Exploring antioxidant and α-glucosidase inhibition in *Eugenia* L. extracts: A comprehensive phytochemical study

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ABSTRACT

This study analyzed extracts obtained from the leaves of *Eugenia uniflora*, *E. involucrata*, and *E. myrcianthes* to determine their chemical composition, antioxidative properties, and α-glucosidase inhibitory capacity. By using liquid chromatography with a diode array detector, we identified chlorogenic acids, flavonoids, tannins, proanthocyanidins, saponins, and triterpenes in the extracts. The antioxidant activities of the extracts were found to be directly related to their total phenolic, flavonoid content and enzyme inhibition. The *E. uniflora* aqueous extract showed significant inhibition of α-glucosidase (IC_{50} 0.98 μg mL^{-1}), indicating its potential as a non-competitive inhibitor for managing Diabetes Mellitus. This study contributes to the existing knowledge on the chemical and biological aspects of *Eugenia* genus.

Keywords: *E. uniflora*; *E. involucrata; E. myrcianthes;* Myrtaceae; Pitanga.

3. Experimental

3.1. Extract processing

Leaves of *E. involucrata* (cherry tree), *E. uniflora* (pitangueira) from the red genotype, and *E. myrcianthes* (peach tree) were collected at the Zoobotanical Garden in the city of Toledo, Paraná, Brazil (24°43'31.8"S 53°44'42.3"W). The plant specimens were identified and registered by the herbarium of the State University of Western Paraná as UNOP 10444, 10445, and 10464.

The leaves were dehydrated naturally at room temperature, ground, and classified using a series of Tyler sieves. The samples were obtained from particles with an average diameter of 0.421 mm through a traditional extraction method using an oil and fat extractor known as Goldfish. Considering that the polarity of the solvent affects the extraction process, and that ethanol enhances the extraction of phenolic compounds (Oliveira et al. 2018; Garmus et al. 2014), ethanol (Neon P.A.) was used at a mass:solvent ratio of 1:10 (w/v). The extraction was conducted at 100 ºC for 90 minutes with continuous heating. Then, a small amount of solvent was added at 130 ºC for 60 minutes,

followed by solvent recovery for 30 minutes. The extracts were evaporated at 60 ºC (using IKA® RV10 control) until the residual solvent was completely removed.

Due to the promising results obtained from in vitro assays of *E. uniflora* (see Results and Discussion section), an aqueous extract was prepared and evaluated. The extraction was performed in a 1:10 ratio (drug:solvent) using turbo extraction in four five-minute cycles. The extraction solvent used was acetone:water (7:3, v/v). The extract was then filtered and concentrated by rotary evaporation at 40 °C and 150 rpm using Laborota 4000 (Heidolph). The concentrate was subjected to freeze-drying at -64 °C and 0.006 mBar using ALPHA 1-2 LDplus (Fisher Scientific, France) and stored at 4 °C.

3.2. Phytochemical screening

3.2.1. LC-DAD-MS analysis

The samples were analyzed using a liquid chromatograph (Shimadzu, Kyoto, Japan) connected to a diode array detector and a high-resolution mass spectrophotometer (LC-DAD-MS) equipped with an electrospray ionization source (Bruker Daltonics, Billerica, MA, USA). A Kinetex C18 chromatography column (150 mm x 2.2 mm, 100 A; 2.6 µm, Phenomenex) was used. The elution profile followed a similar pattern as described by Tolouei et al. (2019). Mobile phases consisted of acetonitrile and ultrapure water with 0.1% formic acid. The flow rate was set at 0.3 µL min^{-1} and the column temperature was maintained at 50 °C. In the electrospray ionization source, nitrogen gas was utilized, with a nebulizer pressure at 4 Bar and drying gas flow rate of 9 L min^{-1}. Both positive and negative ion modes were employed. The samples were dissolved in a mixture of methanol and water (1:1, v/v) at a concentration of 1 mg mL^{-1}, filtered through a Millex 0.22 µm PTFE membrane (Millipore), and then injected (8 µL) into the chromatographic system using an autoinjector. Compound annotation was based on comparison of spectral data with those previously reported in the literature, including UV, accurate mass spectra data, and fragmentation profiles.

3.2.2. Quantification of Total Phenolic Compounds (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method, as described by Singleton and Rossi (1965). The extracts were prepared at a

concentration of 1000 µg mL^{-1} and were evaluated in three separate trials. The absorbance was measured at a wavelength of 765 nm using a spectrophotometer called Kasuaki, Model IL-592. The quantification was based on a standard curve of gallic acid. All measurements were conducted three times, and the TPC was expressed as µg gallic acid equivalent per gram of extract ($\mu g_{EAG}\ g_{ext}^{-1}$).

3.2.3. Quantification of Total Flavonoids Content (TFC)

The spectrophotometric quantification was conducted using the principle of complexing the flavonoid nucleus with a 5% (w/v) solution of aluminum chloride. This methodology was previously described by Woisky and Salatino (1998). The quantification process involved using a calibration curve for quercetin, which was measured at a wavelength of 425 nm using a spectrophotometer. The extracts were evaluated in triplicate at a concentration of 1000 µg mL^{-1}, and the results were expressed as the equivalent amount of quercetin in micrograms per gram of extract ($\mu g_{QUE}\ g_{ext}^{-1}$).

3.3. Antioxidant capacity

The extracts were dissolved at a concentration of 1000 µg mL^{-1} and evaluated to determine their ability to scavenge DPPH (2,2-Diphenyl–1-picrylhydrazyl) and $ABTS^{\bullet+}$ (2,2-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid)) radicals, as well as their ability to reduce the FRAP complex (Iron (III)/tripyridyltriazine), in independent triplicates.

Calibration curves were created using the Trolox standard (Sigma-Aldrich, St. Louis, MO, USA) to quantify the antioxidant activity using the DPPH and $ABTS^{\bullet+}$ methods. For quantification using the FRAP method, ferrous sulfate was used, following the methodology described by Silveira et al. (2018), Re et al. (1999), and Santos et al. (2016) with modifications (Dalmagro et al. 2023). A positive control, quercetin (purity ≥ 95%; Sigma-Aldrich, St. Louis, MO, USA), was used in all antioxidant activity assays at a concentration of 1000 µg mL^{-1}.

3.4. α-glicosidase inhibitory capacity

The activity of α-glucosidase inhibition was determined in 96-well microplates. The extract was dissolved in 100 µL of 0.1 M sodium phosphate buffer (pH 7.5, 0.02%

NaN_3) containing 10% DMSO. The IC_{50} values of the extracts were evaluated using a series of dilutions, starting with a concentration of 5 mg mL^{-1}. 80 µL of α-glucosidase solution (type I, from Saccharomyces cerevisiae) in phosphate buffer (2.0 U mL^{-1}) were added, followed by incubation at 28 ºC for 10 minutes. Then, 20 µL of p-nitrophenyl α-D-glucopyranoside substrate solution (pNPG 10 mM in phosphate buffer) was added (Schmidt et al. 2012). The inhibition of the enzyme was determined by measuring the absorbance of the p-nitrophenol cleavage product at 405 nm for 35 minutes using a Multiskan FC microplate photometer controlled by the SkanIt ver. 2.5.1 software (Thermo Scientific, Waltham, MA, USA).

3.4.1 Type of inhibition of α-glucosidase

The extracts were used to determine the inhibition of α-glucosidase. The Lineweaver-Burk plot was used to analyze the effects of varying concentrations of the reaction substrate (pNPG). This approach was described in a study by Lineweaver and Burk (1934), as well as a more recent study by Şöhretoğlu et al. (2018). To evaluate the inhibition, ten different concentrations of the reaction substrate were tested: 0.20, 0.22, 0.25, 0.29, 0.33, 0.40, 0.50, 0.67, 1.00, 2.00, and 4.00 mM. The same procedure as before was followed. Additionally, other kinetic parameters such as the inhibition constant (K_m) and V_{max} were calculated. The y-intercept of the Lineweaver-Burk graph corresponds to the inverse of V_{max}, while the x-axis intersection represents $-1/K_m$. To determine the inhibition type of the tested extract (competitive, noncompetitive, or mixed type), a comparison of K_m and V_{max} was made in the presence and absence of the inhibitor.

3.5. Statistical analysis

The results were analyzed using analysis of variance (ANOVA). The means were compared using Tukey's test ($p < 0.05$) through the software Statistica 13.0 (Statsoft®, USA).

Table S1. Compounds annotated from the samples by LC-DAD-MS.

Peak	RT (min)	Compound	UV (nm)	MF	Positive (*m/z*)		Negative (*m/z*)		A	B	C	D
					MS	MS/MS	MS	MS/MS				
1	1.3	Quinic acid	-	$C_7H_{12}O_6$	193.0709	-	191.0563	-	X	X	X	
2	1.3	Di-*O*-hexoside	-	$C_{12}H_{22}O_{11}$	-	-	341.1099	191	X	X	X	X
3	1.9	*N*-acetyl leucine	-	$C_8H_{15}NO_3$	174.1119	130	-	-		X		
4	2.7	Gallic acid[st]	271	$C_7H_6O_5$	-	-	169.0145	-	X	X		X
5	15	5-*O*-*E*-caffeoylquinic acid[st]	299, 324	$C_{16}H_{18}O_9$	355.1010	163	353.0887	191, 179, 161			X	
6	17.4	Coumarylquinic acid	283	$C_{16}H_{18}O_8$			337.0933	191, 163		X		
7	17.4	Catechin[st]	279	$C_{15}H_{14}O_6$	291.0868	189, 161, 147	289.0728	221, 188, 159			X	
8	17.6	Coumarylquinic acid derivative	299	$C_{16}H_{18}O_8$	339.1062	220, 165, 147	337.0940	191, 163		X		
9	17.9	Macrocyclic dimeric ellagitannin	265	$C_{61}H_{46}O_{40}$			708.0697 $^{2+}$	765, 633, 613, 597, 450, 427, 301, 275, 273, 169	X	X		
10	18.1	*O*-galloyl PDE-PDE (B-type)	272	$C_{37}H_{12}O_6$	763.1503	425, 407, 299, 287, 179	761.1347	423, 305, 243, 177				X
11	18.3	Chlorogenic acid	288, 325	$C_{16}H_{18}O_9$			353.0883	252, 191, 179, 163, 161	X	X	X	
12	18.8	Actinidioionoside	263	$C_{19}H_{34}O_9$			405.2113	357, 315, 308, 293, 174, 153	X	X	X	

13	19	PFT-PDE (B-type)	276	$C_{30}H_{26}O_{11}$	563.1552	409, 287, 273, 255, 231, 189, 167	561.1423	407, 289, 245, 203			X	
14	19.3	Hydroxy-methoxyphenyl *O*-galloyl-hexoside	263	$C_{20}H_{22}O_{12}$			453.1036	313, 297, 169	X	X		X
15	19.6	Epicatechin[st]	278	$C_{15}H_{14}O_6$	291.0867	207, 189, 179, 161, 147	289.0728	221, 203, 187, 177, 173, 161,			X	
16	19.9	Dihydrochalcon-hexoside	277	$C_{21}H_{24}O_{10}$	437.1429	359, 275, 191, 173, 139	435.1313	342, 273, 171, 121			X	
17	20.1	PFT-PDE (B-type)	277	$C_{30}H_{26}O_{11}$	563.1548	409, 287, 257, 231	561.1417	407, 289, 245, 203			X	
18	20.2	Coumaroylquinic acid	264	$C_{16}H_{18}O_8$			337.0942	191, 173, 163	X	X		
19	21.1	PFT-PDE (B-type)	278	$C_{30}H_{26}O_{11}$	563.1560	409, 287, 257, 231, 189, 179, 167,147	561.1410	407, 289, 245, 203, 179, 164, 151		X		
20	21.4	Afzelechin-afzelechin (B-type)	274	$C_{30}H_{26}O_{10}$	547.1607	405, 393, 287, 275, 255, 227, 189, 167, 149	545.1469	273, 255, 229, 205, 164			X	
21	21.6	Afzelechin-afzelechin (B-type)	275	$C_{30}H_{26}O_{10}$	547.1602	393, 287, 275, 255, 189, 167	545.1471	273, 205, 164			X	
22	21.7	Myricetin *O*-galloyl-hexoside	265, 363	$C_{28}H_{24}O_{17}$	633.1057	319, 153	631.0946	479, 316, 299, 271, 169		X		
23	22	Methoxybenzofuranpropanoic acid *O*-hexoside	264,340	$C_{18}H_{22}O_{10}$	399.1297	237, 191	397.1144	235, 179, 159			X	

24	22.2	Myricetin *O*-hexoside	263, 358	$C_{21}H_{20}O_{13}$	481.0955	319	479.0828	316, 287, 271, 179	X	X		X
25	22.4	Myricetin *O*-hexoside derivative	265, 361	$C_{21}H_{20}O_{13}$	481.0965	319	479.0841	316, 287, 271, 179	X			X
26	22.8	Dihydroxy-isopropylchromone-hexoside	277	$C_{18}H_{22}O_9$	383.1339	365, 347, 287, 263, 247, 233	381.1190	261, 233, 218, 189, 161		X		X
27	22.9	Myricetin *O*-pentoside	266, 363	$C_{20}H_{18}O_{12}$	451.0859	319, 273, 165, 153	449.0737	316, 287, 271, 259, 242, 214, 179	X	X		X
28	23.2	Catechin *O*-gallate	275	$C_{22}H_{18}O_{10}$	443.0984	395, 352, 329, 278, 271, 207, 179, 153	441.0828	289, 221, 205, 183, 169				X
29	23.4	Tetrahydroxy-dimethoxy flavone	272, 351	$C_{17}H_{14}O_8$	347.0769	331, 314, 286, 268, 258,	345.0626	330, 315, 299, 287, 271, 259, 243, 231, 215				X
30	23.7	Di-*O*-galloyl-hexosyl ellagic acid	255, 361	$C_{34}H_{24}O_{20}$			751.0814	449, 301				X
31	24	Myricetin *O*-deoxyhexoside	260, 297, 247	$C_{21}H_{20}O_{12}$	465.1037	319, 273, 153	463.0886	316, 287, 271, 259, 214, 178, 163, 151	X	X		X
32	24.3	Quercetin *O*-hexoside-O-deoxyhexoside	266, 294, 350	$C_{27}H_{30}O_{16}$	611.1626	303	609.1479	300, 271, 255, 178, 151			X	
33	24.8	Methoxybenzofuranpropanoic acid *O*-hexoside derivative	265, 303, 350	$C_{18}H_{22}O_{10}$	399.1288	237, 220, 191	397.1149	277, 247, 235, 219, 205, 189			X	

34	26.7	Quercetin *O*-deoxyhexoside	265, 349	$C_{21}H_{20}O_{11}$	449.1099	303, 283, 257, 229, 201, 155	447.0940	300, 271, 255, 243, 178	X	X	X	X
35	27	Unknow	264, 285, 344	$C_{20}H_{24}O_{11}$	441.1380	237	439.1260	365, 347, 235, 217			X	
36	27.1	Trihydroxy-dimethoxyflavone	288, 345	$C_{17}H_{14}O_7$	331.0811	315, 298, 270, 242	329.0666	299, 271, 243, 199				X
37	27.7	Galloyl-hexosyl-monoterpene	276	$C_{23}H_{32}O_{11}$	485.2013	350, 297, 249, 219, 171, 153	483.1867	313, 169		X		
38	27.7	Trihydroxy-trimethoxyflavone	265, 292, 348	$C_{18}H_{16}O_8$	361.0904	345, 331, 317, 300, 285, 257	359.0778	329, 301, 286, 258, 242, 230, 214				X
39	28.1	Hydroxy-hydroxymethyl-methyl-methoxychromone	260, 300, 348	$C_{12}H_{12}O_5$	237.0764	220, 207, 191, 181, 163	235.0619	218, 205, 189, 177, 161			X	
40	28.4	Myricetin *O*-galloyl-deoxyhexoside	265, 351	$C_{28}H_{24}O_{16}$	617.1132	319, 299, 153	615.0987	463, 317, 271, 178, 169	X	X		
41	28.6	Myricetin *O*-galloyl-deoxyhexoside derivative	263, 349	$C_{28}H_{24}O_{16}$	617.1145	299	615.0989	317, 178	X	X		X
42	29	Kaempferol *O*-deoxyhexoside	264, 345	$C_{21}H_{20}O_{10}$	433.1115	287	431.0994	284, 255, 227		X		
43	29.6	Galloyl-hexosyl monoterpene	275	$C_{23}H_{32}O_{11}$			483.1884	313, 271, 211, 169, 151		X		
44	30.1	Quercetin *O*-galloyl-deoxyhexoside	279, 358	$C_{28}H_{24}O_{15}$	601.1163	299	599.1037	563, 515, 301, 178, 151	X	X		
45	32.6	Steroidal saponin		$C_{39}H_{64}O_{15}$			771.4196	563			X	

46	32.7	Triterpene saponin		$C_{37}H_{60}O_{12}$			695.4005	487, 313	X	X		
47	33.1	Sesquiterpene lactone	266	$C_{15}H_{20}O_3$	249.1488	229, 213, 203, 189, 171, 161, 153	247.1344	229, 203, 187	X			
48	33.6	Pentacyclic triterpene		$C_{30}H_{48}O_6$	505.3533	495, 333, 191	503.3392	485, 453, 421, 409, 309			X	
49	34.5	Pentacyclic triterpene		$C_{30}H_{48}O_6$	505.3542	471, 440, 433, 423, 405, 213, 201, 189, 187, 173, 159	503.3374	409, 390, 307, 298, 162	X		X	X
50	35.2	Sesquiterpene lactone		$C_{15}H_{20}O_3$	249.1494	201, 189, 175, 163, 155, 147	247.1347	203, 201, 187, 177, 163, 155, 149, 133		X		
51	35.9	Pentacyclic triterpene		$C_{30}H_{48}O_5$	489.3582	407, 201, 173, 159	487.3429	409	X	X	X	X
52	36.8	Sesquiterpene lactone		$C_{15}H_{18}O_2$	231.1382	185, 170, 165, 158, 155, 149			X	X		
53	37	Sesquiterpene lactone		$C_{15}H_{18}O_2$	233.1538	215, 187, 185, 171, 157				X		
54	37.1	Sesquiterpene lactone		$C_{15}H_{18}O_2$	233.1532	215, 173, 159				X		
55	37.5	Sesquiterpene lactone		$C_{15}H_{18}O_2$	233.1538	216, 182, 175, 146				X		

56	38.6	Coumaroyl-dihydroxy triterpene acid	291	$C_{39}H_{54}O_7$	635.3944	528, 435, 407, 261, 201, 187, 173, 147	633.3793	589, 513, 467, 419, 401, 163, 145	X	X		X
57	38.8	Coumaroyl-dihydroxy triterpene acid	291, 315	$C_{39}H_{54}O_7$	635.3944	435, 407, 261, 247, 215, 201, 187	633.3794	589, 513, 469, 163, 145	X	X		X
58	38.8	Ferulyl-pentacyclic triterpene	291	$C_{40}H_{56}O_8$	665.4034	591, 435, 283, 177				X		X
59	39.2	Coumaroyl-dihydroxy triterpene acid	295, 310	$C_{39}H_{54}O_7$	635.3933	535, 516, 435, 409, 363, 327, 299, 201, 189, 147	633.3806	615, 589, 573, 513, 469, 163, 145			X	

RT: retention time; MF: molecular formula; st: confirmed by injection of authentic standard; PDE: prodelphinidin unit; PFT: profisetidin unit; A: *E. uniflora* aqueous extract; B: *E. uniflora* ethanolic extract; C: *E. involucrate*; D: *E. myrcianthes*.

Table S2. Quantification of total phenolics (TPC), total flavonoids (TFC) and antioxidative capacity from *Eugenia* L. extracts.

Extracts	TPC	TFC	Antioxidative capacity		
			DPPH	FRAP	$ABTS^{\bullet+}$
	($\mu g_{EAG}\ g_{ext}^{-1}$)	($\mu g_{QUE}\ g_{ext}^{-1}$)	(μM_{Trolox})	($\mu mol_{Fe}^{2+}\ g_{ext}^{-1}$)	($\mu mol_{Trolox}\ g_{ex}^{-1}$)
E. uniflora (aqueous)	168.98 ± 0.04^{a}	8.31 ± 0.31^{a}	1069.07 ± 3.61^{b}	4714.77 ± 0.93^{a}	6938.21 ± 77.45^{a}
E. uniflora (ethanolic)	94.46 ± 1.04^{b}	6.79 ± 0.43^{b}	-	3304.51 ± 69.54^{c}	2262.03 ± 40.16^{b}
E. involucrata	46.11 ± 0.75^{c}	5.06 ± 0.09^{c}	-	1272.62 ± 22.70^{d}	1445.26 ± 102.74^{b}
E. myrcianthes	102.87 ± 1.80^{b}	8.83 ± 0.08^{a}	1052.32 ± 3.61^{c}	4293.52 ± 43.09^{b}	6132.94 ± 429.07^{a}
Quercetin			1085.82 ± 6.00^{a}	3378.85 ± 2.79^{c}	3044.98 ± 0.01^{b}

Mean ± standard deviation (n=3). (−) sign indicates no capacity. Note: different letters, in the same column, represent significant differences ($p<0.05$) by Tukey's Test.

Table S3. Mean IC_{50} values ± standard deviation of the inhibitory activity on α-glucosidase from *Eugenia* L. extracts.

Extract	IC_{50} (μg mL^{-1})
E. uniflora (aqueous)	0.98 ± 0.02^{d}
E. uniflora (ethanolic)	1.44 ± 0.08^{c}
E. involucrata	43.16 ± 2.05^{a}
E. myrcianthes	4.87 ± 0.92^{b}

Mean ± standard deviation (n=3). Note: different letters, in the same column, represent significant differences ($p<0.05$) by Tukey's Test.

Table S4. V_{max} and K_m values of the inhibitory activity on α-glucosidase from *Eugenia* L. extracts

Sample	V_{max} (ΔOD min^{-1})	K_m (mmol L^{-1})
Control	0.077	7.382
E. uniflora (aqueous)	0.054	7.397
E. uniflora (ethanolic)	0.076	11.187
E. involucrata	0.077	12.242
E. myrcianthes	0.076	44.747

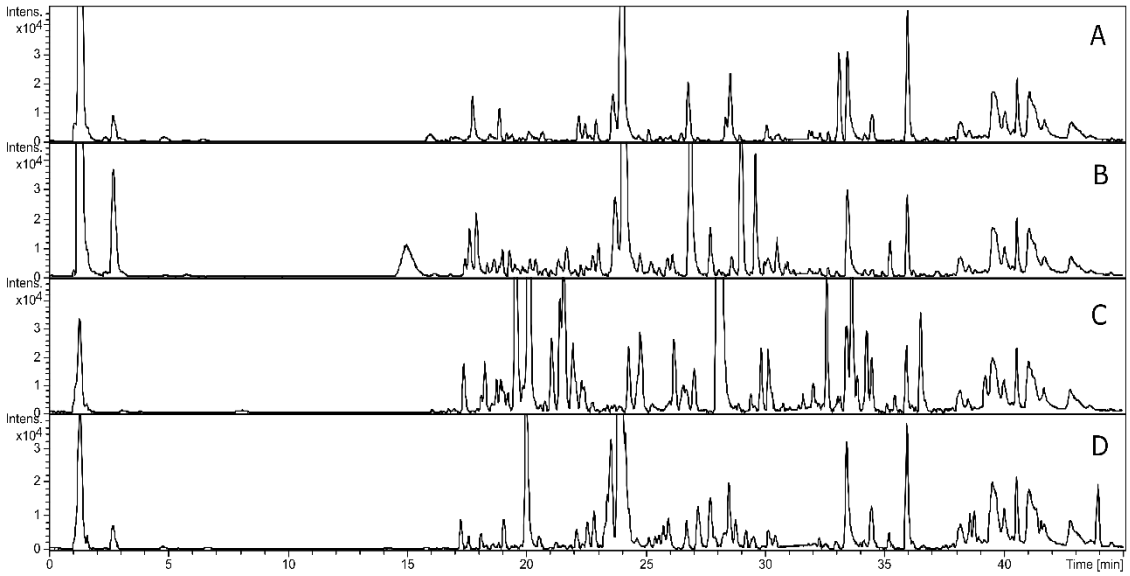

Figure S1. LC-DAD-MS of aqueous extract (A) and ethanolic extract (B) from *E. uniflora*, *E. involucrata* (C) and *E. myrcianthes* (D).

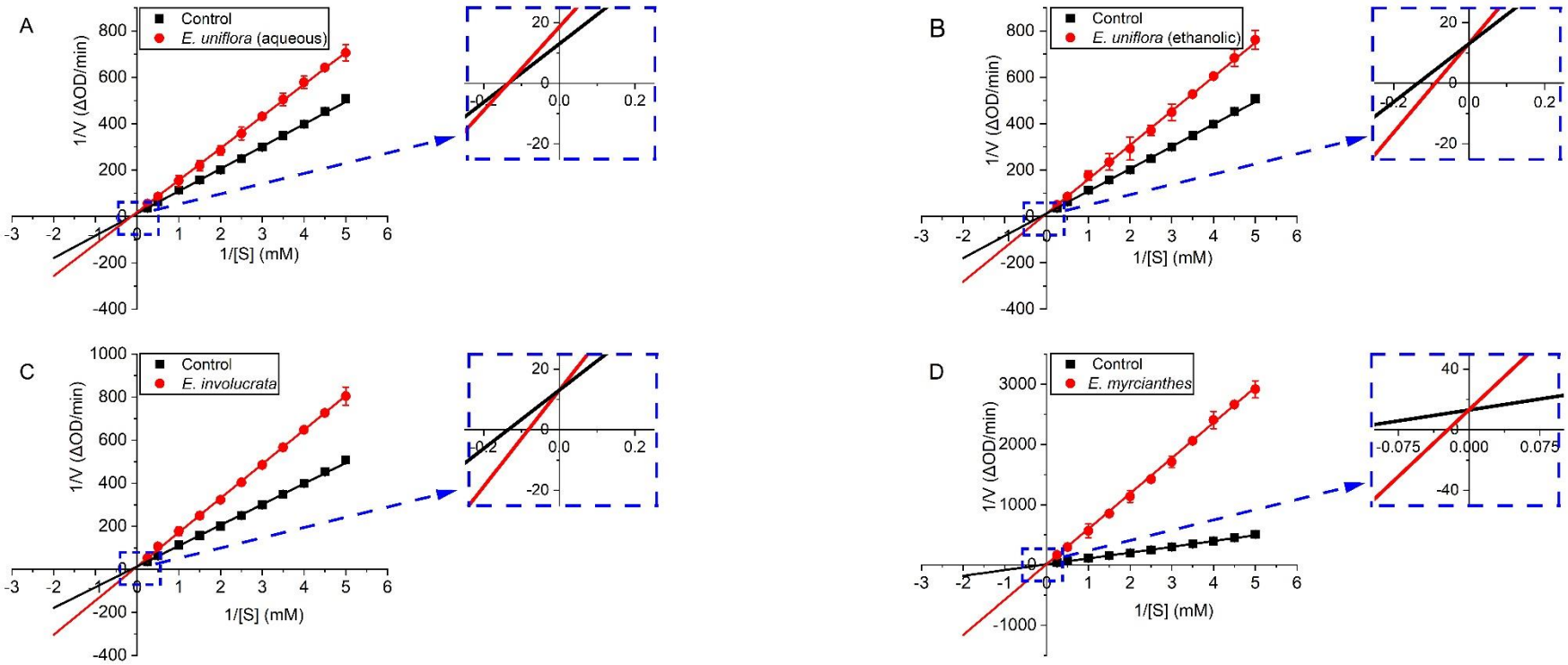

Figure S2. Lineweaver-Burk plots for the inhibition of α-glucosidase by the extracts tested at the IC_{50} concentrations. A: *E. uniflora* – aqueous (0.98 ± 0.02 µg mL^{-1}); B: *E. uniflora* – ethanolic (1.44 ± 0.08 µg mL^{-1}); C: *E. involucatra* (43.16 ± 2.05 µg mL^{-1}) and D: *E. myrcianthes* (4.87 ± 0.92 µg mL^{-1}). Control plots represents the concentration of 0 µg mL^{-1} of extract.

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