration (mg/ml)
Citrus Aurantium c
Citrus Limetta g
Citrus Sinensis f
Citrus Limon b
Citrus Sinensis L e
Citrus Reticulata d
ascorbic acid a

Figure 4.


-4
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PC2 13.9 %
CA
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Flavonoides
polyphenols
αamylase
DPPH
NBT
βcarotene
Cli
CSO
CST
CL
tannins
-0.4
-0.2
0
0.2
0.4
PC1 81.7%
CA
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Figure 5.