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Aqueous Extracts from Nopal (*Opuntia Ficus-Indica*): Antiacetylcholinesterase and Antioxidant Activity from Phenolic Bioactive Compounds

Asma Ressaissi[1,2], Nebil Attia[2], Pedro L.V Falé [1,3], Rita Pacheco[1,4], Vitor H. Teixeira[1], Miguel Machuqueiro[1], Carlos Borges[1,3], Maria Luísa M. Serralheiro[*1,3]

[1]Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal

[2]UR11ES33'Integrated Physiology', Faculty of Sciences of Bizerte, Carthage University, Tunisia.

[3]Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[4] Área Departamental de Engenharia Química. Instituto Superior de Engenharia de Lisboa. Av. Conselheiro Emídio Navarro, 1959-007 Lisboa, Portugal

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Abstract: Cladodes of the cactus plant *Opuntia ficus-indica* (L.) Mill, known as nopal, are used in ethnic gastronomy, such as Mexican cuisine, and for medicinal purposes. This study aimed at obtaining a method to extract bioactive phenolic compounds from nopal. Five nopal aqueous extracts were prepared using different methodologies and their composition was determined by HPLC-DAD and LC-MS/MS. The anti-acetylcholinesterase (AChE) and antioxidant activities were assayed as models of biological effects. The main components identified were piscidic acid, eucomic acid and isorhamnetin derivatives. The highest biological activities were found for aqueous extracts after precipitating the ethanol-insoluble material. The best IC_{50} values obtained were 780±20 µg/ml and 330±40 µg/ml for AChE and DPPH, respectively. *In silico* molecular docking studies indicated that both piscidic acid and isorhamnetin glycosides are able to interact with the AChE active site, but with significantly different binding constants. These results indicate that nopal is a promising plant for the development of polyphenol-based new functional foods.

Keywords: *Opuntia ficus-indica*, nopal, antioxidant activity, acetylcholinesterase inhibition, piscidic acid, docking studies.

INTRODUCTION

Opuntia ficus-indica, a member of *Cactaceae* family best known as "prickly pear", grows wild in arid and semi-arid regions of the globe[1] and is used for human consumption and feedstock forage[2]. Its cladodes (stem pads) are usually known as nopal and have been popularized as a common ingredient in many Mexican cuisine dishes. The cladodes are also used in traditional medicine[3]. Several scientific studies have shown the beneficial effects of nopal on several *in vitro* and animal models[4].

Cladodes contain sugar polymers and fibers as main constituents[3] that are considered as fat excretion agents[5]. The literature refers several methods to extract and purify the phenolic fraction from different food material, microwave assisted extraction[6], extraction-adsorption[7] solvent extraction methods[8], membrane filtration methods[9], two-phase systems based in ionic liquids[10] or aqueous-two phase systems (ATPS)[11] and even the use of enzymes to release the phenolic fractions from mucilage[12] can be mentioned.

Several antioxidant flavonoids were also detected in methanol extract of cladodes[13] and several phenolic acids were observed in methanol extracts of the closely related *Opuntia macrorhiza* (Engelm.) and *Opuntia microdasys* (Lehm.)[14] and also in ethanol extract of cladodes[13]. However, despite its proven biological activities, little is known about the polyphenolic composition of nopal, especially concerning those isolated with aqueous fractions, which are rich in active compounds and their biological activity as acetylcholinesterase inhibitors.

Acetylcholinesterase (E.C.3.1.1.7.) is an enzyme located in the synaptic gaps and neuromuscular junctions[15]. Its inhibition is used clinically to improve the quality of life in Alzheimer Disease (AD) patients[16] and in intestinal disorders[17]. Antioxidant activity in cells is also another important biological activity, as it prevents ROS formation and contributes to the reduction of inflammatory processes[18], which are the basis of several diseases[19]. Antioxidant compounds can be helpful in these situations[18]. The main objective of this work was to develop a method for the extraction and purification of an active polyphenolic fraction from *Opuntia ficus-indica*, using water as the sole extractant. Since polyphenols are generally water soluble, we explored simple and non-expensive aqueous processes like centrifugation, dialysis, filtration and precipitation, and determined their anticholinesterase as well as antioxidant activity.

EXPERIMENTAL

Chemicals: All chemicals were of analytical grade. DTNB (PubChem CID: 6254), acetylthiocholine iodine (PubChem CID: 74629), acetylcholinesterase (AChE) (149 U/mg solid, 241 U/mg protein), Acetylcholine (Ach I) (PubChem CID: 187), tris buffer (PubChem CID: 6503), gallic acid (PubChem CID: 370), quercitin (PubChem CID: 5280343); Folin–Ciocalteu reagent, DPPH (PubChem CID: 2735032) were purchased from Sigma-Aldrich, Barcelona, Spain. Methanol and acetonitrile, both HPLC grade were obtained from Merck (Darmstadt, Germany), VWR International. Folin-Ciocalteu reagent, formic acid, trifluoroacetic acid, $NaNO_2$, $AlCl_3$ were from Merck.

General equipment: Lyophilization was carried out using a Heto PowerDrY LL3300 lyophilizer; purification using dialysis employed 10 kDa cut-off membrane; centrifugation process used a bench centrifuge 5415D; evaporation of ethanol was accomplished using a rotary vacuum evaporator; filtration used Whatman paper no 1.

Plant extracts preparation: Cladodes of *Opuntia ficus-indica* (L.) Mill were harvested from Tunisia in November 2014. Nopal (10 g) was boiled with water (100 mL) for 20 minutes, filtered and lyophilized, referred as **F**; an aliquot of the extract **F** was dialyzed, allowing to obtain the **FD** extract; an aliquot of the lyophilized extract, F, was centrifuged at 5000 x *g* for 5 minutes and the top phase was further used and identified as **FC** extract; to 8 mg of the filtered decoction, F, ethanol (32 mL) was added, stayed 24 h at 8°C, in order to precipitate mucilage and other insoluble compounds in ethanol, the mixture was centrifuged and supernatant was evaporated at 50°C, which was referred as the **FE** extract. Nopal (10 g) was additionally boiled with water (100 mL) during 20 minutes, lyophilized and dialyzed to obtain the final extract, referred as **D**.

High-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) analysis: The HPLC analysis were carried out as described in Falé et al[20]. Piscidic acid was purified from the extract by preparative HPLC, injecting repeatedly a 5 mg/mL extract solution **FE** and recovering the peak at 5.8 min retention time. The gradient for the preparative chromatography was composed of solution A (0.05% trifluoroacetic acid), and solution B (acetonitrile) as follows: 0 min, 5% A, 95% B; 20 min 80% A, 20% B; 25 min, 70% A, 30% B; 30 min, 70%A, 30%A.

The LC-MS and LC-MS/MS analysis were carried out as described in Falé et al[20].

Quantification of phenolic acids: Total polyphenols were determined in the extract using Folin–Ciocalteu method described by Oktay et al.[21]. Total polyphenols were expressed as µg of gallic acid equivalents (GAE)/mg of extract.

Total flavonoids content: To determine the total flavonoid content the method described in Tsai et al.[22] was used. Flavonoids were expressed as µg of quercetin equivalents (QE)/mg of extract.

Acetylcholinesterase inhibition: Acetylcholinesterase enzymatic activity was measured using an adaptation of the method described by Ingkaninan et al[23] and referred in detail in Falé et al[20].

Computational methods: AChE coordinates were obtained from the Protein Data Bank with PDB codes: 1B41 (Homo sapiens)[24]. The missing residues were ignored since they are far from the active site. All protonable residues in AChE were assigned the common protonation state at physiological pH. The molecular interactions between AChE and ligands were calculated using AutoDock suite version 4.2.2[25]. AChE was treated with partial flexibility in some key residues in the active site (Tyr124, Trp286, Tyr337 and Tyr341), since they may play a significant role in the accommodation of the ligands. The torsions for these residues were built around the Cα and Cβ bonds with AutoDock Tools[25,26]. All four ligands were geometrically optimized with Gaussian 09[27] using the B3LYP functional and 6-31G (d, p) basis set[28,29]. Ligand flexibility was accounted by allowing all available torsions to rotate freely. Rotatable bonds were attributed and configured with AutoDock Tools, which resulted in 8 torsions for piscidic acid, 6 for isorhamnetin, and 12 for Isorhamnetin-3-*O*-glucose.The interaction calculations were made on a rectangular prism with 59, 65 and 59 grid points in the x, y and z directions, respectively, with spacing between points of 0.375 Å. This grid was centred on the active site cavity ensuring that the region of interest was fully covered. Several affinity grid maps were calculated to all possible atomic types present in the full set of ligands, where atom types were assigned by Auto Dock tools. The Van der Waals and hydrogen bonding terms were computed using the Auto Dock parameters and electrostatic interactions were evaluated with a distance-dependent dielectric function of Mehler and Solmajer[30]. The search for solutions was performed with the AutoDock Lamarckian Genetic algorithm that combines the global search of the genetic algorithm with the adaptive local search method based on Solis and Wets[31]. The default docking parameters were used with the exception of the number of individuals in the population and the maximum number of energy

evaluations, which were set to 100/8x10^6 for piscidic acid and Isorhamnetin and 500/1.5x10^7 for isorhamnetin-3-*O*-glucose. A total of 2000 LGA runs were made for each pair enzyme–ligand that were reclustered using only the heavy atoms of the ligand and an RMS tolerance of 1.5 Å (2.0 for Isorhamnetin-3-*O*-glucose). The best binding modes were obtained by selecting the lowest energy conformation from the most populated clusters.

Antioxidant activity: Antioxidant activity was measured by the DPPH method, as described by Tepe et al[32] (2005), and referred in detail in Falé et al[20].

Data analysis: The software used was SPSS version 11.5 and the results were expressed as means ± standard deviation. Additional analysis of variance (ANOVA) was performed with P = 0.05.

RESULTS AND DISCUSSION

Composition of cladodes and effect of purification processes on total phenolic and flavonoid content: After removal of the spines, nopal can be consumed in salads, boiled or fried. In the present work, nopal was prepared as a decoction, boiled for 20 minutes. After this process, several purification methodologies were used in order to remove the plant fibres, previously detected in this plant[33] and to obtain extracts with high contents in phenolic compounds.

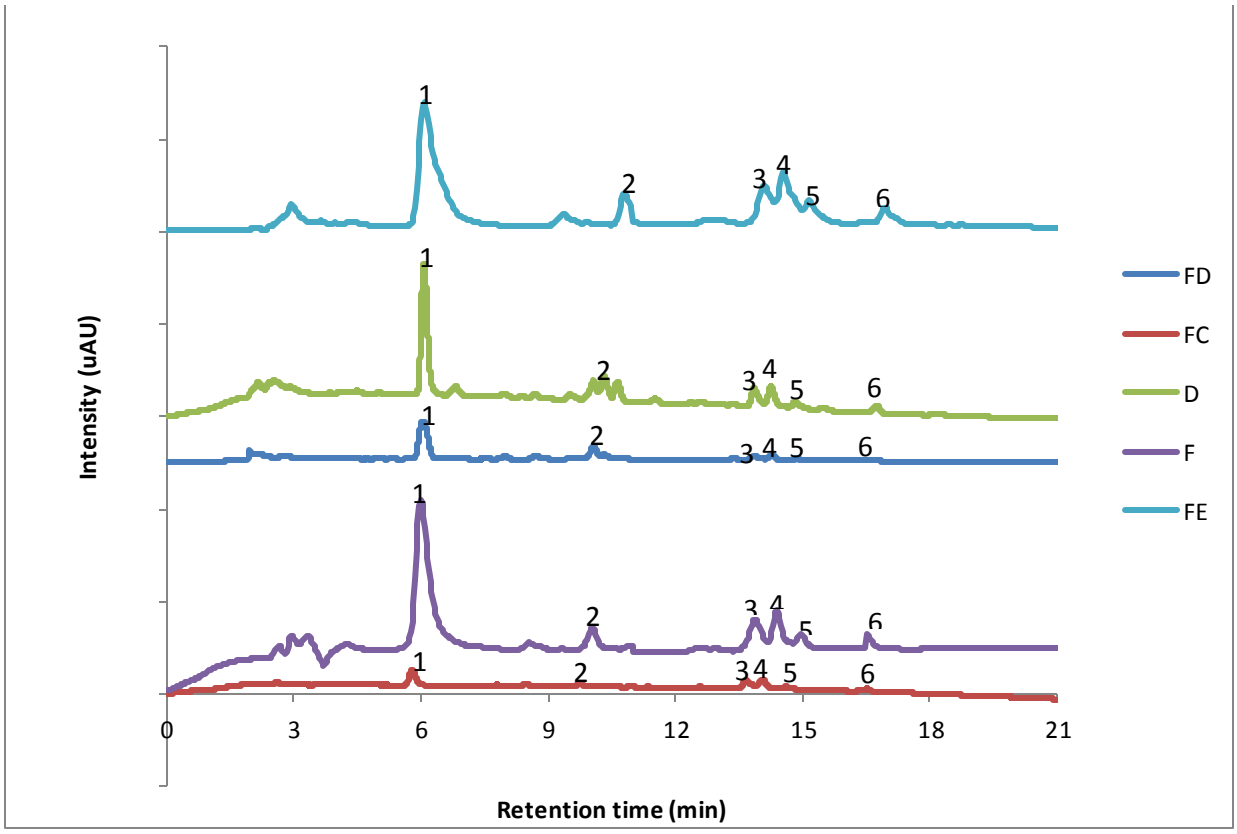


Fig. 1: Chromatograms of aqueous extracts of cladodes of *Opuntia ficus-indica* (L.): (FD) filtration followed by dialysis; (F) filtration; (D) dialysis; (FC) filtration followed by centrifugation; (FE) filtration followed by ethanol extraction.

Table 1: Compounds detected by LC–MS and LC–MS/MS in negative ion mode.

Peak	Compound	$[M-H]^-$ *(m/z)*	Retention time LC	Fragment Ions *m/z* (Rel Ab. %)
1	Piscidic acid	255	5.79	237(4), 209(26), 193(44), 165 (100).
2	Eucomic acid	239	9.73	221(23), 195(11), 193(38), 179(100), 177(67), 149(73), 133(4), 107(10).
3	Isorhamnetin -3-*O*-glucosyl-rhamnosyl-rhamnoside	769	13.66	737(24), 623(16), 605(100), 357(20), 315(87), 313(70), 300(24), 299(33).
4	isorhamnetin-3-*O*-glucosyl-rhamnosyl-pentoside	755	14.03	723(21), 623(17), 605(83), 423(7), 357(11), 315(100), 300(18).
5	Isorahmnetin-3-*O*-glucosyl- pentoside	609	14.61	577(2), 477(5), 459(11), 315(100), 300(39).
6	Isorhamnetin-3-*O*-rutinoside	623	16.49	357(1), 315(100), 300(15).

The composition of the aqueous extracts prepared as decoction and purified by filtration (**F** extract), filtration and dialysis (**FD** extract), dialysis alone without filtration (**D** extract), filtration followed by centrifugation (**FC** extract), and filtration followed by ethanol extraction (**FE** extract), were analysed by HPLC-DAD and LC-MS. In the chromatographic profiles (Fig. 1), we observed that the same compounds were extracted in all processes, however with different quantities. Compound identification was carried out by LC–MS/MS in negative ion mode and shown in Table 1. The extracts of nopal were composed of piscidic acid (**1**), eucomic acid (**2**), and four isorhamnetin glycosylated derivatives (**3-6**). These compounds were also detected in different studies where other solvents were used[34].

In Figure 2, the effect of the purification procedures on the extracts' composition can be seen. The aqueous extraction, after filtration (**F)** contained a higher amount of piscidic acid than the dialyzed (**FD**) or the centrifuged (**FC**). Using dialysis after filtration (**FD**)**,** caused a decrease in the amount of piscidic acid by 30%. Dialysis without filtration (**D**) and ethanol precipitation, after filtration, (**FE**) did not have a significant effect on piscidic acid content.

However, the centrifugation step (**FC**) withdraws most of the phenolic compounds from the extracts, probably partitioning them to the pellet. The flavonoid derivatives (**3-6**) are sensitive to general centrifugation and dialysis and, interestingly, with the ethanol precipitation, the amount of some flavonoid derivatives were higher compared to the simple filtration. To analyse the effect of the different purification processes on the total phenols and total flavonoids content, these compounds were quantified (Fig 3). The concentration of polyphenols in the **FE** and **F** extracts oscillated between 2.07±0.17 µg of GAE/mg of extract and 112.77±0.27 µg of GAE/mg of extract, respectively.

The statistical analyses, at 95% confidence level, showed that the difference between the extracts is significant. Previous studies have reported concentrations of polyphenols in nopal leaves of the same magnitude[34]. Aqueous extracts of *O. ficus-indica* obtained with different extraction methods gave similar values of total phenol amount[13,35]. Figure 3 also shows the content of flavonoids, reported in µg of quercetin equivalents (QE)/mg of extract. The concentration of flavonoids in the extracts varied between 9.04 µg and 81.28 µg QE/mg of extract for **FC** and **FE**, respectively.

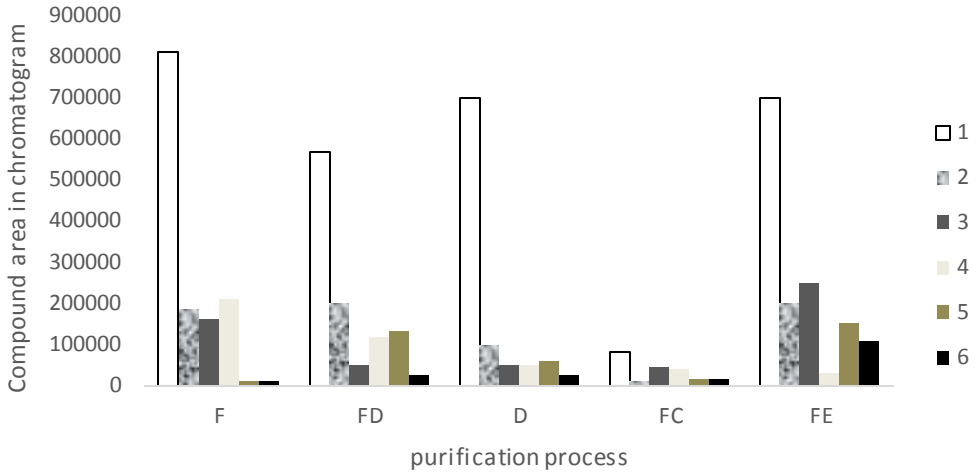


Fig. 2: Effect of the extraction and purification process on the phenolic composition: 1-pisicidic acid; 2-eucomic acid; 3-isorhamnetin-3-O-glucosyl-rhamnosyl-rhamnoside; 4- isorhamnetin-3-*O*-glucosyl-rhamnosyl-pentoside; 5- Isorahmnetin-3-*O*-glucosyl- pentoside; 6- Isorhamnetin-3-*O*-rutinoside.

Even though the flavonoids contents in **F** and **FE** were comparable, the remaining extracts displayed significantly different amounts, at P=0.05. These values were similar to those previously reported[34] and are in agreement with the chromatograms (Fig. 1 and 2). In Fig. 3, we observe a clear loss of compounds after centrifugation (**FC**) or filtration (**FD**), compared to dialysis alone (**D**). These results are in agreement with those obtained by HPLC-DAD (Fig. 2). Only the precipitation with ethanol (**FE**) gives a flavonoid content similar to the filtered extract (**F**).

This decrease in total phenolic content after purification indicates that the polymers fraction also contained flavonoids and other phenolic compounds. These remained inside the dialyses membrane, attached to the higher molecular compounds, or precipitated together with the polymers during ethanol precipitation process. To confirm this hypothesis, we analysed by mass spectrometry the precipitated fraction of the **FE** extract and piscidic acid was still detected (data not shown).

Although some piscidic acid could be found in this fraction, it appeared in the chromatogram (Fig. 1) in an amount similar to the extract without precipitation (**F**). The phenolic compounds found in these extracts were in concordance with those found by Astello-Garcia et al.[34] in ethanol extracts from several *Opuntia* species, including *O. ficus-indica*, where glycosylated flavonoids and eucomic acid were detected as well. A review on the chemical composition of *O. ficus indica* cladodes indicated several flavonoids and several phenolic acids, but neither piscidic nor eucomic acids are referred[4].

The purification of piscidic acid from the extracts by preparative chromatography, allowed its quantification in the different fractions. These values varied between 10 µg/mg (**FC**) to 174 µg/mg (**F**) of extract (Fig. 3). The amount of this acid decreases 13%, 38%, and 94% in **D**, **FD**, **FC**, respectively, and also 13% when ethanol was used as precipitating agent (**FE**).

Bioactivity of the cladodes extracts: effect of the purification processes: The extract and the purified fractions were analysed in order to evaluate their AChE inhibitory activity, antioxidant activity and the effect of the extraction process on these activities. The results are shown in Table 2.

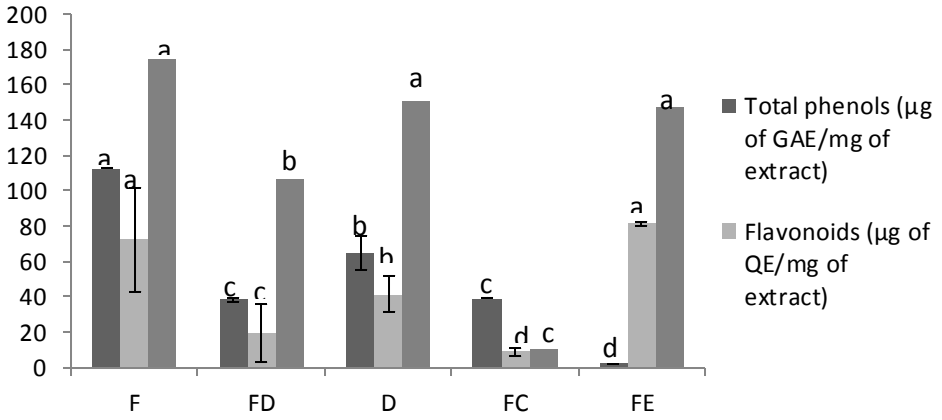


Fig. 3: Total Phenols, flavonoids and piscidic acid content of nopal extracts. (Significant difference at P=0.05 for different letters).

Table 2: IC_{50} (mg/mL) values for Acetylcholinesterase inhibition (AChE) and EC_{50} (mg/mL) DPPH scavenging activity of cladodes' extracts

Extract	AChE	DPPH
F	5.68±0.69[a]	1.30±0.15[a]
FD	2.88±0.024[b]	2.13±0.52[b]
FC	>5 mg/mL[*]	4.56±0.98[c]
D	1.125±0.037[c]	0.33±0.04[d]
FE	0.78±0.02[d]	1.15±0.01[e]
Piscidic acid	0.133±0.01	0.18±0.04

***24.8% inhibition with 5 mg/mL.**

Significant difference at P=0.05 for different letters.

Acetylcholinesterase inhibition: In this study, the inhibition of AChE was used as a model for biological activity studies and to correlate this activity with the purification procedure. The AChE inhibition activity of cladodes was analysed for the extracts obtained through different purification methods and compared with the values obtained for the extract without any purification, the extract **F**. The filtered extract (**F**) had an IC_{50} value of 5.68 mg/mL while, after discarding the polymers (**FD**, **D** and **FE**), the inhibitory activity increased. Removing the polymers from the aqueous extract with ethanol (**FE**), results in an increase of 86% in the AChE activity (Table 2).

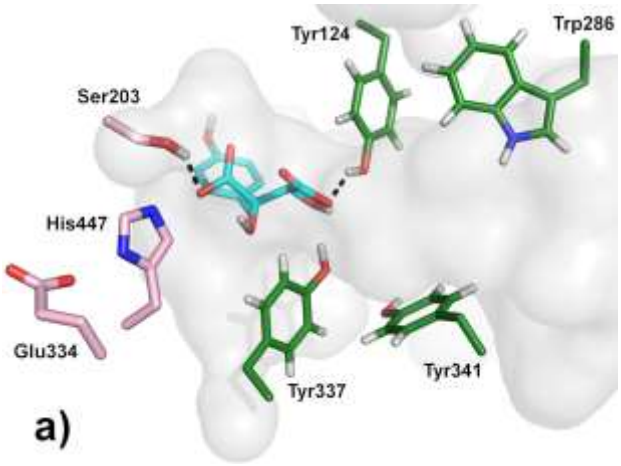
Having into account the amount of piscidic acid present in each extract and its enzyme inhibitory capacity, 43% of the inhibitory activity in **FE**, 46% in **FD** and 25% in **D** extracts can be ascribed to the presence of piscidic acid. The other compounds such as eucomic acid and isorhamnetin derivatives account for the rest of the inhibitory activity as other studies revealed that these flavonoid derivatives have antiacetylcholinesterase activity[36].

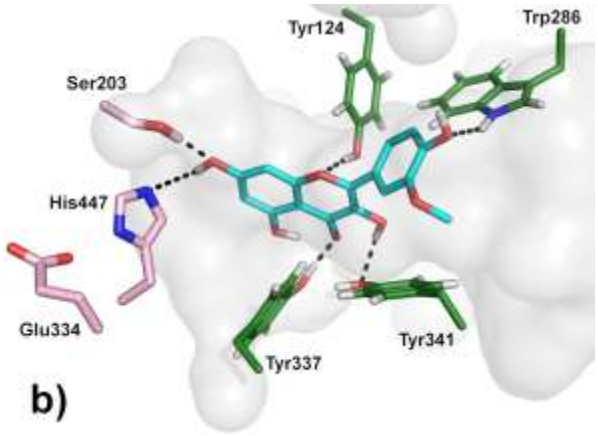
In order to evacuate the possibility of the inhibition effect of the extract be due to the fitting of the phenolic compounds into the enzyme activity site, docking studies were carried out. The active site of AChE is narrow which limits significantly the access to the catalytic triad by large molecules. Small molecules like piscidic acid (Figure 4a) can reach the enzyme pocket but, despite interacting near the active site, they seem not able to establish a strong binding. The quite poor inhibition constant of this

compound, 500 M (Table 3) can also be explained by its strong polarity, which results in a smaller stabilization from desolvation[37].

Isorhamnetin on the other hand, is able to interact across the access tunnel of AChE (Figure 4b), in a similar fashion as the commercially available inhibitor donepezil[38]. The sizeable interaction map and good shape complementarity result in a significant inhibition constant (~7 μM) which help us to confirm that this flavonoid present in the aqueous extracts is able to penetrate the AChE active site and exhibit the enzyme inhibitory activity detected experimentally.

In this work, we identified several sugar derivatives at the 3-*O* position of isorhamnetin. All these derivatizations result in large compounds with obvious difficulties to access the active site of AChE. For simplicity, we explored the enzyme binding ability of a simple 3-*O*-glucosylation of isorhamnetin, which results in the Isorhamnetin-3-*O*-glucose compound[39]. This modification decreases significantly the resulting inhibition power (Table 3), most probably due to its large size hindering the access to the pocket (Figure 4c). For larger derivatives of isorhamnetin, it is expected similar or worse effects on their inhibition constants. However, since all the 3-*O* modifications of isorhamnetin are sensitive to hydrolysis, the resulting flavonoid may probably be the active agent once inside the organism[40].





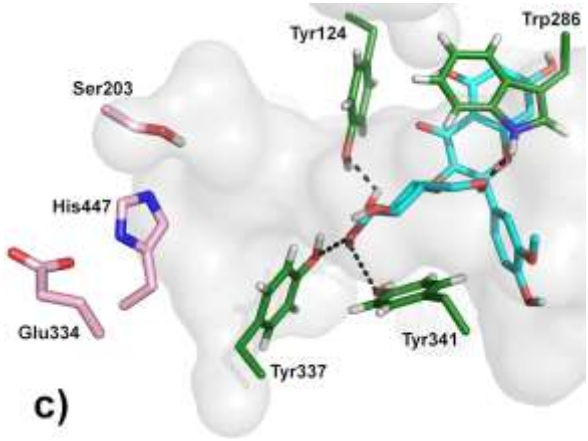


Fig 4: The lowest energy solution from the docking calculations: (a) piscidic acid; (b) Isorhamnetin; (c) isorhamnetin-3-*O*-glucose. Ligands are represented in cyan sticks. The remaining sticks are: the catalytic residues in pink and the flexible side chains of the active site pocket are depicted in green. The hydrogen bonding with the ligand is represented with black dashed lines. The active site pocket was calculated with hollow software[39] and represented as a grey surface.

Table 3: Free energies of binding to AChE by the studied compounds and their corresponding inhibition constants (K_i) at 300K.

Compounds	Binding Free Energy (kcal/mol)	Estimated K_i (μM)[a]
Donepezil [b]	– 7.4	3.9
Piscidic acid	– 4.5	500.2
Isorhamnetin	– 7.0	7.3
Isorhamnetin-3-*O*-glucose	– 6.3	23.0

a) The molecular docking K_i values were obtained from the binding free energies using $K_i = \exp(\Delta G/(RT))$.

b) 37

Although the **F** extract has the highest amount of phenols and flavonoids (Fig. 3) the enzyme inhibition needed a higher quantity of extract due to its higher ratio of fibres/polyphenols. These results reveal that the presence of fibres diminishes the activity of the extracts as enzyme inhibitor. After removal of the fibre, the enzyme inhibitory activity of the extracts is increased. There are no reports about AChE inhibitory activity for this plant, but the values obtained in this work were similar to those obtained with other infusions[20,41] and the IC_{50} of the **FE** extract is lower than some of the other aqueous extracts determined by our group.

Antioxidant activity: Oxidative stress inside the cell may be the cause of several diseases, among which neurodegenerative diseases[42,43], cardiovascular disorders[44] can be mentioned. The use of antioxidants to prevent the formation of oxidative stress inside cells has been recommended[45]. The antioxidant activities of the extracts were evaluated by the DPPH radical scavenging assay. All extracts exhibited antioxidant activity (EC_{50} values) in a concentration-dependent manner (Table 2). The **D** extract exhibited the highest antioxidant activity with an EC_{50} of 330±40 μg/mL. This may be explained by the high amount of non-antioxidant material extracted with water, while these compounds stay in the retentate after the filtration step, together with the fibres and other mucilages. If the filtration procedure, before the dialysis, was removed (like in **D**), an increase of 75% in the antioxidant activity,

relative to the aqueous extractions with filtration (**FD**), was obtained. The low antioxidant activity values of the extracts, compared to standard BHT (EC_{50} of 25.42±1.46 µg/mL) may be explained by the presence of high amounts of 3-*O*-glycosylated flavonoids. In fact, a previous study using descriptive structure-radical scavenging activity relationships of flavonoids showed that the phenolic *OH* groups positions could be more important, for the radical scavenging activity, than their total number[46]. It has also been shown that glycosylated polyphenols are less effective as antioxidants compared to their free aglycon forms[47]. The **D** extract had a high quantity of piscidic acid (EC_{50} of 183.7±0.04 µg/mL), which also can explain its higher antioxidant activity.

Comparing with other *Opuntia spp*[14], this aqueous extract with dialysis purification gave the higher antioxidant activity. Therefore, this procedure looks very promising in obtaining an extract with high antioxidant activity.

CONCLUSION

Nopal, cladodes of *Opuntia ficus-indica*, showed a composition rich in piscidic acid and isorhamnetin derivatives. The extract demonstrated biological activities such as anti-acetylcholinesterase and antioxidant activities. Piscidic acid is an abundant compound in the extracts and it accounts for approximately 50% of the enzyme inhibitory activity. Molecular docking studies indicated that isorhamnetin as a key player with a much stronger inhibitory activity than piscidic acid, which could account for the remaining effect. The enzymatic inhibition increased 86% when the polymers were removed from the aqueous extract by a precipitation with ethanol. This work describes an easy and non-expensive separation procedure that may be considered appropriate to obtain a highly active polyphenol fraction from nopal aqueous extracts for functional food development.

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***Corresponding Author: Maria Luísa M. Serralheiro;**

Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal