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Phenolic composition, antioxidant and enzyme inhibitory activities of Cucurbitaceae fruits

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ABSTRACT

Fruits have been known as great source of bioactive natural compounds with potential biological effects. The objective of this work was to perform digestive enzyme inhibition assays with methanolic extracts of six fruits from the family Cucurbitaceas. The extracts were also analyzed for antioxidant capacity (DPPH·), qualitative and quantitative composition of chemical compounds and nutrients. The fruit extracts showed similar antioxidant activities and had substantially different total polyphenolic contents. In addition, the six fruit extracts examined, at a concentration of 100 µg/mL, significantly inhibited β glucosidase and lipase activity, *in vitro*. These results may be related to the profile of polyphenolic compounds. In conclusion, it can be said that cucurbits fruits are a valuable horticultural product, based on their rich and beneficial chemical composition.

INTRODUCTION

The cucurbits are a very diverse group of plant species that form the family *Cucubitaceae*. Many of them have economic value as those of the genre Cucurbita (pumpkins), *Cucumis* (melons and cucumbers), *Citrullus* (watermelons), *Luffa* (sponge), *Sechium* (*Sechium edule*, known as xuxu) among others. The fruits are eaten fresh and are also used in marmalades, juices. These plants are also used in folk medicine to treat various diseases, such as diabetes, gout, leucorrhea, jaundice, pneumonia, psoriasis etc.[1] An interest in cucurbits production has increased considerably in recent years, with a growing number of reports on their potential health benefits. Recent research suggest that bitter melon (*Momordica charantia*) extracts may ameliorate high fat diet induced obesity and hyperlipidemia in animal model.[2] The hypoglycaemic effect of this plant was demonstrated in experimental models of normal and diabetic animals and in patients with type 2 diabetes.[3,4] Others reports also showed that bitter melon extracts inhibits glucose absorption, promotes the use of hepatic glucose, and increases the number of insulin-positive cells in the pancreas.[5-7] In this same sense, Choi et al., 2007 found that, treatment with *Cucurbita moschata* extract (orally administered for 8 weeks) activated PPAR-α, increased β-oxidation and inhibited adipocyte differentiation in a dose-dependent manner in mice.[8] Previous studies suggest that the inhibition of the metabolic enzymes by plants or fruits is attributed to the phenolic compounds, in particular tannin like components.[9] The *Cucurbitaceaes* contains biologically active chemical constituents such as flavonoids, phenolic compounds, saponins, fixed oils, triterpenes, and steroids. It also contains substances of nutritional interest such as vitamins and minerals.[10,11] However, studies showing the presence of enzymatic inhibitors in fruit extracts from the family of cucurbitaceas are lacking. Therefore in this study the inhibitory effects of the methanolic extract on β-glucosidase and porcine pancreatic lipase were investigated using *in vitro* model. Basic chemical composition and quantitative polyphenolic compounds composition as well as antioxidant capacity of fruit were also evaluated.

MATERIALS and METHODS

Materials

β-Glucosidase, Lipase, conduritol β- epoxide and esculin hydrate were purchased from Sigma. Aluminium-backed TLC layers of silica gel were purchased from Merck. Agar was purchased from Britania (Buenos Aires, Argentina). Sodium acetate, ferric chloride hexahydrate and glacial acetic acid were purchased from Cicarelli (San Lorenzo, Argentina). The fruits were grown in Argentina and were purchased in local markets. In the case of watermelon *(Citrullus lanatus* (Thumb.), two commercial varieties were studied: Charleston Gray and Sugar Baby. The *Cucumis melo* L. (melón), two varieties were evaluated in this work: *Reticulatus* (written or reticulated) and the variety *Inodorus* (cultivars dew of honey and yellow type). In addition the *Sechium edule*, also known as xuxu, was studied.

Fruit Extracts

The fruit were lyophilized as previously described.[12] Extracts were prepared from the dried powdered fruit material macerated with different solvent, evaporated under vacuum at 40°C.

Determination of mineral content

Moisture content of fruit samples were determined by AOAC method.[13] The ashes were made by destruction of the organic matter by dry route at 450-500 ° C, according to the gravimetric method described in SAMLA**.**[14] Obtained ash was used to measure different types of minerals which have important functions in the body they are calcium, phosphorus, iron, potassium and sodium. The determinations of Ca and Mag were made by volumetry of complexes titrating with EDTA and as indicators Carbonic calcon (Ca) and Eriochrome black T (Mg).[15]

Phytochemical prospection tests

For this analysis, a preliminary phytochemical study of fruit extracts was made; the purpose was to find the main secondary metabolites associated with biological activities (alkaloids, flavonoids, tannins, steroids,and terpenes). Phytochemical prospecting tests were performed with the hexane, acetone, ethyl acetate, methanolic and hydroalcoholic extracts.[16]

Determination of total polyphenolic and flavonoid contents

The total phenolic content of fruits was determined by Folin Ciocalteu method. The amount of total polyphenolics was calculated as gallic acid equivalents (GAE) as it was described in previous works.[17] The total flavonoid content was determined by the method described previously and expressed as gram of rutin equivalent (RE)/ 100 g of extract.[12,17]

Determination of total carotenoids contents

The determination of the total carotenes was carried out according to the method described by with some modifications.[18, 19] A 2-g fruit sample with 20 mL of acetone–ethanol solution was homogenized. The filtrate was transferred into a graduated cylinder and solvent added to a final volume of 100 mL. A 50-mL volume of hexane and 25 mL of H_2O were added and shaken vigorously before standing for 30 min to allow separation of phases to occur. The spectrophotometer was blanked with hexane and the absorbance of the hexane phase was measured at 470 nm. The results are expressed as μg of β-carotene equivalent / 100 g, using the molar extinction coefficient of 2500 of β-carotene and applying the following equation:

$$\mu g \text{ de } \beta\text{-carotene equiv} / 100g = (A \times V \times 106 / A_{1cm}^{1\%} \times 100 \times PM\ x_{(g)})$$

A = Absorbance of the sample
V = Total volume of the extract
A = Absorptivity coefficient of β-carotene (2500)
Pmx = Sample weight in grams

Free radical scavenging activity on DPPH

The DPPH free radical scavenging assay was used for the evaluation of the antioxidant activity of the crude extracts, as previously described.[12,17] the reaction mixture containing 1 mL of DPPH solution with different concentrations of the extract in ethanol (10, 50,100, 500 and 1000 μg/mL), was shaken and incubated for 30 min at room temperature and the absorbance was read at 517 nm against a blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and the results are expressed as a percentage.

TLC bioautographic assay for detecting β-glucosidase and Lipase Inhibitors

According to various researchers, bioautographic assays using TLC plates play an important role in the search for bioactive compounds from natural extracts, giving quick access to information concerning both the activity and of the fraction or the metabolite isolated, responsible for the observed activity.[20] In our case, it was used as a preliminary test (data not shown).

Staining solution for β-glucosidase

The technique was developed according to the methodology described in previous works.[21] For the staining solution for glucosidase, agar was dissolved at 80°C in sodium acetate buffer. The solution was allowed to cool to 60°C and ferric chloride solution was added. Later, glucosidase solution in acetate buffer was added and the obtained solution mixed by inversion.

Detection of β glucosidase activity

Samples of fruit extracts were applied to silica gel TLC plate. The amount of fruit extracts loaded was 100 to 500 ug of extract / spot. For the test an aliquot of β- glucosidase staining solution was distributed over the TLC layer. After the staining solution had solidified, the TLC plate was incubated and immersed in solution of esculin (37°C, 2 h). On the plate clear spots indicate inhibition of the glucosidase activity, against a dark brown background. Conduritol epoxide (ranging from 1.25 to 0.001 mg) was used as a positive control of the reaction.[21]

TLC bioautographic assay for detecting Lipase Inhibitors

The test was developed according to the technical specifications published by Hassan et al., 2011.[22] For that, Lipase was dissolved in Tris–HCl buffer; bovine serum albumin was added to the solution in order to stabilize the enzyme during the assay and then the stock solution was kept at 4°C. Samples of fruit methanolic extracts were applied to silica gel TLC plate. After drying, the plate was sprayed with α-naphthyl acetate (150 mg in ethanol) and enzyme solutions. The TLC plate was then incubated at 37°C for 20 min. To reveal, the solution of Fast Blue B salt (50mg in 100mL distilled water) was sprayed onto the TLC plate, where white spots on a purple background indicate lipase inhibitory activity. Orlistat (ranging from 1.5 to 0.001 mg) was used as a positive control of the reaction.

β-glucosidase activity

In this study, B-glucosidase activity was determined according to Kwon et al. 2006, using p-nitrophenyl-β-D-glucopyranoside 5 mmol.L-1 in citrate-phosphate buffer (0.1 mol L^{-1}), as substrate (pH 7.0).[23] In the test, 50 µL of the fruit methanolic extract and 100 µL of enzyme were incubated in bath at 37 ° C for four time periods after addition of 50 µL of the substrate. The reaction was stopped adding 1 mL of NaOH (0.05 mol L^{-1}), and the product was measured at 405 nm using a UV-Visible spectrophotometer.[24] The β-glucosidase inhibitory activity was expressed as the percentage of inhibition then calculated from Eq 1.

$$\%INH = 100 \{1- [(B - D) / (A - C)]\}$$

Where B is the absorbance of the sample, D is the absorbance of the blank (without enzyme), A is the absorbance of control 1 (without inhibitor) and C is the absorbance of control 2 (without inhibitor and without enzyme).

Lipase activity

The inhibition properties of fruit extract (0–50 mg/mL) against lipase were assayed according to the procedure described by Souza et al., 2010. with some modifications.[25] In each analysis, the mixture of 100 µl of lipase, 50 µl of the fruit extracts and 50 µl of 4 mmol L-1 *p*-nitrophenyl laurate substrate in Tris-HCl (0.05 mmol L^{-1} pH 8.0) buffer containing 0.5% Triton-X100 was incubated for four periods of time. The reaction was stopped, transferring the tubes to an ice bath and adding 1.000 µl of Tris-HCl (0.05 mmol L^{-1} pH 8.0) buffer. After incubation, the amount of *p*-nitrophenol released (yellow coloration) in the reaction was measured at 405 nm using a UV-Visible spectrophotometer. The lipase inhibitory activity was expressed as the percentage of the decrease in A 410 when lipase was incubated with the fruit extracts compared to the negative control (solvent only), and was calculated from Eq. (Eq. 2):

$$I\% = \frac{(A-a)-(B-b)}{(A-a)} \times 100$$

(A): in the absence of the extract (possible inhibitor), which corresponds to the control enzyme assay.
(A): in the absence of the extract and the enzyme (white substrate)
(B): in the presence of the extract with the enzyme and substrate
(B): in the absence of the enzyme (white extract + substrate).

Statistics
Statistical analysis was done by using one way analysis of variance using the SPSS program. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS and DISCUSSION

Phytochemical Screening Result

Melon (*Cucumis melo* L.) is a plant species of interest for its specific biological properties and for its economic importance. It belongs to the *Cucurbitaceae* family, which also includes cucumber (*Cucumis sativus* L.), watermelon *Citrullus lanatus* (Thunb.), *Sechim edule* (xuxu) and Luffa (sponge), among others. In general, these

Constituent	Samples		
	Citrullus lanatus	*Sechium*	*Cucumis melo* L.

plants produce tetracyclic oxygenated triterpenoids called cucurbitacines, with bitter taste and purgative effect.[10,11] In this study, the qualitative analyzes of the chemical compounds showed a positive result for flavonoids, steroids, terpenoids and, tannins; also were detected ketoses and monosaccharides.

Minerals content in Cucurbitaceae fruits

It is known that minerals are essential in human nutrition, some play essential role in bone making others are vital in body maintenance; some of minerals are part of enzyme molecules. The mineral contents of fruit *Cucurbitaceas* species are shown in Table 1. In this study, differences among the species and varieties were observed based on the mineral compositions. The N, PC, ash, Ca and Mg values of fruit species varied from 0.77% (*C. lanatus, v.* sugar baby) to 2.99% (*Sechium edule*); 18.68 % (*Sechium edule*) to 4.25 % (*C. melo* L., v. Reticulatus); 3.55 % (*C. lanatus, v.* Charleston Gray) to 9.02 % (*C. melo* L., v. Reticulatus); 0.023 % (*C. lanatus, v.* sugar baby) to 0.107 % (*Sechium edule*) (*Sechim edule*); and 0.208 % (*Sechim edule*) to 0.37 % (*C. lanatus, v.* Inodorus, yellow type), respectively (Table 1). These variations may be due to the variety, type of the cultivars, soil and geographical condition in Argentina.

Table 1. Mineral elements in fruits

			edule			
	Charleston Gray	Sugar Baby		Inodorus honey dew type	Yellow	Reticulatus Written or reticulated
N %	1.24	0.77	2.99	0.95	1.28	0.68
PC %	7.75	4.81	18.68	5.94	8.00	4.25
Ash %	3.55	5.30	5.44	5.50	7.63	9.02
Ca %	0.059	0.023	0.107	0.078	0.088	0.13
Mg%	0.245	0.258	0.208	0.25	0.37	0.35

PC= dried protein

Total carotenoids, polyphenolic and flavonoid contents in fruits

The carotenoids of 6 *Cucurbitaceas* species and varieties were investigated. The average values of total carotenoids in assessed varieties are recorded in Table 2. *C. lanatus* and *C. melo* L v. *Reticulatus* had the highest carotenoids content in fruit extracts (23.15 to 31.12 µg de β-carotene equivalent/100g FL). *C. melo* L. cultivars honey dew and Yellow type had the lowest total carotenoids content (8.43 to 9.29 µg de β-carotene equivalent/100g FL). From these results it is observed that there is a significant difference in the ability of cucurbitaceas to synthesize carotenoids in relation to the variety.[18, 19] The total flavonoids content in all six samples were high, but significant variations were observed between the samples ($P < 0.05$). Higher values were verified to variety *Reticulattus*, presenting 700 mg/100 g FL, followed by 694 mg/100 g FL for the v. Yellow type and 452 mg/100 g FL for the v. Charleston Gray. Finally the *Sechium edule* contains 88 mg/100 g FL and the v. Baby sugar presents 41mg/100 g FL (Table 2). As shown in Table 2, the total polyphenolic (TP) content differed between cucurbit samples. *Citrullus lanatus***,** Cultivar *Yellow type* and *Reticulatus* had the highest polyphenols content in ethanol extracts (1930–1630 mg/100 g FL); *Sechium edule* and Cultivar *honey dew* had the lowest total polyphenols content (694-200 mg TP/100 g FL). TP content of some *Cucurbits* species, ranged from 897 to 136.96 mg gallic acid equivalent (GAE)/g FW according to the literature values.[26] Therefore, our values are related to those obtained by other researchers with the fresh fruit.

Table 2. Total Carotenoids, total Flavonoids and total Polyphenolics content of fruit samples

Samples	**COMPOUNDS**		
	Total Carotenoids	**Total Flavonoids**	**Total Polyphenols**
Citrullus lanatus			
V.Charleston Gray	31.12 ± 1.44	452 ± 1,80	1,808 ± 11.11
V. Sugar Baby	23.15 ± 1.26	41 ± 2.70	1,930 ± 8.50
Sechium edule	ND	88 ± 3.18	200 ± 7.30
***Cucumis melo* L.**			
V. Inodorus			
Cultivar honey dew	9.29 ± 0.76	136 ± 4.14	694 ± 3.34
Cultivar Yellow type	8.43 ± 1.43	694 ± 3.02	1,630 ± 8.54
V. Reticulatus			
Written or reticulate	24.52 ± 1.54	700 ± 3.31	1,790 ± 7.30

Table 2. the data are expressed in mg or µg of lyophilized fruit.

Antioxidant assay

Table 3 shows radical scavenging activity of the *Cucurbits* extracts using DPPH radical. Compared to the reference antioxidant (Trolox), extracts possess similar or lower radical scavenging activity in the DPPH test.[27] Based on the achieved results, it can be seen a dose-dependent response for all samples studied. *Citrullus lanatus* gave the highest DPPH value of inhibition, 68 % at concentration of 10 mg/mL, among the samples in the same amount Trolox. Extracts of *Sechium edule* and honey dew showed nearly identical levels of radical scavenging activity to yellow type extract; nevertheless, total amounts of polyphenols in *Sechium edule* or honey dew were nearly half those of Yellow type, implying that antioxidant activity is not necessarily parallel with the amount of polyphenols.

Table 3. DPPH radical scavenging activity of fruit extracts.

Samples	**% Antioxidant capacity**		
	100 µg/mL	500µg/mL	1000 µg/mL
Citrullus lanatus			
V.Charleston Gray	10.31 ± 0.44	24.47 ± 1.30	68.24 ± 1.27
V. Sugar Baby	9.32 ± 0.21	29.09 ± 2.97	67.18 ± 1.30
Sechium edule	34.23 ± 0.76	58.24 ± 0.92	59.52 ± 0.71
***Cucumis melo* L.**			
V. Inodorus			
Cultivar honey dew	10.02 ± 0.98	26.22 ± 1.23	61.50 ± 1.02
Cultivar Yellow type	11.40 ± 0.54	28.25 ± 0,72	58.54 ± 0.87
V. Reticulatus			
Written or reticulate	10.37 ± 0.67	37.88 ± 0.93	58.41 ± 1.21

Table 3. Values are mean of three replicate determinations (n= 3) ± standard deviation.

Anti- β glucosidase Activity of fruit Extracts

Several studies have shown that many foods and herbs have potential beneficial effects on metabolic disease control by inhibiting the β glucosidase and Lipasa activity. In this study, six methanolic extracts were prepared from commercial species of cucurbitaceas found in Argentina and their anti-glucosidase and anti-lipase activity was investigated. The results of the present study revealed that all fruit extracts tested decreased β glucosidase activity in two, *in vitro* assay. As shown in Table 4, the inhibition percentage ranges from 28.23 % to 36.37 %. Among those studied, four of the extracts showed a relatively high anti- β glucosidase activity of more than 30%.

Anti- Lipase Activity of fruit Extracts

The inhibitory activities towards pancreatic lipase are also reported in Table 4. Among the six fruit extracts examined, all extracts, significantly inhibited PPL *in vitro* (in both test). Among those examined, all of the extracts showed a relatively similar anti-lipase activity of more than 20%. The significant inhibition of PPL was observed up to 26.67% with *Citrullus lanatus*, 28.38% with *Cucumis melo* L., 24.40 % with *Sechium edule*, respectively. Orlistat, a well-known anti-lipase agent, significantly inhibited the PPL activity up to 44%.

Table 4. Percent inhibition of digestive enzymes by fruit extracts.

Samples	% Inhibition	
	β Glucosidase	Lipase
Citrullus lanatus		
V.Charleston Gray	32.61 ± 0.48	26.67 ± 0.78
V. Sugar Baby	30.32 ± 0.26	24.09 ± 1.27
Sechium edule	28.23 ± 0.66	24.40 ± 1.02
***Cucumis melo* L.**		
V. Inodorus		
C. honey dew	30.05 ± 1.11	23.25 ± 1.43
C. Yellow type	33.50 ± 0.84	26.65 ± 1,12
V. Reticulatus		
Written or reticulate	36.37 ± 0.97	28.38 ± 1.03

Table 4. Data are average of triplicates ± standard deviation.

Several investigators suggest that bitter melon can reduce body weight in high fat diet induced obesity in animals.[23] In addition, isolated compounds from *M. charantia* like charantin, insulin like peptide, and alkaloid-like extracts possess hypoglycemic properties similar to its crude extracts.[2-8, 10] Therefore we suggest that these results could be attributed to the chemical profile of the polyphenolic compounds present in the fruit extracts. According to our knowledge, this is the first study on inhibitory activity of *Cucurbitaceas* (melon, water melon and xuxu) against β glucosidase and pancreatic lipase. In conclusion, all fruits are rich in total phenolic compounds, carotenoids and minerals also showed a good antioxidant capacity. Furthermore, the fruit extracts was able to inhibit key enzymes relevant to the digestion of triacylglycerols and to cleave the glycosidic bond, (lipase and β glucosidasa) *in vitro*. The observed effects are likely induced by more than one bioactive compound present in cucurbits extracts. Further studies with animals are needed to evaluate the potential beneficial effects of the extracts, *in vivo*.

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