Phytochemistry and Biological Activity of Spanish *Citrus* Fruits

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Abstract

The evaluation of the potential inhibitory activity on α-glucosidase and pancreatic lipase by *Citrus* spp. fruits of Spanish origin (lemon, orange, grapefruit, lime, and mandarin) together with their phytochemical and antioxidant capacity evaluation (DPPH•, ORACFL, ABTS+, FRAP and O2•-) aiming for new applications of the fruits for nutrition and health was carried out. As far as we are aware, 3-O-cafeoylferuoylquinic acid and two hydrated feruloylquinic acids have been described in orange and 3,5-diferuoylquinic acid in grapefruit, for the first time. Although grapefruit showed higher phytochemical contents of flavanones and vitamin C, the potential for inhibitory effects on lipase was higher for lime and lemon, and lime presented also the best results of *in vitro* α-glucosidase inhibition. On the other hand, higher antioxidant capacity was reported for grapefruit, lemon and lime, well correlated to their phytochemical composition. Based on the results, it could be concluded that Citrus fruits are of great value for nutrition and diet-related diseases such as obesity and diabetes, and consequently, a new field of interest for food industry regarding new bioactive ingredients would be considered.

Keywords: Citrus, lipase, α-glucosidase, flavonoids, vitamin C, antioxidants

1. Introduction

It has been strongly demonstrated that the increasing trend in obesity is accompanied by a growing incidence of diabetes. The inhibition of pancreatic lipase in the case of obesity, and of α-glucosidase in the case of diabetes, is the current therapeutic approach for the treatment of both diseases, since these enzymes play an essential role in lipid and glucose metabolisms.[1, 2] In this sense, some *Citrus* fruits represent a good source of bioactive compounds with certain antidiabetic and lypolytic effects,[3, 4] being nowadays studied with increasingly interest.

Citrus fruits are among the most important horticultural crops, and are consumed mostly as fresh product or juice because of its nutritional value and special flavour. Total *Citrus* production in Spain was 5773619 tonnes in 2011,[5] being the sixth producing country in the world after Brazil, China, U.S., Mexico, and India. Most world and Spanish production is accounted for oranges (*Citrus sinensis* L.), but significant quantities of lemons (*Citrus limon* Burm. f), grapefruits (*Citrus paradisi,* Macfad), mandarins (*Citrus reticulata* Blanco), and limes (*Citrus aurantifolia* Christm.) are also grown. It has been strongly demonstrated that these *Citrus* species are thought to possess beneficial effects on health due to their phytochemical composition, mainly flavonoids and vitamin C, having promising prospects in disease prevention, such as obesity, diabetes, blood lipid lowering, cardiovascular diseases, neurodegenerative disorders and certain types of cancer.[3, 6-10]

Some of these *Citrus* fruits and juices have also been used for functional foods and drinks with potential application in diet-related diseases with different health conditions.[11-13] However, as far as we aware, it does not exist in bibliography a comprehensive comparison of enzymatic effects and antioxidant capacity of different *Citrus* fruits rich in polyphenols in the same study, as we propose. Hence, the aim of this work is to evaluate the antidiabetic and antilypolytic effects (α-glucosidase and lipase inhibitory effects) of 5 *Citrus* whole fruits (lemon, orange, grapefruit, lime, and mandarin) of Spanish origin providing a thorough description on the polyphenolic composition (flavones, flavanones, hydroxycinnamic acids and vitamin C), and correlate it to their antioxidant capacity ($DPPH^{\bullet}$, $ORAC_{FL}$, $ABTS^{+}$, FRAP and $O_2^{\bullet -}$).

2. Results and Discussion

2.1. Phenolic compounds

The HPLC-DAD-ESI/MSn analysis of the hydromethanolic extract of *Citrus* fruits revealed a wide range of different phytochemicals being flavones and flavanones the major compounds (Table 1). Hydroxycinnamic acids were also present (Table 1).[14] According to molecular masses, fragmentation patterns, characteristic spectra, and bibliographical sources,[15-17] the following flavanones were identified: *O*-tryglycosil-naringenin, eriodictyol 7-*O*-rutinoside (eriocitrin), naringenin 7-*O*-rutinoside (narirutin), naringenin 7-*O*-neohesperidoside (naringin), hesperitin 7-*O*-rutinoside (hesperidin), and isosakuranetin-7-O-rutinoside (didymin). Grapefruit had higher quantity of total flavanones, mainly represented by exceptional amounts of naringin, previously reported in the fruit.[18] Great flavanone amounts were also obtained for lemon and mandarin fruits, being eriocitrin the major flavanone in lemon, and narirutin in mandarin. Is important to emphasize the role of these bioactives in health, being directly related to anti-inflammatory activities, anticancer effects, and prevention of atherosclerosis, among others.[19]

With respect to flavones, apigenin 6,8-di-*C*-glucoside, luteolin 7-neohesperidoside 4-*D*-glucoside, diosmetin 6,8-di-*C*-glucoside, diosmetin 7-rutinoside and limocitrin 3-rutinoside were identified, in concordance with previous reports.[16] Lemon and lime fruits displayed all the flavones identified, and the higher amounts of total and individual flavones (Table 1). Apigenin 6,8-di-*C*-glucoside and diosmetin 6,8-di-*C*-glucoside were the major flavones in lemon and lime fruits, being apigenin 6,8-di-*C*-glucoside the major in orange and mandarin too. In contrary to flavanones, grapefruit with less quantity of flavones, reported only apigenin 6,8-di-*C*-glucoside and limocitrin

3-rutinoside. These flavones have been previously described to have an important role in the prevention of cancer and cardiovascular disease.[20]

Several hydroxycinnamic acid derivatives were also detected in *Citrus* species, some of them for the first time, according to MS data and fragmentation patterns: 4-*O*-coumaroylquinic acid, two compounds tentatively identified as isomers of dicaffeoylquinic acid (with MS^- 515, and MS_2 353), 3-*O*-cafeoylferuoylquinic acid, 3-*O*-feruoylquinic acid hydrated (with MS^- 367+18=385), 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 5-*O*-feruoylquinic acid hydrated, ferulic acid, sinapic acid, and 3,5-*O*-diferuoylquinic acid. Is important to emphasize that, to this date, this is the first available report in orange of 3-*O*-cafeoylferuoylquinic acid and both hydrated feruoylquinic acids, and of 3,5-diferuoylquinic acid in grapefruit. As far as we are aware, hydrated forms of feruoylquinic acid do not exist in the nature, so probably these compounds were hydrated in the extraction procedure, with a hydromethanolic extractant. Orange fruit displayed the largest number of hydroxycinnamic acids, being mandarin the richest in total derivatives (Table1). These hydroxycinnamic acids and their derivatives have demonstrated to possess *in vitro* and *in vivo* antioxidant activities.[21]

2.2. Total Phenolic Compounds (TPC) by Folin Ciocalteau-Ciocalteu's Reagent

TPC results are expressed as mg per 100 mL of gallic acid equivalents (GAE). *Citrus* fruits reported great TPC quantities in decreasing order as follows: grapefruit (202.36 ± 1.32), lemon (180.73 ± 5.93), orange (104.05 ± 9.31), mandarin (100.57 ± 1.87), and lime (94.78 ± 1.86). However, TPC values must be interpreted with caution since Folin Ciocalteau reagent can react, not only with phenolics, but also with a variety of non-phenolic reducing compounds including tertiary aliphatic amines, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents leading to an overestimation of the phenolics content. Furthermore, different phenolics may have various responses to the Folin-Ciocalteu's Reagent, presenting lower absorption resulting in underestimations of compounds too,[22] so this results should be evaluated together with those obtained by the analysis of phenolic compounds by HPLC-DAD-ESI/MSn (Table 1).

2.3. Vitamin C

It is widely demonstrated that *Citrus* fruits possess significant amounts of vitamin C, as the sum of ascorbic acid (AA) and dehydroascorbic acid (DHAA).[23] AA, DHAA and total vitamin C content (AA + DHAA) of *Citrus* fruits were expressed in mg/100g (Table 2). Grapefruit with the highest amounts of AA, DHAA and total vitamin C, was separated from the rest of the *Citrus* fruits with significantly lower quantities. These differences between *Citrus* fruits according to variety also can be induced by all the factors that affect vitamin C and AA content including cultural practice, maturity, climate, fresh fruit handling, processing factors, blanching, packaging, and storage conditions.[23] These results of ascorbic and dehydroascorbic acid were noticeable higher than previously reported for other fruits or vegetables, like Sweet Pepper (*Capsicum annuum* L.).[24] Furthermore, *Citrus* fruits with higher TPC content also reported more vitamin C (R^2= 0.782**, P<0.01). Ascorbic acid is known for a number of vital biological activities including synthesis of collagen, neurotransmitters, steroid hormones, and carnitine, responsible for the conversion of cholesterol to bile acid.[25] Apart from this, other major clinical investigations were conducted to understand the benefits in prevention of the common cold, iron absorption, ulcers, colorectal carcinoma, hypertension, prevention of atherosclerosis, and advanced malignancy.[26] Therefore, *Citrus* fruits represent good sources of vitamin C, associated with beneficial effects for health.

2.4. Antioxidant capacity

The antioxidant capacity of *Citrus* fruits was tested against different reactive species: $DPPH^{\bullet}$, $ORAC_{FL}$, $ABTS^{+}$, FRAP and $O_2^{\bullet -}$. The $DPPH^{\bullet}$ and $ABTS^{+}$ are non-biological radicals extensively used to test the antioxidant capacity of plant samples. Other widespread methods for the evaluation of the antioxidant capacity of vegetal samples are FRAP, based on the reduction of Fe; and ORAC, based on the ability of peroxyl radical scavenging. Free radicals, like $O_2^{\bullet -}$, are produced in the body as a result of aerobic metabolism, playing an important role in the formation of other reactive species that result in a wide array of biological damages in living cells.[27] So the use of various methods can provide a more complete evaluation of the antioxidant capacity of the *Citrus* fruits.

2.4.1. $DPPH^{\bullet}$

$DPPH^{\bullet}$ is one of the few stable and commercially available organic nitrogen radicals and has an UV−vis absorption maximum at 515 nm. Upon reduction, the solution color fades; and the reaction progress is conveniently monitored by a spectrophotometer.[28] Regarding to $DPPH^{\bullet}$ results, lemon and grapefruit displayed the highest activity against this radical, followed by lime and mandarin ($P<0.05$, Table 3). Lemon and grapefruit were also the fruits that displayed higher amounts of flavanones and vitamin C, finding a positive and direct correlation between $DPPH^{\bullet}$ and these phytochemicals content (R^2= 0.793***, $P<0.001$ for flavanones, and R^2= 0.726*, $P<0.01$ for vitamin C). Moreover $DPPH^{\bullet}$ was strongly correlated with TPC (R^2=

0.900***, $P<0.001$). All *Citrus* fruits presented significant lower activities when compared to other antioxidant assays performed. Previously, the best $DPPH^{\bullet}$ scavenger among four *Citrus* fruits (lemon, orange, lime and grapefruit) was lime,[29] in disagreement to these results, showing other plant extracts strong antiradical activity too, with an IC_{50} between 0.36-0.99 µg extract/mL.[30]

2.4.2. $ABTS^{+}$

The free radical scavenging ability of plant samples is also studied using a moderately stable nitrogen-centred radical species: $ABTS^{+}$ radical.[31] All tested *Citrus* fruits exhibited significant activity, with similar results to that obtained for $DPPH^{\bullet}$ scavenging assays as follows: lemon and grapefruit exhibited the highest scavenging activity, and orange the lowest (Table 3). For this reason, we found a strong and direct correlation between the results of this two antioxidant methods (R^2= 0.956***, $P<0.001$). Moreover, total flavanones played a significant role of this antiradical activity (R^2= 0.698**, $P<0.01$), being the *Citrus* fruits with higher quantity of flavanones the most reactive. $ABTS^{+}$ was also correlated with TPC (R^2= 0.874***, $P<0.001$). Previous works showed high antioxidant properties against $ABTS^{+}$ of *Citrus* peel phenolic extracts, like grapefruits, which might be useful in the formulation of nutraceuticals and food preservatives.[31]

2.4.3. FRAP (Ferric reducing antioxidant power)

FRAP method is used to measure the total reducing capability of antioxidants based on their potential to react on ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and produce blue colour of the ferrous form, which can be detected at absorbance 593 nm.[32] The tested *Citrus* fruits displayed high and very similar antioxidant capacity ($P<0.05$) in the FRAP assay (Table 3), as expected;[33, 34] but lemon and grapefruit, as in DPPH$^{\bullet}$ and ABTS^{+}, and lime, exhibited certain higher activity than orange and mandarin. These FRAP results were higher than previously reported for an Italian saffron (*Crocus sativus* L.).[35] Other juices from Citrus varieties cultivated in China also reported high activity.[36] No significant correlation between FRAP and any other test was found, which suggested the different mode of action in this method based on iron reduction, with the previous radical scavenging assays employed.

2.4.4. ORAC$_{FL}$

ORAC$_{FL}$ assay provides a direct measure of hydrophilic chain-breaking antioxidant capacity against peroxyl radical.[37] The values obtained for the fruits in the ORAC$_{FL}$ assay varied distinctly among the samples (ranged between 19.44 and 46.33 mM Trolox/100mg dw (Table 3)), being in this case lime and grapefruit the most reactive samples, followed by lemon and mandarin, and reporting orange the lowest value again ($P<0.05$). Is interesting to know that *Citrus* fruits obtained higher ORAC values than over 100 different kinds of foods, including fruits, vegetables, nuts, dried fruits, spices, and cereals from the United States.[38]

2.4.5. Superoxide radical ($O_2^{\bullet-}$)

Superoxide anion ($O_2^{\bullet-}$) plays an important role in the formation of other ROS such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2), and hydroxyl radical (OH^-), which induce oxidative damage in lipids, proteins, and DNA. These species are produced by a number of enzyme systems in autooxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen.[39] Concerning the $O_2^{\bullet-}$ scavenging results, low IC_{50} values were obtained (Table 3), suggesting a high activity of the *Citrus* fruits against this reactive oxygen species, among which lime, lemon, and orange, were the most active. In fact, total flavones were correlated with $O_2^{\bullet-}$ scavenging activity (R^2= -0.720**, P<0.01). Flavones and flavanones of *Citrus* flavonoids, have been described as good superoxide scavengers,[40] supporting this strong effect. A point worth mentioning is that although all *Citrus* fruits were very active against $O_2^{\bullet-}$ radical, some differences between this method and the rest were found, probably due to the differences in the mode of action of this biological method compared to the rest of chemical radicals.

Citrus fruits can effectively scavenge different types of reactive oxygen species or free radicals under *in vitro* conditions (Table 3). The broad range of results indicates that multiple mechanisms may be responsible for their antioxidant capacity, related to their phenolic composition, mainly flavones and flavanones, and their vitamin C content. Although all the antioxidant methods have different nature and origin between them, *Citrus* fruits followed a similar trend in all the methods in general, suggesting that grapefruit, lemon and lime are the most antioxidants among all used in this study, and reporting orange and mandarin lower results. In summary, the combination of phytochemicals and synergistic mechanisms in the fruit matrix is highly responsible for the potent antioxidant activities of fruits.

2.5. α-Glucosidase inhibition

α-Glucosidase is a key enzyme that catalyses the final step in the digestive process of carbohydrates, therefore the inhibition of this enzyme could delay the digestion of oligosaccharides and disaccharides to monosaccharides, diminishing glucose absorption and consequently reducing prostprandial hyperglycemia.[2] The IC_{50} values were calculated in order to compare the different *Citrus* fruits, as shown in Table

3. Different effects were observed as follows: orange and mandarin did not reach the 50% inhibition of enzyme, while lemon and grapefruit caused slight inhibition, being lime more effective (Fig. 2). Flavones and flavanones were reported to be potent α-glucosidase inhibitors.[41] Moreover, some *Citrus* flavonoids, like hesperidin, naringin and polymethoxylated flavones, have demonstrated potential benefits in the management of diabetes in some animal models, by different biochemical mechanisms.[4] The IC_{50} values and total vitamin C (AA + DHAA) were strongly correlated (R^2= 0.879***, P<0.001), but no Pearson correlations between any flavonoid groups and anti-α-glucosidase activity was found in our results. The α-glucosidase inhibitory activities were consistent with the statement that the different phytochemical profile and the interactions between compounds in the fruit matrix can be also involved in the various activities displayed by them. Lime and lemon fruits are active against α-glucosidase, reporting lime the highest effect among all *Citrus* fruits analyzed. Thus, lime fruit may offer dietary coadjuvants to control hyperglicemia in diabetic patients, however further research in the evaluation of their *in vivo* antidiabetic activity is needed to verify this beneficial effect.

2.6. Pancreatic lipase inhibition

The inhibition of pancreatic lipase, which splits triglycerides into absorbable glycerol and fatty acids, is the main prescribed treatment for obesity in developed countries.[1, 42] In order to find alternative sources for obesity prevention and treatment, we searched for the inhibitory action of the Spanish *Citrus* fruits on lipase activity. Results are shown in Table 3 and Fig.1 as U/L and % of inhibition of lipase enzyme, respectively; taking into consideration that the activity of the lipase standard was 260 U/L. Lemon and lime fruits displayed the highest inhibitory effect on pancreatic lipase

(93.74 and 111.37 U/L, respectively), being also richer in flavones as seen above, and finding a strong correlation between % of lipase inhibition and total flavones content (R^2= 0.969***, P<0.001). This potent inhibitory activity of flavones on lipase enzyme has been previously reported.[43] Moreover, citric acid had been also described as driver of thermogenesis, reducing obesity risk.[44] The remaining orange, mandarin and grapefruit also displayed certain inhibitory effects, being previously demonstrated to improve the lipid metabolism some of their phytochemicals, such as eriocitrin[8] or hesperitin.[45] Consequently, Spanish origin *Citrus* have demonstrated *in vitro* inhibition of pancreatic lipase, especially for lemon and lime. Taking into account that lime and lemon were also the best performed fruit in terms of α-glucosidase inhibition, they may be developed individually or in synergistic formulations as natural alternatives for the treatment of obesity and diabetes through dietary intervention, even though further *in vivo* research is needed.

3. Experimental

3.1. Chemicals

2,2-diphenyl-1-picrylhidracyl radical ($DPPH^{\bullet}$), 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt ($ABTS^{+}$), 2,4,6-Tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate, fluorescein (free acid), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (APPH), sodium phosphate monobasic, sodium phosphate dibasic, Folin Ciocalteu's Reagent, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), trizina hydrochloride, 4-nitrophenil α-D-glucopyranoside, α-Glucosidase from *Saccharomyces cerevisiae*, and potassium phosphate were obtained from Sigma-Aldrich (Steinheim, Germany). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and magnesium chloride hexahydrate were purchased from Fluka Chemika, (Neu-Ulm, Switzerland); sodium carbonate anhydre, sodium benzoate and potassium sorbate were bought from Panreac Química S.A., (Barcelona, Spain). LIPASE-PS™ (Kit) was obtained from Trinity Biotech (Jamestown, NY, USA). Ultrapure water was produced using a Millipore water purification system.

3.2. Fruits

Citrus fruits were purchased at 'Carrefour Planet' Store (Centros Comerciales Carrefour S.A., Murcia), gathered from different producers:

-Lemon: *C. limon* (Burm. f), lemon cv. 'verna' (caliber 3/4-58/72 mm; Cat. 1.; Los Ramos, Murcia)

-Orange: *C. sinensis* (L.) (Cat. 1.; Los Ramos, Murcia)

-Lime: *C. aurantifolia* (Christm.) Swingle, lime. (CIF B-29607751, Málaga)

-Mandarin: *C. reticulata* (Blanco), Honey tangerine cv. 'Murcott' (caliber 54/64mm, Piles, Valencia)

-Grapefruit: *C. paradisi* (Macfad), 'Star Ruby' red grapefruit (caliber 84/97 mm; Cat. 1, Castellón, Valencia)

3.3. Extraction

All whole fruits were cut in 3cm portions, frozen with liquid N_2 and freeze dried. An amount of 100 mg of sample was weighed and added with 1 mL of methanol/water (70:30% v:v). Then, samples were vortexed and sonicated in ultrasonic bath for 60 min. Samples were kept at 4°C overnight, and sonicated again for 60 min. A centrifugation (model EBA 21, Hettich Zentrifugen) step (10000 rpm, 5 min) was used to separate the supernatant from the solid residue. This supernatant was filtered through a 0.45 μm PVDF filter (Millex HV13, Millipore, Bedford, Mass., USA) and stored at 4°C before performing all analytical methods. Three different extractions were made for each method.

3.4. Identification of phenolic compounds by HPLC-DAD-ESI/MSn and quantification by RP-HPLC-DAD

Chromatographic analyses for the identification were carried out on a Luna C18 column (250 x 4.6 mm, 5 mm particle size; Phenomenex, Macclesfield, UK). Water:formic acid (99:1, v/v) in an Agilent HPLC 1100 series equipped with a photodiode array detector and a mass detector in series (Agilent Technologies,Waldbronn, Germany) with the same conditions used previously according to Gironés-Vilaplana *et al.*.[46] The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A) and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03)

For the quantification HPLC-DAD system was used, as described by Gironés-Vilaplana et al.,.[47] Different phenolics were characterised by chromatographic comparison with analytical standards as well as quantified by the absorbance of their corresponding peaks. Flavonols and flavones were quantified as quercetin 3-*O*-glucoside at 360nm, cinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm, and flavanones as hesperidin at 280 nm.

3.5. Total Phenolic Compounds (TPC) by the Folin-Ciocalteu's Reagent

The Folin-Ciocalteu's Reagent method was adapted to a microscale assay according to.[48] Results were expressed as mg per 100 mL of gallic acid equivalents (GAE).

3.6. Extraction and analysis of vitamin C

Vitamin C content was determined by HPLC as described by González-Molina *et al.*.[49] AA (ascorbic acid) and DHAA (dehydroascorbic acid) were identified and quantified by comparison with pattern areas from AA and DHAA. The vitamin C content was calculated by adding AA and DHAA content, and results were expressed as mg/100 g dried weight.

3.7. Antioxidant capacity

The free radical scavenging activities were determined using the DPPH$^{\bullet}$, ABTS^{+} and FRAP (ferric reducing antioxidant power) methods adapted to a microscale according to Mena *et al.*.[50] The antioxidant activity was evaluated by measuring the variation in absorbance at 515 nm after 50 min (DPPH$^{\bullet}$), at 414 nm after 50 min

($ABTS^+$) of reaction with the radical, and finally at 593 nm after 40 min for FRAP assay. Assays were measured by using 96-well micro plates (Nunc, Roskilde, Denmark) and Infinite® M200 micro plate reader (Tecan, Grödig, Austria). All reactions started by adding 2 µl of the corresponding diluted sample to the well containing the stock solution (250 µl). Final volume of the assay was 252 µl. Antioxidant activity was also determined using the ORAC-FL assay, according to Ou *et al.*.[37] Results were expressed as mM Trolox/100mg dried weight.

Superoxide radical ($O_2^{\bullet -}$) scavenging activity was also determined spectrophotometrically in a 96-well plate reader by monitoring the effect of controls and blends on the $O_2^{\bullet -}$ induced reduction of NBT at 560 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure.[51] The experiments were performed in triplicate, and results were expressed in IC_{50} (Concentration of sample to inhibit 50% of radical).

3.8. α-glucosidase inhibitory activity

α-glucosidase inhibitory activity was assessed by modification of a previously reported procedure.[52] Briefly, each well contained 100 µl of 2 mM 4-nitrophenyl α-D-glucopyranoside in 10 mM potassium phosphate buffer (pH 7.0) and 20 µl of the samples, diluted 1/2 in buffer. The reaction was initiated by the addition of 100 µl of the enzyme solution (56.66 mU/mL). The plates were incubated at 37ºC for 10 min. The absorbance of 4-nitrophenol released from 4-nitrophenyl α-D-glucopyranoside at 400 nm was measured. The increase in absorbance was compared with that of the control (buffer instead of sample solution) to calculate the inhibitory activity and the IC_{50}.

3.9. Lipase inhibitory effect

Lipase activity was determined as previously described by Gironés-Vilaplana *et al.*,[46, 47] and adapted to a microscale 96-well micro plates (Nunc, Roskilde, Denmark) in Infinite® M200 micro plate reader (Tecan, Grödig, Austria). The recorded rate of increase in absorbance at 550 nm due to the formation of quinone diimine dye was used to determine the pancreatic lipase activity in the samples prepared. The pancreatic lipase activity in fruits was expressed in U/L.

3.10. Statistical analysis

Data presented are mean values (n=3) ± Standard Deviation. All data were subjected to analysis of variance (ANOVA) and a Multiple Range Test (Tukey's test), using IBM SPSS statistics 21 software (SPSS Inc., Chicago, IL). Pearson's correlation analysis was performed to corroborate relationships between selected parameters.

4. Conclusions

Nowadays, scattered publications dealing with the bioactive composition of Citrus fruits and their potential effects on health are found. The hydromethanolic extracts of Citrus fruits revealed a wide and diverse range of phytochemicals, mainly flavones, flavanones, and vitamin C (AA+DHAA); and significant antioxidant capacity and biological activity. Grapefruit displayed the highest phytochemical contents in terms of flavanones and vitamin C. To the best of our knowledge, this is the first available report of 3-O-cafeoylferuoylquinic acid and both hydrated feruoylquinic acids in orange, and of 3,5-diferuoylquinic acid in grapefruit. Although grapefruit, lemon and lime performed better in terms of antioxidant capacity methods and correlated well with flavanones and vitamin C, the lemon and lime were the best candidates for antidiabetic and antilypolytic purposes (α-glucosidase and lipase inhibition), also correlated to vitamin C and flavones content, respectively. Therefore multiple biological activities indicates the value of lemon and lime Citrus as sources of bioactive compounds for new product developments (i.e. combinations of fruits to enrich new foods or beverages), with potential applications in diet-related diseases such as obesity and diabetes. However, more in vivo research and safety evaluations should be underway to allow scientifically backed statements and recommendations for dietary intake.

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Figure captions

Figure 1. Lipase inhibition (%) of Spanish *Citrus* fruits (100 mg dried fruit/1 mL of methanol:water 70:30 v:v).

Figure 2. α-glucosidase inhibition of Spanish *Citrus* fruits

Table 1. Bioactive composition (flavones, flavanones and hydroxycinnamic acid derivatives) identified and quantified in *Citrus* fruits (mg/100g dried weight)

Compound	Rt	$[M-H]^-$	MSn	Lemon	Orange	Lime	Mandarin	Grapefruit
Flavanones (280 nm)								
FV1 *O*-tryglycosil-naringenin	22.3	741	579, 279	-	28.57 ± 0.81	-	78.41 ± 1.53	33.37 ± 4.79
FV2 Eriodictyol 7-*O*-rutinoside	30.9	595	577, 287	938.30 ± 16.45	68.05 ± 2.68	257.34 ± 0.47	-	-
FV3 Naringenin 7-*O*-rutinoside	37.7	579	271	-	139.90 ± 0.13	48.80 ± 0.20	488.81 ± 14.08	309.26 ± 4.63
FV4 Naringenin 7-*O*-neohesperidoside	42.2	579	271	-	-	-	-	2530.65 ± 26.77
FV5 Hesperitin 7-*O*-rutinoside	45.8	609	301	372.89 ± 4.03	335.55 ± 5.80	188.54 ± 1.24	123.59 ± 2.38	-
FV6 Isosakuranetin-7-O-rutinoside	61.4	593	285	-	-	-	319.92 ± 15.39	226.84 ± 0.06
TOTAL				1311.19 ± 20.48	572.07 ± 3.80	494.67 ± 1.91	1010.73 ± 0.46	3100.11 ± 26.86
Flavones (360 nm)								
FL1 Apigenin 6,8-di-*C*-glucoside	22.8	593	503, 473	45.99 ± 0.19	19.60 ± 0.74	65.38 ± 0.93	50.13 ± 0.39	10.96 ± 0.93
FL2 Luteolin 7-neohesperidoside 4-*D*-glucoside	26.3	756	623, 594, 286	22.32 ± 1.56	8.70 ± 2.17	12.05 ± 0.65	-	-
FL2 Diosmetin 6,8-di-*C*-glucoside	28.1	623	503, 413, 383	63.58 ± 0.21	6.27 ± 0.29	44.88 ± 1.34	5.61 ± 0.51	-
FL4 Diosmetin 7-*O*-rutinoside	44.8	607	299	21.56 ± 0.21	3.39 ± 0.43	13.35 ± 0.46	-	-
FL5 Limocitrin 3-rutinoside	47.7	653	345	21.32 ± 0.11	-	11.34 ± 2.85	-	16.22 ± 0.34
TOTAL				174.76 ± 2.07	37.96 ± 0.72	147.00 ± 3.43	55.74 ± 0.11	27.17 ± 0.33
Hydroxycinnamic acid derivatives (320 nm)								
HA1 4-*O*-coumaroylquinic acid	5.7	337	173	-	21.41 ± 0.01	-	-	-
HA2 Dicaffeoylquinic acid (1)	6.1	515	353	-	21.40 ± 0.36	24.49 ± 2.15	23.64 ± 0.66	12.63 ± 0.71
HA3 Dicaffeoylquinic acid (2)	7.0	515	353	-	-	31.36 ± 0.72	32.92 ± 1.24	18.62 ± 2.26
HA4 3-*O*-caffeoyl-4-*O*-feruoylquinic acid	8.2	530	513	-	26.40 ± 0.06	-	-	-
HA5 3-*O*-feruoylquinic acid hydrated	9.6	385	367, 173	-	21.01 ± 0.03	-	-	-
HA6 3-*O*-caffeoylquinic acid	12.3	353	191, 179	62.65 ± 0.32	14.60 ± 0.39	44.90 ± 0.32	69.01 ± 0.35	41.65 ± 0.70
HA7 5-*O*-caffeoylquinic acid	16.1	353	191	18.52 ± 0.73	55.09 ± 0.17	6.44 ± 0.06	57.10 ± 4.50	87.66 ± 1.95
HA8 5-*O*-feruoylquinic acid hydrated	18.9	385	367, 173	-	-	-	-	29.09 ± 0.04
HA9 Ferulic acid	19.2	175	169	25.78 ± 0.02	-	-	-	-
HA10 Sinapic acid	21.3	447	285	25.03 ± 0.83	-	-	-	-

HA11	3,5-*O*-diferuoylquinic acid	32.0	561	367, 173	-	-	-	58.81 ± 1.10	-
	TOTAL				131.98 ± 1.26	188.99 ± 0.65	106.98 ± 2.61	241.48 ± 5.64	160.56 ± 1

Table 2. Ascorbic acid (AA), dehydroascorbic acid (DHAA) and total vitamin C (AA+DHAA) of *Citrus* fruits (mg/100 g dried product)

Citrus fruit	AA	DHAA	VITAMIN C
Lemon	57.66 ± 5.12 a	97.60 ± 9.20 a	155.26 ± 14.32 a
Orange	84.82 ± 0.86 b	36.24 ± 0.96 a	121.06 ± 0.50 a
Lime	81.57 ± 3.82 ab	46.70 ± 0.52 a	128.27 ± 3.30 a
Mandarin	64.03 ± 5.54 ab	67.26 ± 8.79 a	131.29 ± 14.33 a
Grapefruit	114.52 ± 10.81 c	283.19 ± 34.87 b	397.71 ± 42.51 b
LSD P<0.05	6.162	22.791	28.411

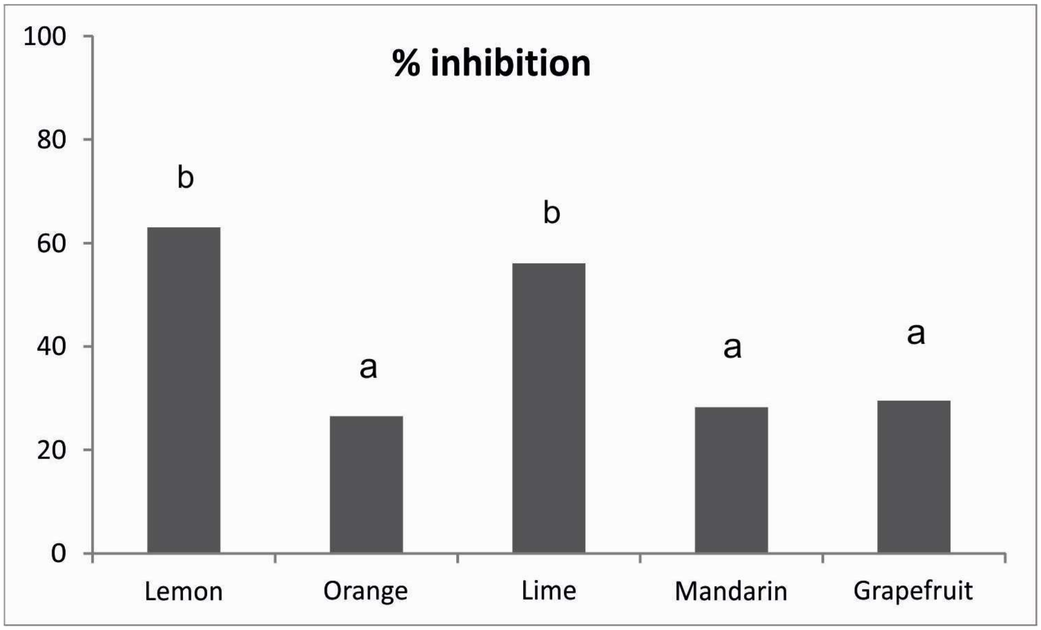
Means (n=3) in the same columns followed by different letters are significantly different at $P < 0.05$ according to Tukey's test.

Table 3. Antioxidant capacity, α-glucosidase inhibition, and lipase activity in *Citrus* fruits.

Fruit	**DPPH$^{\bullet}$** mmol Trolox/100g d.w.	**ABTS^{+}** mmol Trolox/100g d.w.	**FRAP** mmol Trolox/100g d.w.	**ORAC** mmol Trolox/100g d.w.	**$O_2^{\bullet -}$** IC_{50} (mg/mL)	**α-glucosidase** IC_{50} (mg/mL)*	**Lipase** U/L
Lemon	3.92 ± 0.11 c	9.00 ± 0.58 c	7.53 ± 1.02 a	31.26 ± 3.42 b	1.33 ± 0.20 a	36.59 ± 1.60 b	93.74 ± 7.39 a
Orange	1.54 ± 0.20 a	4.83 ± 0.17 a	5.95 ± 1.11 a	19.44 ± 0.30 a	1.79 ± 0.27 a	-	186.89 ± 6.75 b
Lime	2.53 ± 0.15 b	6.14 ± 0.59 ab	7.35 ± 1.37 a	45.12 ± 3.49 c	1.54 ± 0.12 a	10.96 ± 0.31 a	111.37 ± 4.17 a
Mandarin	2.50 ± 0.17 b	6.47 ± 0.30 b	5.13 ± 0.33 a	31.70 ± 2.50 b	2.96 ± 0.03 b	-	182.20 ± 8.62 b
Grapefruit	4.22 ± 0.19 c	8.69 ± 0.42 c	7.07 ± 0.20 a	46.33 ± 1.77 c	2.54 ± 0.25 b	62.10 ± 2.32 c	179. 10 ± 13.14 b
LSD P<0.05	0.182	0.424	0.756	2.109	0.160	1.035	6.973

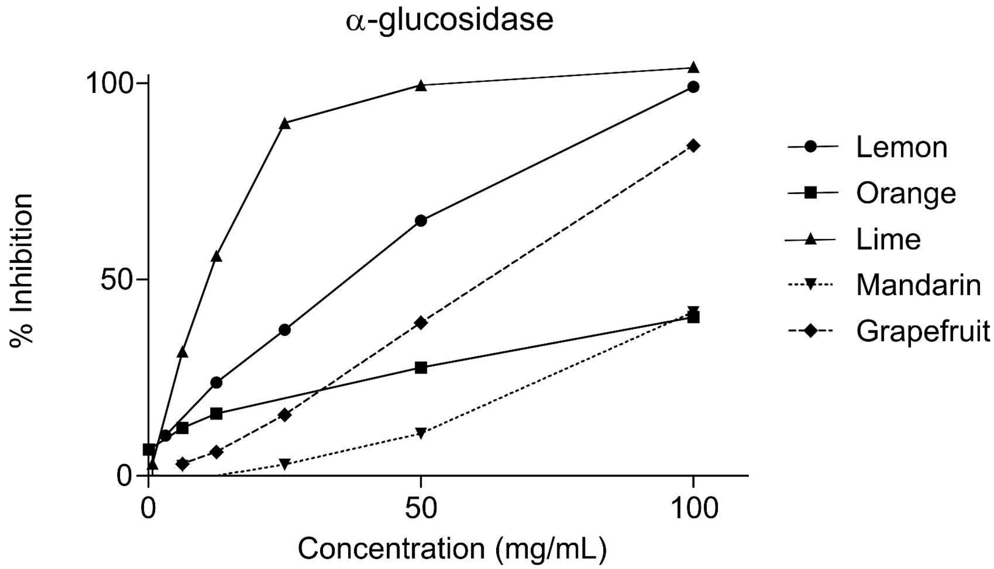
Means (n=3) in the same columns followed by different letters are significantly different at $P < 0.05$ according to Tukey's test. * Samples without data did not inhibit 50% of enzyme.

Figure 1



Different letters means significantly different at $p<0.05$ with a LSD=2.745 according to Tukey HSD Multiple Range Test.

Figure 2


α-glucosidase
100
50
0
% Inhibition
0
50
100
Concentration (mg/mL)
Lemon
Orange
Lime
Mandarin
Grapefruit

Grapefruit, lemon and lime displayed high antioxidant capacity and interesting inhibitory activity on glucosidase and lipase of interest for nutrition and health.