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3-Methoxycarpachromene and Masticadienonic acid as New Target Inhibitors from Pistacia atlantica leaves against trypanothione reductase of Leishmania parasites: In Vitro and In Silico Studies

Sarra Maamri^{*1, 2}, Benarous, Khedidja¹, Yousfi, Mohamed¹

- ¹ Laboratoire des sciences fondamentales, université Amar Telidji, 03000 Laghouat, Algérie.
- ² Department de biologie, Université M'hamed Bougara, Boumerdes , Algérie.

* Correspondence: s.maamri@univ-boumerdes.dz

Abstract: This study aimed to identify new drug molecules against Leishmania parasites, leishmaniasis's causal agent, using Pistacia atlantica leaves as source. The evaluation of the anti-leishmania potential against the promastigote form of Leishmania. infantum and Leishmania. major was performed. A new in silico study was accomplished using molecular docking, with Autodock vina program, to find the binding affinity of two important phytochemical compounds from this plant (Masticadienonic acid, 3-Methoxycarpachromene) towards the trypanothione reductase as target drugs, responsible for defence mechanism against oxidative stress and virulence of this parasites. Results: Several concentrations showed a significant decrease in cell viability (P<0.0001), with IC50 values of 0.3 mg/ mL for L. infantum and 0.12 mg/ mL L. major; The molecular docking confirms the significant relationship between Leishmania survival and the inhibition of this crucial enzyme. There were promising and new positive results on binding modes of selected ligands and the trypanothione reductase for the first time. Through this work, we propose 3-Methoxycarpachromene and Masticadienonic acid as anti Trypanosomatidae species drug.

Keywords: Pistacia atlantica leaves; L. infantum; L. major; promastigote; antileishmanial; Masticadienonic acid, 3-Methoxycarpachromene; molecular docking.

1. Introduction

Leishmaniasis is a non-contagious infectious vector-borne disease [1], is still responsible for extensive morbidity and mortality in the world [2]. A paramount public health concern: is endemic in 98 countries; Approximately 2 million new cases are registered annually; about 50,000 deaths each year [3]. Two epidemiological forms, cutaneous and visceral leishmaniasis, are diffuse in Algeria, which ranks second, after Afghanistan, to the prevalence of cutaneous leishmaniasis [4].

Until now, no effective vaccine is available for leishmaniasis treatment [5]. Chemotherapy is the foremost approach to trait this infection [6].Current drug treatments for all forms of leishmaniasis get a severe impact on humans:

Renal failure, Hepatotoxicity, leucopenia, neurotoxicity and cardiotoxicity...etc [7-9]. But lack of potential alternatives forced them to be dependent on these chemo-therapeutic drugs [10]. Several scientific reports declared a therapeutic failure linked to the emergence of drug-resistant strains [11-14].

In this context, regarding these multiple factors, it is necessary to search for a new alternative drug to treat leishmaniasis. More attention has been paid to the natural herbal compounds to avoid the inconveniences of chemotherapy [10]. Molecular docking has become an important tool for high-throughput virtual screens and drug discovery [9]. Up to 24 Leishmania enzymes (52 distinct protein structures from the Protein Data Bank

(PDB)) have been exploited as potential enzyme drug targets utilising the Molegro Virtual Docking software [15]. Table 1 presents the targets enzymes for Leishmania infantum and Leishmania major from literature. The Trypanothione reductase, one of the most important targets for leishmania treatment, given those parasites lacking this enzyme, are avirulent and highly sensitive towards reactive oxygen species [16]. Besides, reducing this enzyme activity to 50% or less of normal ranges decreased several Leishmania spp's abilities to proliferate within activated macrophages [17-19]. This fact makes trypanothione reductase an attractive target for the development of new potential drugs.

Target Enzyme	References	Number of published papers (google scholar)				
Leishmania infantum						
Tyrosine aminotransferase	[20]	298				
Trypanothione synthetase	[21]	1 340				
DNA topoisomerases	[22]	1400				
Trypanothione reductase	[23]	1 790				
	Leishmania	ı major				
Glyoxalase I	[24]	547				
Trypanothione reductase	[25]	3 830				

Table 1. Targets enzymes for L.infantum and L. major from literature

Pistacia atlantica Desf (P. atlantica) is the famous taxa of the Pistacia genus belongs to the family Anacardiaceae that grows in the Middle Eastern regions and the Mediterranean. Traditionally, the plant parts were employed for therapeutic purposes due to their healing potentials [26], such as stomach aches, indigestion, throat infections, and peptic ulcers, a repellent of insects, against chest diseases, expectorant, and anti-asthma [27]. Previous researchers have described various biological activities for different P. atlantica extracts: antimicrobial, antifungal, antiviral, antiplasmodial and antileishmanial [28]. Different parts of P. atlantica have been investigated for various phytochemical studies. Most of the papers are devoted to terpenoids. For example, they marked the presence of α -pinene, β -pinene, limonene, Terpinolene, Camphene, Terpinen-4-ol, Bornyl acetate, Sabinene, p-Mentha-1 (7), 8 diene, Masticadienonic acid, Masticadienolic acid and Morolic acid in the different plant parts[29, 30]. Great importance has also been attributed to Phenolic Compounds. The Gallic acid, Quercetin-3-glucoside, catechin, epicatechin, Naringenin; apigenin, caffeic acid, ferulic acid, and 3-Methoxycarpachromene are present in the plant parts [29, 31]. The in-silico analysis literature shows a important number of natural antiparasitic compounds like polyphenolic and Terpenoids [15, 32, 33] .The present study aimed to evaluate P. atlantica leaf extract antileishmanial activity (on humans collected isolates), then to identify new selective inhibitors of trypanothione reductase using an in silico approach. From the above-cited terpenoids and phenolic compounds, we have selected Masticadienonic acid and 3-Methoxycarpachromene (Table 2) as ligands because there is not any docking of these ligands with the selected target.

2. Results and Discussion

2.1. Phenolic content and antioxidant activity by the DPPH method

The leaf extract of P.atlantica recorded a high yield of about 27.3 %. The determination of total polyphénols and flavonoids in the extract were carried out spectro-photometrically from calibration curves of gallic acid and rutin, respectively. The amount of total phenolic compound was 45.11 ± 0.05 (mg GAE/g dw), The content of flavonoids was $41.0.2 \pm 0.07$ (mg RE/g dw). Our result marks a difference compared to that obtained by Ben Ahmed et al. 2017 [34], total leaf phenolic compound from the female gender collected in the same sampling area is considerably higher than ours, but the flavonoids were less than that ours. This dissimilarity could be due to the difference in the organic solvent. Other than our results seem to be in the same order according to the result found in the ethanolic of a leaf extract from Tunisian P. atlantica, particularly the flavonoids amount [35].

Phenolic compounds are well-known antioxidants that have long been of interest because of their benefits to human health, as they address and prevent many diseases.

It is interesting to note that flavonoids constitute half of this chemical class in aglycone, glycosides and methylated derivatives formes [36]. General free radical scavenging activity has been broadly utilised as a robust and rapid measure for assessing herbal extracts' general in vitro antioxidant capacity [37], particularly attributed to phenolic compounds i.e. flavonoids and phenolic acids [38]. The extract scavenging properties of DPPH was calculated from the linear regressions of the percentage of inhibition and concentration.

The IC50 leaf extract is $58.24 \pm 0.02 \ \mu\text{g} / \mu\text{M}$ DPPH; this value is lower than those of Trolox, $93.48 \pm 0.07 \ \mu\text{g} / \mu\text{M}$ DPPH. The mechanism of radical scavenging, the reaction between the antioxidant and DPPH, depends on the antioxidant's structural conformation [39]. Thus, this activity depends not only on the content of phenolic compounds but also on the structure and interaction between the different compounds [39].

Regarding the activity against DPPH free radical, the result is near that obtained by Toul et al. (2016) and Peksel et al. (2010), cited in Table 2 [40,41]. The extract has lower activity than other crude extract compared to the work carried by other studies quoted in Table 2 [40,42-44]. It is also different from those who used the same extraction solvent but whose fractionation steps were different [40,42-44]; this is probably related to the biochemical composition of the extracts, which is mainly dependent on the nature of extraction solvent as well as the steps of confrontations by organic solvents.

cited in other studies						
Extraction Solvent	IC₅₀/ reactionel volume	DPPH Concentration / reactionel volume	IC50 (μg / M DPPH)	Sampling area	References	
Ethyl Acetate fraction	58.24μg / M DPPH /(100 μL)	500µM/(1 mL)	58.24	Aïn Oussara Alegria	Present study	
methanol	2.4(μg/ mL) / 0.75(mL)	(20 mg/L)/ (1.5 mL)	23,66	Istanbul, Turkey	[40]	
Ethyl Acetate	4.12(μg / mL)/ (0.75 mL)	(20 mg/L)/ (1.5 mL)	40,61	Istanbul, Turkey	[40]	
acetone-water(70/30)- Ethyl Acetate	0.124 (mg/ mL)(50μL mL)	(0.025 g/L)/ (1950 μL)	50,15	Tlemcen Alegria	[41]	
Ethanol –Water (70/30)	4.6(μg/ mL)/ 3(mL)	(0.3mg/ mL)/(1 mL)	18,14	Iran	[42]	
Ethyl Acetate fraction	1.54 (µg/ mL) /(3 mL)	(0.3mg/ mL)/(1 mL)	6,07	Iran	[44]	

Table 2. Comparison of the activity against DPPH free radical from P.atlantica leaf extract cited in other studies

2.2. Antileishmanial effects against promastigote form

The effects of leaf extract on cell viability were tested on the promastigote form of two different Leishmania species using the MTT assay. Several concentrations showed a significant decrease in cell viability (P<0.0001), Promastigotes of L. infantum and L. major were inhibited, with IC₅₀ values of 0.3 mg/ mL, and 0.12 mg/ mL, respectively (Fig. 1). Tukey's Multiple Comparison Test confirms that the results are significant and that Leishmanicidal activity of the extract is dose-dependent (p<0.0001). These results prove essential as a crude extract compared to the standard drug Glucantime when the IC₅₀ value was 0.05 mg/ mL [45].



Figure 1.Dose-response curves of P.atlantica leaf extract on promastigotes of Leishmania

2.3. Molecular docking

In the aim to confirm the experimental results, a molecular docking study was conducted to simulate the interactions of studied compounds in the catalytic site of trypanothione reductase. These simulations predicate that the binding energies of the enzyme are -8.4 kcal/mol for 3-Methoxycarpachromene and -6.2 kcal/mol for Masticadienonic acid (Table 3). These low binding free energies confirm the stability of the studied complexes. 3-Methoxycarpachromene interacts with three hydrogen bonds (Glu466: 1.85A°; His461:2.58A°; Asn340: 2.44 A°) and different hydrophobic types interactions (Π-Alkyl, Π-sigma, Π-cation, and Alkyl-alkyl with Asn340, Arg472, Cys469, Thr457, Ile339, and Ala343. This ligand is better than Masticadienonic acid in both binding energy and number of interactions (Figure 2 and 3).

It should be noted that trypanothione reductase structure is identical for all the characterised species of Trypanosomatidae (67% similarity of primary sequence from Trypanosomatidae, 82% identity between Leishmania spp. and >80% among Trypanosoma spp) [46]. So this result proves to be important in the treatment of all diseases linked to Trypanosomatidae species.

Ligand	Free binding energy (kcal mol ⁻¹)	Closest residues	Hydrophobic interactions	Hydrogen bonds	Length (Å)
3-Methoxycar pachromene	-8.4	Glu466, His461, Asn340, Arg472, Cys469,Thr457, Ile339, Ala343	П-Alkyl, П-sigma, П-cation, Alkyl-alkyl	Glu466 His461 Asn340	1.85 2.58 2.44
Masticadieno nic acid	-6.2	Trp21, Leu17, Gly49, Val53, Val58, Ile106, Ser14	П-Alkyl, alkyl-alkyl	Ser14 Gly49	2.96 2.9

Table 3. The results of interactions between compounds and trypanothione reductase



Figure 2.Best pose of docking for 3-Methoxycarpachromene in the catalytic site of trypanothione reductase



Figure 3.Best pose of docking for Masticadienonic acid in the catalytic site of trypanothione reductase

2.4. ADMET study

The result generated from the Lipinski, and ADMET filtering analyses are represented in Table 4. The two ligands fulfilled the requirement for Lipinski analysis of the rule of-five with corresponding favourable predicted ADMET parameters. The predicted physiochemical properties for bioavailability of the lead compounds were further represented in Figure 4. The ADME/tox and pharmacokinetic properties from the filtering analyses suggested the 3-Methoxycarpachromenewith a high probability of human intestinal absorption, subcellular distribution while the Masticadienonic acid presented low intestinal absorption.



3-Methoxycarpachromene

Masticadienonic acid

Figure 4.Summary of pharmacokinetic properties of the studied complexes.

Models	3-Methoxycarpachromene	Masticadienonic acid
A. Absorption		
Blood-Brain Barrier	No	No
Human Intestinal Absorption	high	low
Skin Permeation	-5.60 cm/s	-3.68 cm/s
B. Metabolism		
P-gp Substrate	Non Substrate	Non Substrate
CYP450 1A2 Inhibitor	Inhibitor	Non Inhibitor
CYP450 2C9 Inhibitor	Non Inhibitor	Inhibitor
CYP450 2D6 Inhibitor	Non Inhibitor	Non Inhibitor
CYP450 2C19 Inhibitor	Non Inhibitor	Non Inhibitor
CYP450 3A4 Inhibitor	Inhibitor	Non Inhibitor

Table 4. ADMET profiling enlisting absorption, metabolism and toxicity related drug like parameters of the two selected ligands

3. Materials and Methods

3.1. Plant Collection and Identification

The leaf of P atlantica Desf. were sampled from Ain Oussara in Algeria in 2019 (GPS coordinates 35° 27' 15.355" N 2° 54' 27.394" E). The leaves were authenticated by voucher specimen in the Fundamental Sciences Research Laboratory's herbarium at Laghouat University, Algeria. The collected sample was first cleaned, and then it is dried in the shade at room temperature.

3.2. Extract Preparation and quantification

After drying, the collected leaves were crushed manually. Tow gram of the obtained powder was extracted according to the method described Djeridane, et al, 2006 [39]. The dried extract residue was then dissolved in 5 mL of absolute methanol and kept at -10°C.

3.3. Evaluation of antioxidant activity (DPPH assay)

DPPH (2, 2 -diphenyl-1- picrylhydrazyl) free radical scavenging activity of leaf extract was done via a slightly modified method of Brand Williams et al. (1995) [49]. describe by Djeridane, et al, 2006 [39]. A new radical DPPH solution in methanol was prepared before the measurements of absorbance. About 1 mL DPPH solution (500μ M) was mixed with 1 mL of leaf extract dilution. The mixture was shaken vigorously at room temperature and allowed in the dark for 30 min. When an antioxidant compound reacts DPPH• with, it donates hydrogen atom, which reduces the free radical. The decrease in colour (from purple to light-yellow) was measured at 517 nm on a UV-visible light spectrophotometer (UV1601).

The leaf extract's antioxidant potential was expressed as an IC50 value defined as the concentration (in μ g/ M DPPH) of the extract that inhibited DPPH radicals' formation by 50%. The result was compared to the Trolox as a positive control, and the experiment was conducted in triplicate

3.4. Antileishmanial effects against promastigote form

We sought to evaluate the antileishmanial potency of leaf extract on Leishmania isolates for the most widespread species transmitted in Algeria. This investigation's selected strains are responsible for the visceral form or the cutaneous forms of this disease: L. infantum MON1and L. major MON25, respectively. They were sampled from infected humans.

We adopted the colourimetric cell viability MTT assay to evaluate the antileishmanial effect. According to the procedure mentioned by Shokri et al.[50], at first, 100μ L of 106 the promastigotes cells/mL (taken from logarithmic growth phase) was put into a 96-well microtiter. Then, 100μ L of different concentrations (0.005-7.3 mg/mL) of leaf extract was added to each well and incubated at 25° C±1°C for 72 hours. After incubation, 10μ L of MTT solution (5mg/mL) was added to each well and incubated at 25° C for 3 hours. The promastigotes were cultured in the complete medium with no drug used as a positive control. Finally, the absorbance was photometrically measured by an ELISA reader (ELX800) at 492nm.

The 50% inhibitory concentration (IC50) was determined by sigmoidal dose-response regression analysis using Graph Pad Software, La Jolla, CA, USA).

The concentration of inhibitors was calculated in mg of ethyl acetate crude extract per mL (mg / mL).

3.5. Molecular docking

We achieved a molecular docking using trypanothione reductase enzyme, one of the most important targets for leishmania treatment (Table 1); after detailed screening in the Protein Data Bank (PDB), we have found many PDB files of this enzyme, we have chosen the enzyme with PDB ID: 5EBK because it is complexed with inhibitors, in addition, the inhibition mechanism is well described in the work of Saccoliti et al., 2017[51]. From the above-cited terpenoids and phenolic compounds, we have selected Masticadienonic acid and 3-Methoxycarpachromene (Table 5) as ligands, for the reason that flavonoids and high molecular weight terpenoids are both extractible by ethyl acetate, besides, there is not any docking of these ligands with the selected target. The ligands were obtained from the PubChem database [52], and assembled with Discovery Studio visualiser v4.0. We have prepared the protein by removing all unnecessary water molecules, heteroatoms, ligands, and co-crystallised solvent. Polar hydrogens and partial charges were added to the structure using Autodock tools (ADT) (version 1.5.4). We performed the molecular docking (blind docking) using the AutoDock Vina program [53] in an eight CPU station. The software uses rectangular boxes for the binding site; the center of the box has been set and displayed using ADT. The enzyme's grid box was set with 1 Å separated grid points positioned in the middle of the active site for the studied protein. Regarding the flexibility of the side chain during this specific docking, flexible torsions in the ligands were assigned, and the acyclic dihedral angles were allowed to rotate freely [54]. The default settings were used, except that the number of output conformations was set to one. The number of docking runs was set at 10 runs. The number of solutions obtained is equal to 10 conformations for each ligand and enzyme. All these solutions are very well handled. The "random seed" is random. The preferred conformations were those of lower binding energy within the active site. Finally, the generated docking results were directly loaded into Discovery Studio visualiser, v 4.0.



Table 5. The selected ligands from Chemical ingredients of P.atlantica cited in the literature

3.6. ADMET study

To evaluate the two studied compounds' drug-likeness prediction, they were subjected to Lipinski filter in which an orally bio-active drug is expected not to violate more than one of the criteria for drug-likeness namely: cLogP, hydrogen donor and acceptor molecular mass, and molar refractive index [55]. The predicted Absorption Distribution Metabolism, Excretion and Toxicity (ADMET) study were analysed using SwissADME server (http://www.swissadme.ch/index.php) [56], which is reported as an important tool in drug discovery. We have inserted The SDF file and canonical SMILES of the two terpenoids into the server online to calculate ADMET properties using default parameters.

3.7. Statistical analysis

Data are expressed as mean \pm standard deviation (S. D). Statistical analysis involved a one-way analysis of variance (ANOVA). A value of p < 0.05 was considered statistically significant.

4. Conclusions

The findings are very promising. The 3-Methoxycarpachromene and Masticadienonic acid display a strong inhibitory activity on trypanothione reductase, which alters the parasitic defense mechanism against oxidative stress this study's results, we propose a therapeutic strategy to treat the Leishmania infection. The potent metabolites 3-Methoxycarpachromene and Masticadienonic acid might be an effective strategy to solve Antimony-resistant strains. Those natural molecules also might become drug candidates as anti Trypanosomatidae species drug.

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