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Original Research Article

Alpha-Amylase Inhibitory and Antioxidant Activity of Red Galls Induced by *Forda riccobonii* in *Pistacia atlantica* Desf. Leaves: *In vitro* and *In silico* Studies

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ABSTRACT

Pistacia atlantica has been widely used as a herbal remedy in the Mediterranean and the Middle East since antiquity. *P. atlantica* has been used as an antidiabetic drug plant in Arabic folk medicine. This study aims to determine the hypoglycemic and antioxidant activities of the gall part induced by *Forda riccobonii* in *P. atlantica* leaves from Algeria. Hypoglycemic activity was determined by measuring alpha-amylase inhibition activity using spectrophotometric analysis of extracts. Antioxidant activity was determined by DPPH, ABTS scavenging assay and FRAP reducing assays. A molecular docking study was carried out on compounds previously isolated from leaves using AutoDock vina software. The highest total phenolic content (655.49±18.36 mg GAE/g DM) and flavonoid content (381.30±15.47 mg QE/g DM) were detected in the butanol fraction. Based on the DPPH assays the ethyl acetate and butanol fractions had high antioxidant activities with EC_{50} 2.46 and 9.94 µg/mL respectively. According to the ABTS assay, the best antioxidant activities were for the 35% MeOH-H_2O (4.22 µg/mL) and ethyl acetate fractions (7.23 µg/mL). The *α*-amylase inhibitory activity was highest in the ethyl acetate fraction (IC_{50} 64.53±2.50 µg/mL) followed by the positive control acarbose (78.98±6.23 µg/mL) and butanol fraction, (151.85±3.01 µg/mL). The *in silico* study of compounds 5,6,7,4'-tetra-hydroxy-flavonol-3-*O*-rutinoside and 3',5,7-tri-hydroxy-4'-methoxy-flavanone gave the best docking score (-8,3491 Kcal/mol and -8,1583 Kcal/mol, respectively). Finally, the *in silico* studies have confirmed significant *α*-amylase inhibitory activity with these molecules. As well as, the ADMET properties of these molecules appeared to be non-carcinogenic and non-hepatotoxic. Therefore, the gall's extract of *P. atlantica* could be used in the treatment of diabetes.

***Keywords*:** *Pistacia atlantica* galls, *Forda riccobonii*, Antioxidant activity, alpha-amylase, Molecular Docking

Introduction

Diabetes mellitus is a metabolic disorder presented by chronic hyperglycemia. The main form of diabetes that accounts for more than 90% of cases worldwide is type II diabetes.[1] The research and development of new antidiabetic medicine, from a practically inexhaustible source of biologically active molecules, medicinal plants, has led to several ethnobotanical, phytochemical and pharmacological studies of medicinal plants traditionally used in the treatment of diabetes.[2] Antidiabetic drugs work in different mechanisms, such as stimulating insulin secretion (glinides and sulfonylureas), improving insulin receptor sensitivity and peripheral glucose uptake (metformin and thiazolidinediones), suppressing liver glucose production (biguanides), delaying digestion and absorption of intestinal carbohydrates to maintain postprandial glucose levels (*α*-amylase and *α*-glucosidase inhibitors).[3] Inhibiting *α*-glucosidase and *α*-amylase activity is a crucial strategy to lower postprandial blood glucose, although some of these inhibitors, used in clinical diabetes management, are notorious for their gastrointestinal side effects.[1]

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Pistacia atlantica Desf., commonly referred to as "*Betoum*", is one of many plants that grows naturally in Algeria and belongs to the Anacardiaceae family.[4] *Pistacia atlantica* extracts are known to have many pharmacological properties including antiviral,[5] antimicrobial,[6] antitumor,[7] antihypertensive, antioxidant,[8] anti-inflammatory,[9] antihyperglycemic,[10] antihyperlipidemic,[11] anticholinesterase,[12] and cytotoxic activities.[13, 14]

The term "gall" in relation to trees refers to abnormal growths or swellings that develop on the leaves, stems, branches, and even roots of trees.[15] These galls are often caused by the tree's response to the action of certain organisms, such as insects, mites, fungi, or bacteria. The organisms induce the tree to form these structures as a protective response or as part of their life cycle.[16] Galls are also used in the Sahara (Algeria) as an expectorant, against asthma and chest diseases.[13] The marginal red galls formed in the leaves of *P. atlantica* were, induced by *Forda riccobonii* insects.[17]

P. atlantica has been used as an antidiabetic drug plant in Jordanian folk medicine.[18] The antihyperglycemic and antioxidant activities of marginal galls extracts formed on the leaves of *P. atlantica* (from Algeria), have not yet determined. Based on the ethnobotanical use of the plant galls, our study focuses on the hypoglycemic activity of crude extracts of different organic solvents, of the galls formed on *P. atlantica* leaves, by *in vitro* inhibition of alpha-amylase activity, followed by an *in silico* study (ligand-protein interaction) of compounds previously isolated from *P. atlantica*. The total phenolic, total flavonoid content, and antioxidant activity were also reported.

Materials and Methods

Chemicals and reagents

Analytical grade chemicals and reagents were purchased from Honeywell Fluka Germany (acetone ≥99% and ethyl acetate ≥99%), Riedel-de-Haën Germany (hexane ≥95%, chloroform ≥99.5%, butanol ≥99.5%, ascorbic acid ≥99%) and Sigma-Aldrich Germany (gallic acid ≥98%, quercetin ≥95%, Trolox ≥98%, DPPH, ABTS, FRAP and Folin ciocalteau).

Plant material

Marginal red galls formed in the leaves of *P. atlantica*, (Figure 1), were collected during July 2019 at the Ain-oussera station (*Oued Boussedraia:* 35°36'E, 2°95'N), located 210 Km south of Algiers. Professor Salima Benhouhou, a botanist from the National Institute of Agronomy (Algeria) confirmed the identity of the plant material. After collection, the samples were dried at room temperature (25°C). A voucher specimen (PAUG-52S/08/10) was deposited in the Herbarium of the Fundamental Sciences Laboratory at the University of Amar Telidji Laghouat (Algeria).

Extract preparation

Forty grams (40 g) of gall powder was macerated with 400 mL of acetone at room temperature (25 °C) for 24 hours. The extract was then filtered through Whatman No. 1 filter paper. Acetone extract was selected for solvent-solvent fractionation by increasing polarity solvents, described by Ondua.[19] The acetone extract fractionation was carried out successively using: chloroform, hexane, ethyl acetate, methanol-water 35% and *n*-butanol. The recovery fractions were then evaporated under reduced pressure using a rotary evaporator at 40°C. The fractions were dissolved in methanol and kept in a refrigerator (4 °C) until used.

Total Phenolics (TPC) and Flavonoids Content (TFC)

The total phenolics content of the crude extract was determined by the *Folin-Ciocalteu* method using a spectrophotometer, as described by Sánchez-Rangel.[20] The Folin reagent was diluted 10 times and 250 µL was added to 50 µL of sample or standard with suitable dilutions. After 2 min, 1 mL of a 4% Na_2CO_3 solution, was added to the reaction medium. After 30 minutes of incubation at room temperature (25°C) the absorbance was measured at 760 nm. The phenolic content was determined from a standard curve of different concentrations of gallic acid (0.05 mg/mL to 0.5 mg/mL). The results were expressed as mg/g gallic acid equivalent (GAE) by dry matter (DM). The calibration curve equation, and the coefficient of correlation were:

$y(Abs) = 3.7576 \times (Con.)$; $r^2 = 0.9997$

The flavonoid content of the extracts was determined using the Adebiyi method,[21] with aluminium trichloride ($AlCl_3$) as reagent. One mL of each diluted solution was mixed with 1 mL of 2% $AlCl_3$, these solutions were kept in the dark for 30 minutes at room temperature (25 °C). the absorbance was measured at 430 nm. Quercetin (0.01 mg/mL at 0.1 mg/mL) was used as a standard to plot the calibration curve. The flavonoid content of each extract was expressed as mg/g quercetin equivalent (QE) by dry matter (DM). The calibration curve equation and the coefficient of correlation were:

$y(Abs) = 4.4612 \times (Con.)$; $r^2 = 0.9998$

Antioxidant activities

DPPH radical scavenging assay methods

The DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical-scavenging activity was determined using the method of Brand-Williams.[22] One mL, of crude extract dissolved in methanol, was added to 1 mL of freshly prepared DPPH solution (100 µM). After 30 min of incubation, at room temperature (25°C) and in the dark, the absorbance was read against the blank at 517 nm using a spectrophotometer (UV-Vis) (Thermo Spectronic *Helios Alpha* 9423 UVA, Germany). The concentration of extract leading to 50% reduction of DPPH (EC_{50}) was also determined. Trolox and ascorbic acid were used as positive controls.

ABTS radical scavenging assay methods

The ABTS (2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging capacity of the samples was measured with modifications method described by Re.[23] The ABTS•+ radical was prepared by adding 7mM of ABTS with 2.45mM of persulfate potassium incubated in the dark and at room temperature (25 °C) for 24 hours. A series of dilutions were prepared in methanol (1 mL), was added to 1 mL of a solution of ABTS. The mixture was incubated for 15 min in the dark. Then the absorbance was measured against the blank at 734 nm by spectrophotometer (UV-Vis) (Thermo Spectronic *Helios Alpha* 9423 UVA, Germany). The extract concentration that led to 50% reduction of ABTS (EC_{50}) was also determined. Trolox and ascorbic acid were used as positive controls.

FRAP assay methods

The FRAP (Ferric Reducing Antioxidant Power) assay was carried out according to the Berker procedure.[24] Four hundred (400 µL) of the extract at different concentrations was mixed with 1 mL of hydrochloric acid (HCl 1 M) and 200 µL of SDS (1%) and 300 µL of a solution of potassium ferricyanide $K_3Fe(CN)_6$ (1%). The mixture was incubated in a water bath at 50°C for 20 minutes and then cooled to room temperature (25°C). A solution of ferric chloride 0.1 % (200 µL) was added to the reaction mixture. The absorbance was measured at 750 nm using UV-Vis spectrophotometer (Thermo Spectronic *Helios Alpha* 9423 UVA, Germany) against a blank. The results were expressed by Trolox Equivalent Antioxidant Capacity (TEAC).

Alpha-amylase inhibition

The α-amylase inhibition activity was performed using the method described by Benmohamed.[8] Briefly, the samples were dissolved in methanol to give a concentration range from 62.5 µg/mL to 4000 µg/mL. Different concentrations of samples (25 µL) was mixed with 50 µL of enzyme (1U) and incubated at 25 °C for 10 min. Then, 50 µL of substrate (0.1%) was added to the mixture and incubated at 37°C for 10 min. After the second incubation, the colorimetric reagent was added, with 25 µL of hydrochloric acid (HCl 1 M) and 100 µL of potassium iodide (KI). A blank was prepared that contains all reagents except the enzyme. The absorbance was measured at 630 nm after incubation using a 96 well microplate reader (Perkin Elmer, EnSpire Multimode Plate Reader, USA). The α-amylase inhibition was expressed as the percentage of inhibition and the EC_{50} values were calculated for each sample.

Molecular docking studies

In this part, two coumarins and two flavonoids were isolated from *P. atlantica* by Toul.[25] The molecular docking approach was used between these molecules and the human pancreatic alpha-amylase protein (PDB ID: 2QV4) for determining their interaction with the binding site.

The ligands are mol1: 7-ethoxycoumarin, mol2: 7-hydroxy-5-methoxycoumarin, mol3: 3',5,7-trihydroxy-4'-methoxyflavanone and mol4: 5,6,7,4'-tetrahydroxyflavonol-3-*O*-rutinoside, where their structures are presented in Figure 2. They were obtained from PubChem database[26] and assembled with Discovery Studio visualizer v4.0 to PDB files. Acarbose is a known inhibitor of alpha-amylase and was used as the reference ligand. It was redocked over itself in the crystal structure of the human pancreatic alpha-amylase complexed with acarbose (PDB ID: 2QV4) to validate the accuracy of the docking protocol.

Protein structure was downloaded from the Protein Data Bank (PDB, http://www.rcsb.org.pdb). The PDB ID: 2QV4 file represents the 3-D structure of human pancreatic α-amylase in complex with acarbose at 1.97 A° resolutions. The protein structure was prepared by eliminating unnecessary molecules, including water molecules, ligands, and heteroatoms. Kollman charges and polar hydrogens were incorporated into the structure using AutoDock Tools (ADT) version 1.5.6. Molecular docking was carried out using the AutoDock Vina program[27] on a five-CPU station featuring an Intel® Core™ i5 Processor. The software employed square boxes to define the binding site, with the box's center specified and displayed using ADT. The grid box was set at 30x30x30 dimensions with coordinates x=12.3847, y=48.1361, z=26.2092, and a 0.375 Å separation between grid points positioned at the active site's midpoint for each protein. Default simulation settings

were utilized, and the preferred conformations were those with the lowest binding energy within the active site. Finally, the docking results were visualized using Discovery Studio Visualizer (version 4.0) software.

ADME-T analysis

To study the effect of four ligands studied on the inside human body, the evaluation of pharmacokinetic parameters was done. The predicted absorption distribution metabolism, excretion (ADME) study were analyzed using the SwissADME server (http://www.swissadme.ch/index.php),[28] whereas, toxicity was predicted using ProTox II server (http://tox.charite.de/protox II/).[29] The default settings were set to calculate ADMET properties.

Figure 1: Galls formed on *Pistacia atlantica* leaves

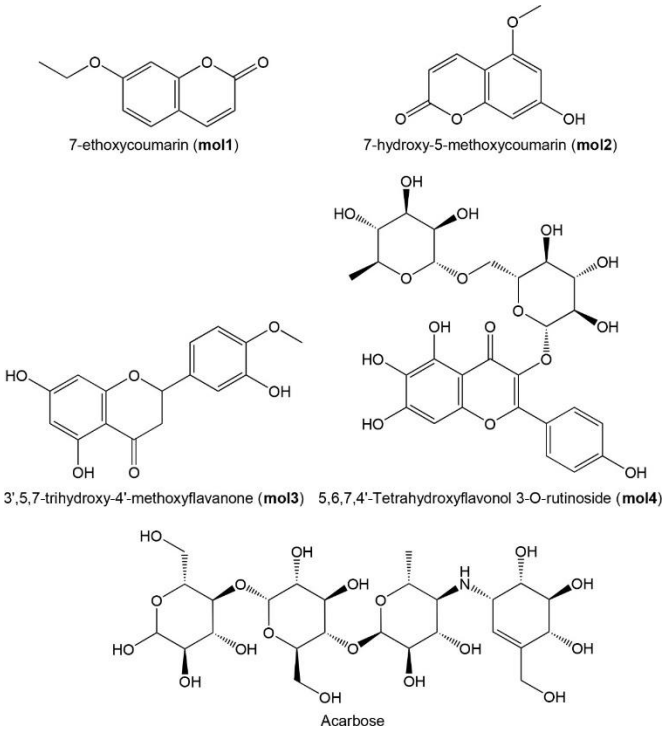

Figure 2: Structures 2D of ligands used in the molecular docking from *Pistacia atlantica*

Statistical analysis

All results are reported as the mean ± standard deviation of triplicate experiments. The significance of the differences between the test extracts in these experiments was assessed using analysis of variance (ANOVA) and the Student *t*-test, with significance determined at a probability level of $p \leq 0.05$.

Result and Discussion

The dried acetone extract of *P. atlantica* galls was successively extracted with solvents of different polarity: chloroform; water; hexane; 35% MeOH-water; butanol and ethyl acetate. Six fractions were obtained: ethyl acetate fraction (F1), butanol fraction (F2), aqueous fraction (F3), hexane fraction (F4), 35% Methanol-Water fraction (F5), chloroform fraction (F6). The hexane fraction contains fats and pigments. The chloroform fraction contains moderately non-polar compounds. The ethyl acetate fraction contains moderately polar compounds. The butanol fraction contains very polar compounds. The yield of each fraction is shown in Table 1.

The highest extraction yields were reported for the chloroform (F6) and butanol fractions (F2) (3.78% and 2.18%) followed by the Et-O-Ac fraction (F1) (1.04%), while low yields were noted for the 35% MeOH-H_2O (F5) and hexane fractions (F4) 0.09% and 0.10% respectively. The fruits of *P. atlantica* have a higher extraction yield than gall, such as 11.65% for the butanol fraction and 9.85% for the acetate fraction.[30] The metabolite content of each species and the nature of the solvent used in extraction, with different polarities, allow compounds to be separated according to their levels of solubility in the extraction solvent.[31]

Total Phenolics (TPC) and Flavonoid Contents (TFC)

The determination of TPC was carried out by the spectrophotometric method using the Folin-Ciocalteu reagent. While the determination of TFC of our fractions, was carried out by the $AlCl_3$ method. The results are shown in Figure 3 and Table 1.

The total phenolic content (TPC) ranged between 16.42 ± 0.27 and 655.49 ± 18.36 mg GAE/g dray matter (DM). The highest TPCs were detected in the butanol fraction (F2: 655.49 ± 18.36 mg GAE/g) and the Et-O-Ac fraction (F1: 566.07 ± 11.98 mg GAE/g). The 35% MeOH-H_2O fraction had the lowest concentration (F5: 16.42 ± 0.27 mg GAE/g). This result indicates that the galls of *P. atlantica* contain very polar phenolic compounds. The flavonoid content (TFC) varied between 0.74 ± 0.07 and 381.30 ± 15.47 mg QE/g DM. The highest concentration was present in the butanol fraction (F2) with a value 381.30 ± 15.47 mg QE/g DM. The chloroform fraction (F6) has the lowest concentration 0.74 ± 0.07 mg QE/g DM. These results are not unexpected because flavonoids are mainly polar compounds and are therefore more soluble in polar extractants.[32] Extracts of leaves and buds from *P. atlantica*, had the highest phenolic contents with 255.78 ± 4.73 and 233.94 ± 6.20 mg GAE/g DM, respectively. The galls had higher concentrations which may be related to the influence of the infectant on the metabolism of the plant.[33] Several factors can influence the phenolic content; geographical and climatic factors, as well as the degree of maturation of the plant have a strong influence on the content of phenolic compounds.[34]

Antioxidant activities

The antioxidant capacity of the different fractions was determined by DPPH, ABTS and FRAP methods. The EC_{50} values for each extract are shown in Table 1. The DPPH methods, indicated that the (F1) and (F2) and (F3) fractions had high antioxidant activity with EC_{50} of 2.46, 9.94 and 14.2 µg/mL respectively. According to the ABTS results, the highest antioxidant activity was present in (F5) and (F1) fraction with an EC_{50} of 4.22 and 7.23 µg/mL respectively. The results were comparable to those of Trolox positive controls (1.85 ± 0.08 µg/mL and 2.84 ± 0.42 µg/mL) for DPPH and ABTS respectively. The results of the FRAP methods, shows that the chloroform fraction (F6) has the highest reducing power 2216.20 ± 9.37 mgTEAC/g followed by the butanol fraction (F2) 2025.35 ± 11.63 mgTEAC/g. When the TEAC value increases this means that the extract is a good reducer. The correlation between total phenolic (TPC) and flavonoid contents (TFC), and antioxidant assays is presented in Table 2.

The results showed that there was a strong correlation between the total phenolic content and DPPH radical scavenging ($r = 0.714$, $p < 0.05$). There is significant relationship between the two variables. The high positive correlation between total phenolic content and antioxidant assay confirms that phenolic compounds may contributed to the antioxidant activity.

The antioxidant power of galls by ABTS methods confirmed the antioxidant efficacy tested by DPPH methods in the ethyl acetate fraction (F1). This good activity in the ethyl acetate fraction (F1) in both methods (ABTS, DPPH) can be explained by the richness of flavonoids in hydroxyl groups, indeed these secondary metabolisms, are known by their stabilization of free radicals due to the high reactivity of their hydroxyl groups. Our study indicated that it contained relatively high amounts of phenolic compounds, possibly flavonoids, tannins and terpenoids, which may be responsible for the antioxidant activity. According to Toul[33] the highest DPPH scavenging activity was observed in the crude methanolic extract of leaves (EC_{50} = 0.059 ± 0.001 mg/mL), followed by the butanol fraction of the same part (EC_{50} = 0.060 ± 0.001 mg/mL). The results of this study suggest that leaves and buds with a high concentration of phenolic compounds may play a significant role in scavenging free radicals. Results obtained by Hatamnia[35] showed EC_{50} values ranging between 0.067 mg/mL and 0.406 mg/mL. However, the results from our study indicate a higher antioxidant efficacy than what they reported.

Alpha-amylase inhibition

The inhibitory activity of fractions extracts of *P. atlantica* galls on alpha-amylase were evaluated and the results obtained are presented in Table 1.

It appears from the results obtained that the ethyl acetate fraction (F1) has the best inhibitory activity on *α*-amylase, followed by acarbose and butanol fraction (F2) at respective IC_{50} concentrations 64.53 ± 2.50 µg/mL, 78.98 ± 6.23 µg/mL and 151.85 ± 3.01 µg/mL. However, no activity was noticed in the aqueous fraction (F3), hexane fraction (F4), 35% MeOH-H_2O fraction (F5) and chloroform fraction (F6) (IC_{50} > 400 µg/mL).

The correlation test performed between the inhibition activity of *α*-amylase for the different fractions and the total phenolic and flavonoids contents has shown a strongly positive correlation between these variables (r = 0.849, p < 0.05), which means a significant relationship. It appears that polyphenols of the Et-O-Ac fraction (F1) and the butanol fraction (F2) have participated in the inhibition of *α*-amylase. Pekacar and Deliorman Orhan[10] found that the ethyl acetate sub-extract and methanolic extract of Turkish *P. atlantica* leaves had a strong inhibitory effect on the enzymes *α*-amylase (IC_{50} = 32.78 ± 1.09 and 40.22 ± 3.28 µg/mL, respectively).

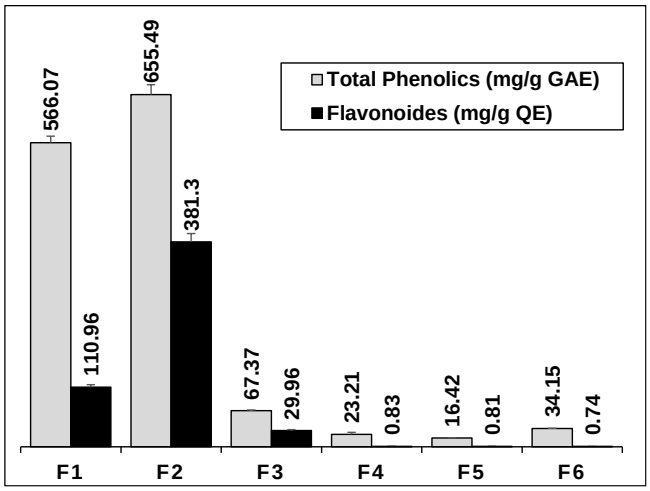

Figure 3: The total phenolics content (mg GAE/g) and flavonoid content (mg QE/g) of different fractions

Table 1: Total phenolics, total flavonoids content, antioxidant activity, and alpha-amylase inhibition of fraction extracts of *Pistacia atlantica* Galls

Fractions (Extracts)		Yield (%)	TPC mg GAE/g	TFC mg QE/g	DPPH EC_{50} (µg/mL)*	ABTS EC_{50} (µg/mL)*	FRAP (mg TEAC/g)*	*α*-amylase IC_{50} (µg/mL)
F1	Et-O-Ac	1.04	566.07 ± 11.98	110.96 ± 4.71	2.46 ± 0.14	7.23 ± 0.81	666.20 ± 4.88	64.53 ± 2.50
F2	Butanol	2.18	655.49 ± 18.36	381.30 ± 15.47	9.94 ± 0.21	71.27 ± 1.57	2025.35 ± 11.63	151.85 ± 3.01
F3	H_2O	0.51	67.37 ± 1.01	29.96 ± 2.01	14.20 ± 0.80	16.69 ± 0.97	823.94 ± 7.21	> 400
F4	Hexane	0.11	23.21 ± 3.40	0.83 ± 0.31	160.70 ± 2.34	187.97 ± 3.52	345.17 ± 3.15	> 400
F5	35% MeOH-H_2O	0.10	16.42 ± 0.27	0.81 ± 0.15	322 ± 3.11	4.22 ± 0.76	322.54 ± 2.95	> 400
F6	Chloroform	3.87	34.15 ± 0.29	0.74 ± 0.07	220.75 ± 2.76	150.33 ± 6.51	2216.20 ± 9.37	> 400
Trolox		/	/	/	1.85 ± 0.08	2.84 ± 0.42	/	/
Ascorbic acid		/	/	/	3.77 ± 0.18	1.38 ± 0.44	/	/
Acarbose		/	/	/	/	/	/	78.98 ± 6.23

* Values are expressed as mean ± SD (standard deviation)

Table 2: Pearson Correlation between antioxidant activities and total phenolic and flavonoids contents, alpha-amylase inhibition

Pearson's *r*	DPPH	ABTS	FRAP	TPC	TFC	*α*-amylase inhibition
DPPH	1					
ABTS	0,235	1				
FRAP	-0,129	-0,582	1			
TPC	0,714	-0,064	0,314	1		
TFC	0,288	-0,251	0,496	0,871	1	
α-amylase inhibition	/	/	/	0.849	0.482	1

The IC_{50} for butanol sub-extract was 100.18 ± 2.92 µg/mL. However, the study conducted by Chelghoum[34] on *α*-amylase inhibition by *P. atlantica* leaves phenolic extracts from "Aflou-Algeria" samples found IC_{50} value of 4.79 ± 0.11 mg/mL. There is significative difference between leaves, fruit and galls of *P. atlantica*. Several studies have shown that extracts of medicinal plants and especially extracts rich in tannin can improve blood sugar levels.[36-38] The study carried out by Kato[39] proved that condensed tannins have the ability to inhibit *α*-amylase and that this ability is closely related to the degree of polymerization of tannins. Additionally, Elya[40] reported that glycosides

present in the crude extracts act as substrates for *α*-amylase, potentially contributing to their inhibitory activity. RP-HPLC analysis led by Pekacar and Deliorman Orhan[10] of ethyl acetate sub-extract of Turkish *P. atlantica* leaves, showed the presence of gallic acid (0.984 ± 0.103 g/100g dry extract); methyl gallate (8.194 ± 0.0.016 g/100g dry extract); rutin (0.510 ± 0.001 g/100g dry extract) and quercetin-3-*O*-glucoside (0.498 ± 0.001 g/100g dry extract). So, in our study, ethyl acetate extract and butanol extract of *P. atlantica* galls are the most active against *α*-amylase. The molecules present in these extracts could be responsible for the inhibition of *α*-amylase. *P. atlantica* is rich in different metabolites, making them promising in the search for new antidiabetic molecules.

Molecular docking

Table 3 presents the docking calculation scores (kcal/mol) and binding interactions obtained after docking all ligands (mol1, mol2, mol3, and mol4) with *α*-amylase (2QV4). The results in Table 3 reveal that the ligand mol3 and *α*-amylase form a substantial number of donor and acceptor hydrogen bonds. The binding free energy scores for most of the ligands ranged from -6.2764 to -8.3491 kcal/mol. In particular, mol4 (5,6,7,4'-tetrahydroxy-flavonol-3-*O*-rutinoside) and mol3 (3',5,7-trihydroxy-4'-methoxy-flavanone) achieved the highest docking scores, with -8.3491 kcal/mol and -8.1583 kcal/mol, respectively. The complex formed by mol4 (2QV4-Mol4) exhibited a lower energy score compared to acarbose (-7.9382 kcal/mol). The binding mode observed for mol3 with the active site residues of *α*-amylase involves three hydrogen bond interactions, all falling within the range of strong interactions. Two of these interactions were observed with polar negatively charged amino acids Glu233 (2.32 Å) and Asp300 (1.94 Å), while the third involved the aromatic amino acid Tyr151 (2.79 Å) (Figure 4).

According to Chu and Wang[41] binding energy serves as a crucial metric when evaluating interactions between enzymes and inhibitors. In this context, low binding energy is indicative of a more stable interaction, whereas high binding energy suggests a less stable interaction. The active sites of *α*-amylase consist of three amino acid residues Asp197, Glu233, and Asp300 (known as the catalytic triad).[42] Among the ligands tested, mol3 was the only one that exhibited hydrogen bond interactions (involving its hydroxyl group) with the catalytic triad of *α*-amylase (Asp197, Glu233, and Asp300). In contrast, acarbose interacted with seven amino acids within the active sites of *α*-amylase. Most of these interactions involved hydrogen bonds with the catalytic triad, particularly Glu233 (at a distance of 1.87 Å) and Asp300 (at a distance of 3.03 Å), as well as several other nearby amino acid residues (Tyr62, His305, Asp353, Asp356) (Figure 5).

Table 3: The results of interactions between ligands and human alpha-amylase (PDB: 2QV4)

Ligand	Repeating ratio%	Free binding energy (kcal. mol^{-1})	Closest residues	Hydrophobic interactions	Hydrogen bonds	Length (Å)
Mol1	100	-6,2764	Trp58, Trp59, Tyr62, His299, Gln63	pi-alkyl, pi-pi stacked, pi-sigma, pi-alkyl	Gln63	2.05
Mol2	100	-6,2792	Tyr62, Leu162, His101, Ala198, Asp197	pi-pi stacked, pi-alkyl, pi-alkyl, Alkyl	Asp197	5.14
Mol3	100	-8,1583	Asp197, Lys200, His201, Tyr151, Glu233, Ile235, Asp300	pi-anion, pi-alkyl, pi-cation	Tyr151, Glu233, Ile235, Asp300	2.79, 2.32, 2.24, 1.94
Mol4	100	-8,3491	Trp59, Tyr62, Leu165, Asp197, Aps300, His305	pi-pi stacked, pi-pi stacked, pi-alkyl	Aps300, His305	2.28, 2.31
Acarbose	100	-7,9382	Tyr62, Glu233, Asp300, His305, Asp353, Asp356, Val354	/	Tyr62, Glu233, Asp300, His305, Asp353, Asp356	2.68, 1.87, 3.03, 2.15, 2.85, 2.39

mol1: 7-ethoxycoumarin, mol2: 7-hydroxy-5-methoxycoumarin, mol3: 3',5,7-trihydroxy-4'-methoxyflavanone, mol4: 5,6,7,4'-tetrahydroxyflavonol-3-*O*-rutinoside

Consequently, based on computational analysis, it appears that mol3 exhibits drug-like properties and holds potential as a drug candidate for *α*-amylase inhibition. Although four compounds found in *P. atlantica* leaves were not necessarily present in the gall, we decided to conduct a docking study to assess their ADME (Absorption, Distribution, Metabolism, and Excretion) properties. The results of this study are presented in Table 4. Two of the examined compounds exhibited a high gastrointestinal absorption index and were considered non-carcinogenic. Specifically, mol3 adhered to Lipinski's rule, signifying its potential as a drug candidate. However, mol4 (5,6,7,4'-tetrahydroxy-flavonol-3-*O*-rutinoside) achieved the highest docking score (-8.3491 Kcal/mol) but exceeded Lipinski's rule due to its higher molecular weight (610.52 g/mol). According to Lipinski[43] compounds with molecular weights exceeding 500 g/mol are expected to be absorbed more slowly, potentially delaying their therapeutic effects. Furthermore, mol3 (3',5,7-trihydroxy-4'-methoxy-flavanone) complies with Lipinski's rule, suggesting that it may offer good oral bioavailability and favourable drug-like properties. Overall, the four studied compounds appeared to be non-carcinogenic and non-hepatotoxic.

Conclusion

Based on the results of the *in vitro* and *in silico* studies, the ethyl acetate extract of *P. atlantica* galls shows good antioxidant activity and antidiabetic activity by inhibition of alpha-amylase. Our *in silico* studies have confirmed significant *α*-amylase inhibitory activity with molecules isolated from *P. atlantica*. Further experiments on animal models are necessary to validate the hypoglycemic activity observed in these extracts.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 4: ADMET profiling enlisting absorption, metabolism and toxicity related drug like parameters of all the four selected monoterpenes

Models	Mol1	Mol2	Mol3	Mol4
A. Absorption				
Blood-Brain Barrier	+	+	-	-
Human Intestinal Absorption	+	+	+	-
Skin Permeation	-5.81 cm/s	-6.39 cm/s	-6.71 cm/s	-10.94 cm/s
P-glycoprotein Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor
P-gp Substrate	Non Substrate	Non Substrate	Yes Substrate	Yes Substrate
B. Metabolism				
CYP450 1A2 Inhibitor	Yes Inhibitor	Yes Inhibitor	Non Inhibitor	Non Inhibitor
CYP450 2C9 Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor
CYP450 2D6 Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor
CYP450 2C19 Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor
CYP450 3A4 Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor	Non Inhibitor
C. Toxicity				
AMES mutagenesis	Non	Non	Non	Non
Carcinogenicity	Non	Non	Non	Non
Hepatotoxicity	Non	Non	Non	Non
Acute Oral toxicity	Non	Non	Non	Non

mol1: 7-ethoxycoumarin,
mol2: 7-hydroxy-5-methoxycoumarin,
mol3: 3',5,7-trihydroxy-4'-methoxyflavanone,
mol4: 5,6,7,4'-tetrahydroxyflavonol-3-*O*-rutinoside

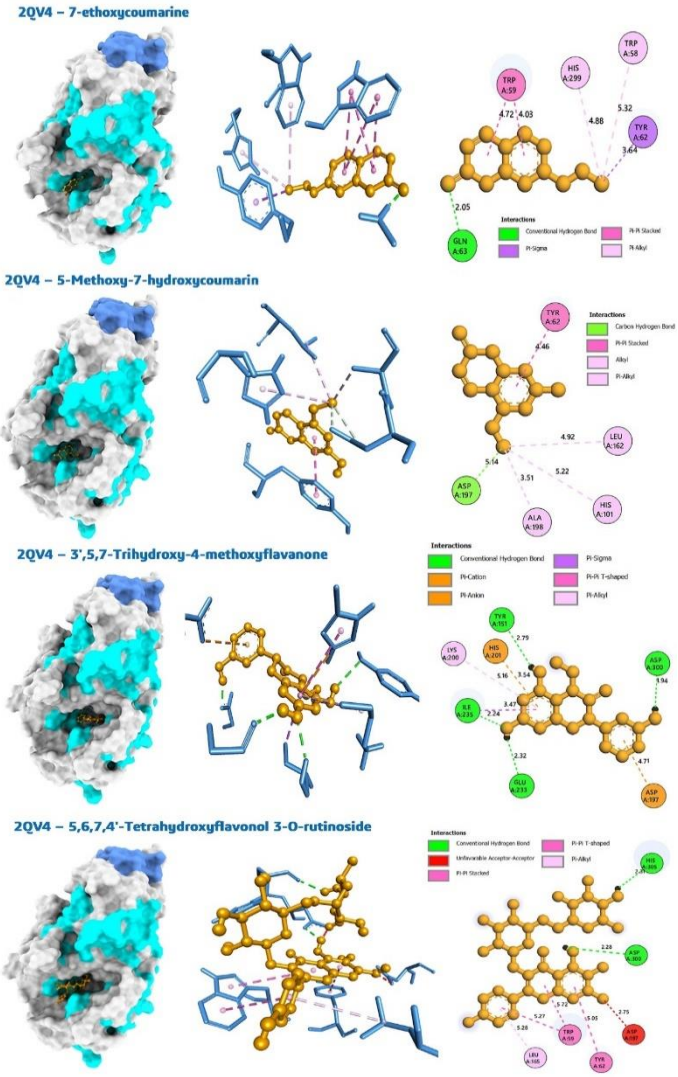

Figure 4: Best docking poses of Binding mode (3D and 2D) of mol1, mol2, mol3, mol4 at the active site of human alpha-amylase (2QV4).

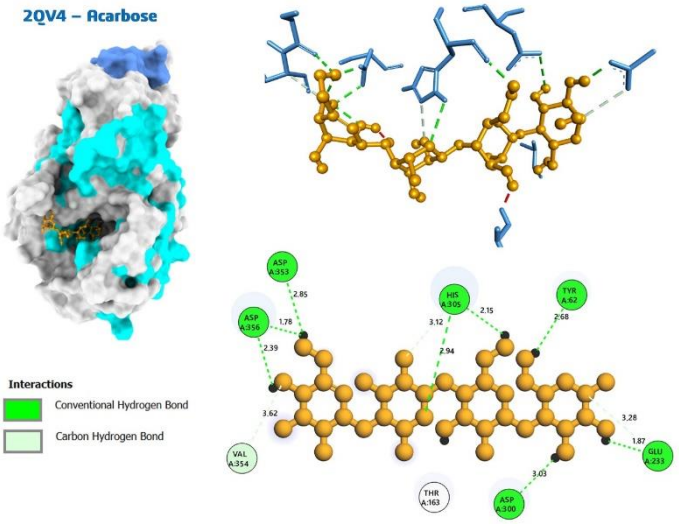

Figure 5: Best docking pose of Binding mode of Acarbose as reference at the active site of human alpha- amylase (2QV4).

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