Pharmacophore-based Virtual Screening From Phytocannabinoids for Anti-obesity Activity

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Abstract

Search for new pharmacological alternatives for obesity is based on the design and development of compounds that can aid in weight loss so that they can be used safely and effectively over a long period while maintaining their function. The endocannabinoid system is related to obesity by increasing orexigenic signals and reducing satiety signals. *Cannabis sativa* is a medicinal plant of polypharmaceutical potential that has been widely studied for various medicinal purposes. The *in silico* evaluation of their natural cannabinoids (also called phytocannabinoids) for anti-obesity purpose stems from the existence of synthetic cannabinoid compounds that have already presented this result, but which did not guarantee patient safety. In order to find new molecules from *C. sativa* phytocannabinoids, with the potential to interact with the pharmacological target cannabinoid receptor 1, a pharmacophore-based virtual screening was performed, including the evaluation of physicochemical, pharmacokinetic, toxicological predictions and molecular docking. The results obtained from the ZINC¹² database pointed to Zinc 69 (ZINC33053402) and Zinc 70 (ZINC19084698) molecules as promising anti-obesity agents. Molecular Dynamics (MD) studies disclose that both complexes were stable by analyzing the RMSD (Root Mean Square Deviation) values, and the binding free energy values demonstrate that the selected structures can interact and inhibit their catalytic activity.

1. Introduction

Obesity is considered a global epidemic disease, is a public health problem that has been growing annually, according to the World Health Organization (WHO). It may lead to comorbidities such as diabetes mellitus, cardiovascular disease, stroke, hypertension and certain types of cancer (endometrial, breast, ovarian, prostate, liver, gallbladder, kidney and colon) [1, 2].

Currently, the search for new targets for the treatment of obesity motivates research worldwide. An example of this was rimonabant, a cannabinoid antagonist that was marketed in Europe, but it was discontinued because presented several side effects on the central nervous system (CNS) [3].

The endocannabinoid system involves the presence of the following components: cannabinoid receptors (rCB), endogenous cannabinoids (endocannabinoids), and enzymes involved in endocannabinoid synthesis and degradation [4].

Cannabinoid receptors belong to the G protein superfamily and are distributed throughout the human body [5] rCB1 primarily regulate central nervous system (CNS) neurotransmission, but are also present peripherally in gastrointestinal organs, adipose tissue, myocardium, vascular endothelium, peripheral nerves, liver, pancreas, and skeletal muscle [6]. It has been studied as a target for various diseases such as pain, anxiety, multiple sclerosis, Huntington's and Parkinson's diseases, and even for obesity.

The rCB1 are known for having endogenous neuromodulatory ligands that belong to the family of lipids, which present different chemical structure than the exogenous tetrahydrocannabinol (THC) ligand.

Among the most important endogenous cannabinoid (ECs) neurotransmitter is arachidonoyl ethanolamide (or anandamide - AEA), [1, 7] being agonists of such receptor [8].

The activation mechanism of rCB1 varies according to its location. If it is presynaptic and with adenylate Cyclase inhibition, it may be generated hyperpolarization with a consequent decrease in neurotransmitter release. If it is postsynaptic, the action of rCB1 is on K⁺ channel regulation and adenylate cyclase inhibition [7,9].

The neuromodulatory action of AEA is due to the following characteristics: they have distinct synthetic pathways; their cell release occurs after membrane depolarization and calcium intake; and its action is terminated with its reuptake and degradation by an intracellular enzyme (fatty acid amide hydrolase) [10].

Regarding the onset of obesity, the AEA can act in two different ways in the CNS. The first occurs via the mesolimbic system, acting to encourage the search for palatable foods (capable of providing pleasure); and the second is through the hypothalamus, where modulates appetite by regulating the release of orexigenic or anorectic substances [11]. Basically, EC signaling in the hypothalamus consists of modulating feeding by decreasing satiety signals and increasing orexigenic signals. This signaling can be observed after fasting, when ECs become activated in the hypothalamus, stimulating appetite [12, 13, 14].

Engeli and collaborators [13] expose the relationship between rCB1 and obesity by conducting a study of fasting ECs and after eating foods in obese and normal humans, submitted to two weeks of the low-fat, high-isocaloric diet. In this study, it was evidenced that the basal AEA of obese was significantly high, while the levels of 2-AG (another endocannabinoid) were similar to those of normal individuals, thus associating endocannabinoid system with the pathogenesis of the metabolic disease, where rCB1 modulates pancreatic, adipose, skeletal muscle and liver metabolism. Burdyga and collaborators [14] report that rCB1 is expressed in greater amounts during fasting, while food intake promotes the decrease of these receptors.

Typical terpenosphenols of *C. sativa* are called phytocannabinoids. They have 21 carbons in their chemical structure and are divided into eleven subclasses, which include their metabolites, derivatives and transformation products. They are: cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), delta-9-tetrahydrocannabinol (Δ 9-THC), delta-8-tetrahydrocannabinol (Δ 8-THC), cannabicyclol (CBL), cannabielsoin (CBE), cannabinol (CBN), cannabinoidiol (CBDN), cannabitriol (CBT) and various cannabinoids [15]. Among these are those that are psychoactive (Δ 9-THC) and those that are non-psychoactive (the other classes) [16].

In this way, considering the mentioned studies that evaluated the relationship between the endocannabinoid system and obesity, as well as the existence of synthetic cannabinoids developed for the treatment of obesity, and also the intense search for the development of new treatment alternatives for such disease, this article aimed to perform a molecular modeling study of phytocannabinoids of *C. sativa* to provide new promising molecules with interest in the treatment of obesity.

2. Experimental Section

2.1 Design and optimization of molecules

The design of the chemical structure of the 104 phytocannabinoid molecules presented by Pertwee [15] was performed in the *ChemSketch v2007 2.1* program and their structures were geometrically optimized in the *HyperChem v8.0* program, using the semi-empirical method *RM1 (Recife Model 1)*, in order to get structures with minimal conformational energy.

2.2 Prediction of biological activity and pharmacophore derivation

Prediction of biological activity was performed on the *PASS (Prediction of Activity Spectra for Substances)* server [17] (<http://www.way2drug.com/PASSOnline/>) to identify those molecules that have CB1 receptor antagonist activity. From the 32 best results, then, the pharmacophoric pattern was derived in the *PharmaGist* server [18] (<http://bioinfo3d.cs.tau.ac.il/PharmaGist/>) to obtain the biological activity regions of the cluster [19].

2.3 Virtual screening

Performed on the *ZINCPharmer* web server (<http://zincpharmer.csb.pitt.edu/>) [20] which identifies molecules by the similarity of hydrophobic, aromatic, hydrogen donor and acceptor regions, positive and negative ions. Molecules selected from the *ZINCpharmer* web server have the same pharmacophoric regions previously predicted for phytocannabinoids. Compounds that combine with a well-defined pharmacophore will serve as potential leaders for the possible discovery of rCB1 acting drugs through molecular modeling.

The filter used for screening was: subset zinc purchasable; Max Hits per conf: 1; Max Hits per Mol: 1; Max total hits: 1000; e Max RMSD: 1,5; $0 \leq \text{Molecular Weight} \leq 500$, $0 \leq \text{Rotatable Bonds} \leq 8$.

2.4 Physicochemical properties

Physicochemical properties were identified on the *PubChem* server (<http://www.ncbi.nlm.nih.gov/pccompound>) for phytocannabinoids and on the *ZINC* server (<http://zinc.docking.org/>) for the screened molecules on the *ZINCpharmer* server. These data allow the assessment, by Lipinski's Rule of Five [21], of the oral absorption of each molecule through its molecular weight, log P, and the number of hydrogen donors and acceptors.

2.5 Prediction of pharmacokinetic and toxicological properties of phytocannabinoids and Zinc molecules

To predict the pharmacokinetic properties of absorption, distribution, metabolism and excretion (ADME), the *QikProp* software [22] was used to calculate the parameters: stars, percent human oral absorption (% HOA), cellular permeability in Caco2 (pCaco-2) and MDCK (pMDCK), human serum albumin binding (plogKhsa), Van der Waals surface area (PSA), and blood-brain barrier permeability (pLogBB).

Toxicological properties were determined by nephrotoxicity, hepatotoxicity, carcinogenicity and mutagenicity predictions in the *DEREK NEXUS 2.1* software [23], using as a prediction filter for humans.

2.6 Molecular Docking Simulation

Docking analysis was performed using *GOLD 5.7.1 (Genetic Optimization for Ligand Docking)* software to predict the binding of molecules with better physicochemical, pharmacokinetic and toxicological profiles to deal with rCB1. The crystallographic structure of rCB1 complexed with an antagonist (taranabant) was obtained from the Protein Data Bank (PDB) server under the PDB ID: 5U09, with a resolution of 2.6 Å. Validation of binding at the binding site, that is, reproduction of the ligand conformation occurs by redocking calculating Root Mean-Square-Deviation (RMSD), which must have been less than 2 Å. The highest score anchors are those whose chemical structures have the most favorable binding free energy to bind to the binding site [24].

Protein preparation involved the addition of hydrogens, the extraction of water molecules, as well as the deletion of all ligands present in the structure, with ligand A09 being taranabant. Ligand A09 was selected in the binding site definition step for the program to detect the binding cavity, restricting the selection of atoms to solvent and keeping the surface accessible. The phytocannabinoids and screening molecules evaluated (Zinc molecules) were pre-optimized in HyperChem under the RM1 semi-empirical method and inserted into the following binding site coordinates: $x = 21.66$, $y = 3.21$ and $z = -9.06$.

2.7 Molecular Dynamics Simulation

The MD simulation for systems was conducted using the AMBER 18 program [25], from the coordinates obtained in the molecular docking. The electrostatic potential of each of the compounds was calculated with the Gaussian 09 program [26], with the HF/6-31G* [27]. The PROPKA 3.0 [28] was used to compute the protonation states of the amino acid residues at neutral pH. The general Amber force fields GAFF [29] and MMFF99SB [30], were used for the parameterization of the ligands and the amino acid residues of the enzyme, respectively. The compounds chosen were solvated using a cubic water-box with the explicit solvation model TIP3P [31]. The distance of 12.0 Å between the cell wall and the solvated atoms of the system was used, and Cl⁻ ions were added to neutralize the overall charge of the complexes, whereas the MD minimization, heating and simulation stages were performed in the SANDER module [32] of AmberTools 18 was used in the MD calculations. Water molecules, hydrogens, ions, and the whole protein structure were minimized by four minimization steps in order to eliminate possible stereo spatial conflicts between the atoms. Subsequently, the systems were gradually heated considering the NVT ensemble from zero (0) to 298 K for a period of 25 ps, with the protein atoms subjected to a restriction constant of 25 kcal/mol.Å², the system was equilibrated by 250 ps considering the NPT ensemble ($p=1.0$ atm and $T = 298$ K). The Langevin algorithm [33], was used to regulate the system temperature, while the particle mesh Ewald (PME) method, was used to calculate long-range electrostatic interactions using a 9.0 Å cutting radius. In the course of the MD simulation, the bonds involving hydrogen atoms were restricted using the SHAKE algorithm [34], and the equations of motion were integrated every 2.0 fs using the Verlet

algorithm [35]. After minimization and equilibrations of the system, 40 ns of MD was produced with an integration time of 2.0 fs and considering the NPT ensemble at 298 K and 1.0 atm of pressure for each complex. Finally, the stability of the systems was performed out by using the root mean square deviation (RMSD) in relation to the initial structure, calculated with the PTRAJ program, present in the AMBER 18 program, and the trajectories were visualized with the Chimera 1.14 program [36]. The calculation of the interaction energy between the ligand and the macromolecule was performed with the MMPBSA.py program, implemented in the AMBER 18 program.

2.7.1 Free binding energy calculations

To evaluate the affinity between the ligands and the binding free energy of the analyzed compounds was determined using the molecular mechanics–generalized Born surface area (MM-GBSA) and molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) methods. In both methods, the last 10 ns of MD simulations, which contains 1,200 frames were used to perform the binding free energy calculations. The snapshots were carefully extracted by using the CPPTRAJ module and submitted to the MMPBSA.py module [37] of AmberTools 18. The final frames of trajectory were selected for binding free energy calculations due to the conformational stability of protein-ligand systems analyzed by the RMSD plots. The binding free energy values were computed by using MM-PB(GB)/SA approaches [38] implemented on the MMPBSA.py module [39].

3. Results And Discussion

3.1 Chemical structure and biological activities prediction of phytocannabinoids

Cannabinoid molecules evaluated in this study were designed based on the work of Pertwee in the *Handbook of Cannabis* [15]. Of the 104 initial phytocannabinoids, only 41 showed a prediction of activity for rCB1 antagonism, and their chemical structures are presented in Figure 1.

Table 1 - Values of P_a and P_i , regarding biological activities, predicted on the PASS server, of 41 phytocannabinoids with $P_a > P_i$ for cannabinoid antagonist activity.

Phytocannabinoid	RCB1 antagonism		Phytocannabinoid	RCB1 antagonism	
	$P_a^{[a]}$	$P_i^{[b]}$		$P_a^{[a]}$	$P_i^{[b]}$
Rimonabant	0.959	0.001	21	0.171	0.004
01	0.513	0.002	22	0.168	0.004
02	0.125	0.005	23	0.201	0.004
03	0.132	0.005	24	0.057	0.021
04	0.394	0.003	25	0.303	0.003
05	0.082	0.011	26	0.052	0.024
06	0.409	0.003	27	0.224	0.004
07	0.470	0.002	28	0.240	0.004
08	0.112	0.006	29	0.273	0.003
09	0.109	0.006	30	0.273	0.003
10	0.039	0.037	31	0.235	0.004
11	0.039	0.037	32	0.173	0.004
12	0.065	0.017	33	0.040	0.036
13	0.071	0.014	34	0.044	0.031
14	0.258	0.004	35	0.087	0.010
15	0.623	0.002	36	0.045	0.029
16	0.117	0.004	37	0.513	0.002
17	0.046	0.029	38	0.064	0.018
18	0.231	0.004	39	0.041	0.034
19	0.042	0.034	40	0.050	0.026
20	0.153	0.005	41	0.049	0.026

[a] Probability of activity. [b] Probability of inactivity.

The PASS server has been used to predict the biological activity for the initial 104 molecules, in terms of P_a (probability of being active) and P_i (probability of being inactive). Table 1 shows that 41 of them showed positive indicative results for antagonist activity on rCB1 receptor ($P_a > P_i$), and the others showed no indication of biological activity. In order to search for a common pharmacophore in between them, in the next step, only 32 molecules (maximum limit of molecules accepted by web server) with the best results were used: 01, 02, 03, 04, 05, 06, 07, 08, 09, 12, 13, 14, 15, 16, 18, 20, 21, 22, 23, 24, 25, 26, 27,

28, 29, 30, 31, 32, 35, 37, 38, e 40. The most relevant results were those with $P_a > 0.4$, highlighted in bold in Table 1.

3.2 Pharmacophoric pattern of phytocannabinoids with the prediction of antagonist activity on rCB1

Determination of regions on molecule structures that are most likely related to their biological activity, i.e. derivation of their pharmacophoric patterns, was performed on the PharmaGist server [19] with the 32 best-sorted molecules in the previous step. The alignment of such molecules presented the best score of 15.993 with the following points in the pharmacophoric pattern: 01 hydrogen-accepting region (HBA) (yellow sphere) and 04 hydrophobic regions (HPO) (green spheres) (Figure 2).

Sharma and collaborators [40] defined as a characteristic pharmacophore of peripheral-acting rCB1 antagonists a four-point model with the following regions: 1 HBA, 1 HPO and 2 aromatics (AR). In the same study, other pharmacophoric hypotheses were evaluated, including a five-point model with: 1 HBA, 2 HPO and 2 AR. Wang and collaborators [41] also obtained two 5-point pharmacophoric patterns where the first comprised 1 HBA, 2 AR and 2 HPO; while the second 1 HBA, 1 AR and 3 HPO. The information shows us that there is no defined pharmacophoric pattern for cannabinoid antagonists.

The pharmacophoric pattern obtained in this study resembles Wang's second model, except for the aromatic region. However, it is noteworthy that the pharmacophoric patterns obtained in the literature, concerning cannabinoid antagonists, were built using individual molecules as a reference, and do not refer to the alignment of various antagonists. The proposition of a novel pharmacophore may prove useful in the search for safer cannabinoid antagonists for anti-obesity activity.

3.3 Virtual Screening

In order to perform a pharmacophore-based screening, we used our previously described pharmacophore (1 HBA and 4 HPO), by using the purchasable database from the ZINCPharmer web server and 1000 hits were obtained as results, with RMSD ranging from 0.40 to 0.88Å [20].

Subsequently, these hits were submitted to biological activity prediction on the PASS server. Results obtained showed that 78 molecules (Figure 3) were favorable to cannabinoid antagonist activity, besides presenting indicatives of anti-obesity activity, like Rimonabant, as shown in Table 2.

Table 2 - Values of P_a and P_i , regarding biological activities predicted on the PASS server, of 78 Zinc molecules that showed activity prediction for rCB1 antagonism and anti-obesity.

Molecule	Zinc molecule	RCB1 Antagonist Activity		Anti-obesity Activity	
		Pa ^[a]	Pi ^[b]	Pa ^[a]	Pi ^[b]
Rimonabant	ZINC1540228	0.959	0.001	0.805	0.005
01	ZINC13961796	0.079	0.012	0.164	0.162
02	ZINC13961797	0.101	0.007	0.259	0.089
03	ZINC21053963	0.067	0.016	0.711	0.006
04	ZINC429727	0.043	0.032	0.271	0.082
05	ZINC7453189	0.115	0.006	0.169	0.156
06	ZINC20616264	0.075	0.013	0.430	0.033
07	ZINC12438613	0.041	0.034	0.231	0.104
08	ZINC4938689	0.076	0.013	0.326	0.059
09	ZINC40788974	0.144	0.005	0.341	0.054
10	ZINC77954021	0.077	0.013	0.351	0.051
11	ZINC44375240	0.072	0.014	0.299	0.069
12	ZINC80871235	0.049	0.026	0.271	0.082
13	ZINC4939048	0.046	0.029	0.202	0.124
14	ZINC40785807	0.053	0.024	0.369	0.046
15	ZINC40785808	0.063	0.018	0.299	0.069
16	ZINC2917038	0.050	0.025	0.178	0.146
17	ZINC40791167	0.103	0.007	0.627	0.010
18	ZINC35459881	0.069	0.015	0.371	0.046
19	ZINC40788781	0.179	0.004	0.473	0.025
20	ZINC46031287	0.120	0.005	0.469	0.026
21	ZINC40786024	0.061	0.020	0.714	0.006
22	ZINC40786018	0.055	0.022	0.732	0.005
23	ZINC35537899	0.206	0.004	0.518	0.019
24	ZINC80873789	0.052	0.024	0.290	0.073
25	ZINC75346785	0.058	0.021	0.268	0.084

26	ZINC58899851	0.142	0.005	0.213	0.116
27	ZINC71992509	0.038	0.038	0.394	0.040
28	ZINC64657983	0.131	0.005	0.450	0.029
29	ZINC20559878	0.216	0.004	0.460	0.027
30	ZINC40143414	0.102	0.007	0.564	0.014
31	ZINC40324123	0.051	0.025	0.321	0.061
32	ZINC36329383	0.062	0.019	0.359	0.049
33	ZINC73813351	0.048	0.028	0.361	0.049
34	ZINC12891825	0.095	0.008	0.403	0.038
35	ZINC72302752	0.106	0.007	0.332	0.056
36	ZINC06799655	0.046	0.029	0.270	0.078
37	ZINC02776799	0.042	0.033	0.163	0.163
38	ZINC02732822	0.062	0.019	0.656	0.008
39	ZINC65024931	0.081	0.011	0.268	0.084
40	ZINC64818443	0.059	0.021	0.189	0.135
41	ZINC75495649	0.049	0.027	0.308	0.065
42	ZINC40790855	0.080	0.012	0.495	0.022
43	ZINC39543673	0.137	0.005	0.662	0.008
44	ZINC42567713	0.054	0.023	0.323	0.060
45	ZINC40790660	0.053	0.024	0.515	0.019
46	ZINC40790853	0.081	0.011	0.497	0.021
47	ZINC72078107	0.107	0.007	0.277	0.079
48	ZINC44203802	0.121	0.005	0.403	0.038
49	ZINC78775193	0.069	0.015	0.229	0.105
50	ZINC40324133	0.051	0.025	0.262	0.087
51	ZINC40324129	0.050	0.025	0.301	0.068
52	ZINC40790659	0.040	0.036	0.466	0.026
53	ZINC08610833	0.090	0.009	0.230	0.104
54	ZINC09821343	0.048	0.028	0.171	0.153

55	ZINC06590068	0.042	0.033	0.181	0.143
56	ZINC93543397	0.069	0.016	0.395	0.040
57	ZINC77878134	0.044	0.031	0.247	0.095
58	ZINC05524999	0.112	0.006	0.261	0.088
59	ZINC42369595	0.160	0.004	0.220	0.111
60	ZINC30863592	0.086	0.010	0.348	0.052
61	ZINC85907100	0.124	0.005	0.289	0.073
62	ZINC15679622	0.052	0.024	0.243	0.097
63	ZINC04539076	0.179	0.004	0.214	0.115
64	ZINC23043320	0.080	0.012	0.792	0.005
65	ZINC69300690	0.104	0.007	0.208	0.119
66	ZINC00217472	0.082	0.011	0.281	0.077
67	ZINC33053400	0.070	0.015	0.600	0.012
68	ZINC25139189	0.043	0.032	0.247	0.095
69	ZINC33053402	0.123	0.005	0.583	0.013
70	ZINC19084698	0.163	0.004	0.536	0.017
71	ZINC12181893	0.066	0.017	0.362	0.048
72	ZINC92234847	0.076	0.013	0.222	0.109
73	ZINC20946885	0.053	0.024	0.337	0.055
74	ZINC20947372	0.129	0.005	0.420	0.034
75	ZINC64968042	0.061	0.019	0.263	0.087
76	ZINC40391844	0.083	0.011	0.382	0.043
77	ZINC15593474	0.066	0.017	0.164	0.162
78	ZINC71388757	0.067	0.016	0.194	0.130

[a] Probability of activity. [b] Probability of inactivity.

Overall, 78 molecules (or hits) obtained by virtual screening on the ZINC platform, showed positive predictions for anti-obesity activity, despite its low prediction for rCB1 antagonism. Of these, 24 had P_a results > 0.4, for anti-obesity activity, which are: 03, 06, 17, 19-23, 28-30, 34, 38, 42, 43, 45, 46, 48, 52, 64, 67, 69, 70 and 74.

3.4 Analysis of physicochemical properties

Physicochemical properties were evaluated according to Lipinski's Rule of Five (RO5), [21] which represents a fundamental guideline for getting to know the oral absorption of a drug candidate, regarding its permeability and solubility, as well as of what is necessary for pharmacological action. According to this, has a good prediction for oral absorption those molecules that present results with at most a single violation of the evaluated parameters/properties, as follows: molecular weight (g/mol) (MW) ≤ 500 , log P ≤ 5 ; hydrogen bond donors (HD) ≤ 5 and hydrogen bond acceptors (HA) ≤ 10 .

The data required in the analysis of 32 phytocannabinoids (the same selected for the pharmacophore derivation) was obtained from the Pubchem server. All of the 32 molecules evaluated at this stage, except molecule 14, showed results indicating good oral absorption (Table 3).

Table 3 - Physicochemical properties of 32 phytocannabinoids obtained from the PubChem web server.

Phytocannabinoid	Molecular weight (g/mol)	Log P	Hydrogen Donors	Hydrogen Acceptors	Violations of the Rule of 5
Rimonabant	463.79	6.1	1	2	1
01	314.47	7.0	1	2	1
02	358.48	7.0	2	4	1
03	358.48	6.5	2	4	1
04	286.41	5.9	1	2	1
05	330.42	6.0	2	4	1
06	258.36	4.9	1	2	0
07	300.44	6.4	1	2	1
08	344.45	6.0	2	4	1
09	302.37	4.4	2	4	0
12	494.72	8.5	1	4	1
13	490.68	9.2	1	4	1
14	640.95	11.6	2	4	2
15	300.44	5.2	1	2	1
16	344.44	7.73	1	4	1
18	314.47	6.5	2	2	1
20	286.42	5.4	2	2	1
21	328.49	6.8	1	2	1
22	258.36	4.5	2	2	0
23	300.44	6.0	2	2	1
24	310.44	7.0	2	2	1
25	310.44	6.1	1	2	1
26	354.47	6.2	2	4	1
27	282.38	5.0	1	2	0
28	254.33	4.1	1	2	0
29	324.46	6.4	0	2	1
30	296.41	5.6	1	2	1

31	268.36	4.5	1	2	0
32	326.44	5.8	2	3	1
35	314.47	5.9	0	2	1
37	314.47	7.0	1	2	1
38	348.49	4.1	3	4	0
40	314.47	6.4	1	2	1

Bold results indicate violations of RO5.

According to the results presented in Table 3, only phytocannabinoid 14 violated RO5, with molecular weight and log P above the evaluated parameters, which indicates that its physicochemical profile does not favor its oral administration. The other molecules are in agreement with RO5 and also with the physicochemical characteristics of rimonabant [42] in which the only violation is related to log P (equal to 6.11).

Concerning the ability of a molecule to present good CNS permeability, the physicochemical property that is most related to this issue is the lipophilicity, which may be expressed by log P, i.e. the partition coefficient of a given molecule calculated in the system 1-octanol/water. With this in mind and taking into account, a generic analysis in the context of RO5, values of $\log P \leq 4$ indicates that a molecule may be inactive in the CNS, and $\log P > 4$ indicates that may be active in the CNS.^[27] Given this information, all phytocannabinoids presented in Table 3 have physicochemical potential to permeate the blood-brain barrier, an expected result for these metabolites that are known for their action in the CNS.

For the 78 Zinc molecules obtained from the virtual screening procedure, their physicochemical properties (Table 4) were collected from the ZINC server itself (<http://zinc.docking.org/>). All screened molecules were either within Lipinski's RO5.

Table 4 - Physicochemical properties of 78 Zinc molecules obtained from the ZINC¹² web server.

Zinc molecules	Molecular weight (g/mol)	Log P	Hydrogen Donors	Hydrogen Acceptors	Violations of the Rule of 5
Rimonabant	463.79	6.1	1	2	1
01	378.432	2.9	1	7	0
02	382.464	2.6	1	7	0
03	403.548	3.0	0	6	0
04	337.423	3.5	2	5	0
05	280.331	2.9	0	5	0
06	360.502	3.8	2	6	0
07	349.415	3.0	1	5	0
08	351.45	4.0	1	5	0
09	352.825	3.4	1	5	0
10	257.337	3.0	1	4	0
11	359.216	3.9	2	5	0
12	342.761	3.4	3	5	0
13	368.502	5.1	1	4	1
14	319.368	2.4	1	6	0
15	381.439	3.6	1	6	0
16	338.411	3.3	1	6	0
17	331.391	1.8	1	5	0
18	256.349	3.6	1	3	0
19	352.825	3.3	1	5	0
20	391.537	3.7	1	6	0
21	415.884	4.2	1	6	0
22	325.759	2.5	2	6	0
23	257.337	3.3	1	4	0
24	342.761	5.9	3	5	1
25	307.397	4.0	2	4	0
26	392.528	5.0	2	5	0

27	373.243	3.9	2	5	0
28	360.885	5.4	1	4	1
29	332.831	4.6	1	4	0
30	290.794	4.6	2	3	0
31	372.902	4.9	1	3	0
32	358.875	4.5	1	3	0
33	319.449	3.8	1	5	0
34	386.274	3.9	2	4	0
35	365.458	3.2	1	5	0
36	316.405	2.3	1	6	0
37	381.386	3.4	2	6	0
38	368.503	1.4	0	7	0
39	378.476	4.3	1	6	0
40	300.406	3.7	1	5	0
41	354.454	2.9	1	6	0
42	471.948	3.5	1	7	0
43	351.454	2.8	0	6	0
44	330.494	3.1	1	5	0
45	381.823	1.6	1	7	0
46	395.85	1.9	1	7	0
47	336.52	4.8	1	4	0
48	303.328	3.3	1	4	0
49	252.446	4.1	0	2	0
50	370.861	4.7	1	4	0
51	356.834	4.3	1	4	0
52	367.796	1.5	2	7	0
53	448.954	4.7	0	6	0
54	428.536	4.4	0	6	0
55	284.359	3.6	2	4	0

56	279.388	2.1	2	6	0
57	350.378	2.7	0	7	0
58	391.298	7.0	1	4	1
59	291.395	2.8	0	5	0
60	286.379	2.7	1	5	0
61	331.435	3.9	2	4	0
62	461.65	6.5	1	5	1
63	388.393	4.6	1	5	0
64	325.459	2.4	1	5	0
65	399.491	4.1	2	7	0
66	311.429	4.3	1	4	0
67	419.591	3.7	1	6	0
68	298.39	3.3	2	5	0
69	449.617	2.9	0	7	0
70	381.48	3.5	0	7	0
71	367.453	2.8	0	7	0
72	291.439	4.4	1	4	0
73	392.503	3.5	1	6	0
74	426.948	4.2	1	6	0
75	376.5	6.0	1	4	1
76	351.385	4.6	0	6	0
77	300.406	3.8	1	5	0
78	362.408	3.3	2	5	0

Bold results indicate violations of RO5.

Regarding the potential to permeate the CNS according to log P values, the following molecules showed a possible poor permeability, with better results than rimonabant in the Log P parameter: 01-12, 14-20, 22-23, 25, 27, 33-38, 40-46, 48, 52, 55-57, 59-61, 64, 67-71, 73, 77 and 78. Other molecules that presented log P > 4, tend to show a higher CNS permeability, although such characteristics should be confirmed by further pharmacokinetic profile analysis [43].

Lee and collaborators [24] presented the following physicochemical profile of cannabinoid antagonists: $MW \leq 500$, $\log P \leq 7$; $HD \leq 3$ and $HA \leq 6$. It is noted that $\log P$ increases due to the fat-soluble profile of molecules that has CNS permeability characteristics, and the number of hydrogen donors and acceptors decreases. Evaluating phytocannabinoids through this prism it can be observed that molecule 14 would continue with 2 violations and that molecules 12, 13, 14 and 16 would violate the stipulated limit for $\log P$. Evaluating Zinc molecules according to the physicochemical properties of cannabinoid antagonists, no molecule has more than a single violation [44]. It is noteworthy that the physicochemical profile of cannabinoid antagonists is inherent in the molecules that permeate the blood-brain barrier, a fact related to the adverse effects of this class of molecules and unwanted in this study [42].

3.5 Pharmacokinetic properties analysis

The pharmacokinetic properties of phytocannabinoids are listed in Table 5 and were analyzed according to the parameters indicated in the QikProp manual. The evaluated parameters include: stars (similarity to known drugs: high: 0-2; medium: 3; and low: > 4), percentage of human oral absorption (% HOA) (high: > 80%; average: 25-80%; and low: < 25%), binding to human serum albumin (plogKhsa) (between -1.5 (low) and 1.5 (high)), pCaco-2 (intestinal cell) permeability (good: > 500 nm/sec; and poor: < 25 nm/sec) and pMDCK (renal cells) (good: > 500 nm/sec; and poor: < 25 nm/sec); logBB (low permeation in blood-brain barrier when < -1 and easy permeation in blood-brain barrier when > -1) and Van der Waals surface area (PSA > 70: does not exceed BBB; PSA < 70 exceeds BBB) [22].

Table 5 - Pharmacokinetic properties of 32 phytocannabinoids predicted and evaluated by the QikProp software.

Phytocannabinoid	Similarity	Absorption	Cellular Permeability		CNS distribution		
	Stars	% HOA	logKhsa	pCaco-2 (nm/sec)	pMDCK (nm/sec)	logBB	PSA
Rimonabant	2	95	1.37	420	2697	1.48	30.2
01	3	100	1.22	4184	2324	-0.12	27.2
02	0	90	0.99	261	147	-0.80	68.1
03	0	97	0.73	188	103	-0.95	71.7
04	1	100	0.97	4190	2328	0.02	27.2
05	0	100	0.75	249	140	-0.65	68.2
06	1	100	0.73	4176	2319	0.17	27.2
07	1	100	1.09	4182	2322	-0.05	27.3
08	0	94	0.61	188	103	-0.86	71.6
09	0	88	0.29	176	96	-0.61	71.8
12	4	100	2.36	3665	2014	-0.39	47.5
13	4	100	2.42	4189	2327	-0.32	50.2
14	10	100	3.30	3217	1749	-0.80	49.9
15	1	100	1.08	4323	2408	-0.04	26.5
16	0	90	0.84	337	194	-0.61	64.7
18	1	100	1.02	2946	1590	-0.41	34.8
20	0	86	0.78	180	99	-1.10	35.9
21	3	100	1.23	6835	3950	0.04	21.9
22	1	100	0.54	2924	1577	-0.09	35.9
23	1	100	0.89	2943	1588	-0.33	34.8
24	1	100	0.89	2619	1401	-0.53	34.1
25	2	100	1.16	4228	2350	-0.13	26.9
26	0	91	0.91	309	177	-0.76	64.1
27	1	100	0.90	4225	2348	0.01	26.9
28	1	100	0.66	4230	2352	0.16	26.9
29	4	100	1.39	9906	5899	0.55	12.6

30	1	100	1.03	4237	2356	-0.06	26.9
31	1	100	0.78	4232	2352	0.09	26.9
32	0	100	0.89	1566	803	-0.65	47.8
35	3	100	1.31	6361	3655	0.13	15.7
37	3	100	1.22	4266	2373	-0.12	26.9
38	0	100	0.56	1027	509	-0.87	65.9
40	2	100	1.13	5535	3145	-0.06	25.0

%HOA: percentage of human oral absorption; logKhsa: binding to human serum albumin; pCaco-2: intestinal cells; pMDCK: renal cells; logBB: blood-brain barrier permeation; and PSA: Van der Waals surface area.

Prediction of binding to human serum albumin indicates the ability of the molecule to be absorbed and bioavailable to interact with its target receptor. Evaluating molecules from Table 5 in terms of logKhsa, one can see that none showed low aggregation, 09 and 22 showed medium aggregation and others showed results that indicate high aggregation to serum albumin protein, a similar result to rimonabant.

Blood-brain barrier permeation was assessed by logBB (low permeation when < -1 and easy permeation when > -1) [45] and PSA (low brain permeation when $> 70 \text{ \AA}$) [46]. Rimonabant showed a prediction for high permeability in the blood-brain barrier, a factor associated with its side effects. According to logBB values obtained, only molecule 20 showed predictive results for low CNS permeation. On the other hand, according to PSA values obtained, molecules 03, 08 and 09 would have low CNS permeability. All other molecules tend to be well absorbed in oral administration and permeate the CNS due to their values of $\text{PSA} < 70$ [47]. The best results, therefore, were for molecules 03, 08 and 09, which had $\text{PSA} > 70$, despite medium cell permeability; and for molecules 01, 04, 06, 07, 15, 18, 21-25 and 27-40, with high cell permeability and prediction for action in the CNS.

Furthermore, we also predicted and evaluated the pharmacokinetic properties of the 78 molecules selected from the virtual screening using the ZINC database, as can be seen in Table 6.

Table 6 - Pharmacokinetic properties of 78 ZINC molecules predicted and evaluated in QikProp software.

ZINC molecules	Similarity	Absorption		Cellular Permeability		CNS distribution	
	Stars	% HOA	logKhsa	pCaco-2 (nm/sec)	pMDCK (nm/sec)	logBB	PSA
Rimonabant	2	95	1.37	420	2697	1.48	30.2
01	0	100	0.15	1004	669	-0.73	79.9
02	0	100	0.06	1019	698	-0.72	79.3
03	0	100	-0.19	1057	860	-0.41	63.9
04	0	95	0.44	606	287	-0.71	82.3
05	0	97	0.15	770	373	-0.85	63.9
06	1	97	0.45	732	353	-0.66	89.1
07	0	100	0.27	1653	1255	-0.39	71.6
08	0	100	0.48	1282	647	-0.41	69.8
09	0	100	0.55	2288	2962	-0.01	51.3
10	0	100	0.22	2290	1211	-0.17	54.8
11	0	100	0.39	977	2892	-0.24	69.4
12	0	100	0.29	1014	2180	-0.39	77.9
13	0	100	0.52	1221	875	-0.45	67.8
14	0	100	0.19	1736	898	-0.33	68.6
15	1	100	0.59	2636	1410	-0.25	69.5
16	0	100	0.25	1417	721	-0.43	79.2
17	0	100	0.09	1249	1120	-0.39	77.3
18	1	100	0.53	4266	2373	0.09	39.4
19	0	100	0.60	2201	2245	-0.08	51.8
20	1	96	0.37	631	302	-0.91	75.6
21	0	100	0.66	2775	2904	-0.09	70.0
22	0	100	0.16	1239	1273	-0.40	80.6
23	1	100	0.33	2587	1382	-0.13	54.1
24	0	100	0.28	1136	2467	-0.31	77.7
25	0	100	0.58	1997	1045	-0.27	62.4

26	0	100	0.86	1572	1152	-0.42	71.1
27	0	100	0.69	2133	6204	-0.13	53.3
28	1	100	0.84	2810	3479	0.06	49.4
29	1	100	0.69	5116	6650	0.32	46.9
30	1	100	0.511	2856	3182	0.06	50.2
31	0	100	0.501	1910	3724	-0.05	44.6
32	0	100	0.345	1890	3652	-0.03	44.7
33	0	100	0.407	2708	2136	0.03	58.1
34	0	100	0.422	1346	2675	-0.28	64.6
35	0	100	0.231	2230	1860	-0.32	54.4
36	0	93	0.088	633	665	-0.48	77.6
37	1	100	0.715	1477	2156	-0.34	78.3
38	0	84	-0.754	598	457	-0.62	81.4
39	0	100	0.781	1751	906	-0.32	71.2
40	1	100	0.413	792	384	-0.67	71.8
41	1	94	0.286	617	293	-0.89	84.9
42	0	100	0.361	921	1111	-0.61	89.6
43	0	100	0.08	1449	738	-0.45	68.1
44	0	92	-0.353	967	703	-0.61	68.4
45	0	100	-0.048	785	934	-0.65	94.1
46	0	97	-0.106	786	936	-0.55	88.1
47	1	94	-0.037	621	882	-0.69	62.6
48	0	100	0.423	2049	3617	0.06	53.7
49	3	91	0.182	549	316	1.07	5.6
50	0	70	-0.277	107	44	-1.89	96.8
51	0	100	0.355	1751	3684	-0.06	55.9
52	0	90	-0.056	544	629	-0.55	100.6
53	0	100	0.505	1598	3754	-0.07	64.1
54	0	100	0.56	1732	1601	-0.23	62.8

55	1	97	0.322	872	426	-0.61	77.6
56	0	84	-0.174	371	383	-0.73	82.1
57	0	100	0.321	1325	671	-0.67	80.3
58	1	100	0.789	2443	6359	-0.19	54.5
59	1	100	-0.024	2040	1069	-0.28	61.9
60	1	100	0.535	1855	965	-0.23	57.2
61	0	78	-0.055	129	197	-0.46	67.3
62	2	100	1.071	2211	1856	-0.04	56.3
63	0	100	0.72	1406	2083	-0.39	64.1
64	0	100	-0.176	1492	1258	-0.31	60.0
65	0	100	0.341	1190	1053	-0.34	87.8
66	0	100	0.613	2448	1302	-0.18	59.8
67	1	100	0.353	782	379	-0.75	72.8
68	1	89	0.328	398	183	-0.92	84.9
69	0	100	-0.386	1532	784	-0.39	70.1
70	0	100	-0.204	1626	1499	-0.14	70.7
71	0	100	-0.296	1715	1554	-0.14	74.6
72	1	100	0.598	2550	1361	-0.19	51.1
73	0	100	0.307	683	748	-0.43	73.4
74	0	100	0.432	780	2004	-0.26	75.6
75	0	100	0.9	2952	1594	-0.29	51.7
76	0	100	0.104	1045	925	-0.54	73.5
77	1	100	0.392	837	408	-0.65	69.8
78	0	100	0.704	936	821	-0.53	71.74

%HOA: percentage of human oral absorption; logKhsa: binding to human serum albumin; pCaco-2: intestinal cells; pMDCK: renal cells; logBB: blood-brain barrier permeation; and PSA: Van der Waals surface area.

All molecules presented favorable Stars values results, except for molecule 49. Despite this molecule presented stars = 3, which should indicate low reliability, other parameters evaluated in this study were within the stipulated limits for the calculations. Only two molecules showed results indicating low oral

absorption in terms of %HOA: 50 and 61. All other molecules showed results indicating high oral absorption. Concerning aggregation to plasma albumin protein, all results indicate less aggregation to albumin than rimonabant. Most molecules showed indicative results for high cell permeability in pCaco-2 and pMDCK. However, molecules 04, 05, 06, 20, 38, 40, 41, 55 and 67 showed medium permeability in pMDCK; and molecules 50, 56, 61 and 68 showed medium results for both.

Most of them were within the indicative parameters for blood-brain barrier permeability, with results > -1 . Only molecule 50 showed results indicating non-permeation ($\log BB = -1.89$). The others, although presenting a prediction to permeate such barrier, have more satisfactory results than rimonabant (< 1.5), indicating less affinity.

Regarding PSA, molecules that may be administered orally acting peripherally ($PSA > 70$) were: 01, 02, 04, 06, 07, 12, 16, 17, 20-22, 24, 26, 36-42, 45, 46, 50, 52, 55-57, 65, 67-71, 73, 74, 76 e 78. Only the Zinc molecule 49 had PSA lower than that of rimonabant.

Therefore, concerning pharmacokinetic properties, Zinc molecules that fitted within the desired criteria for parameters, and also that presented predictions indicating higher chances of being administered orally and with high chances of acting peripherally ($PSA > 70$ and $\log P < 4$) and act peripherally are: 01, 02, 04, 06, 07, 12, 16, 17, 20, 22, 36-38, 40-42, 45, 46, 52, 55-57, 67-71, 73 e 78.

3.6 Analysis of toxicological properties

Results obtained in the DEREK software were analyzed considering the following toxicological endpoints: mutagenicity, carcinogenicity, peroxisomal proliferation, hepatotoxicity, genotoxicity, neurotoxicity and reproductive effects. This software generates its output results using different confidence levels for each corresponding endpoint: CERTAIN (active), PROBABLE (probable), PLAUSIBLE / EQUIVOCAL (evidence is not strong / toxicity is not certain), DOUBTED (inactive but doubtful due to lack of experimental evidence) and IMPROBABLE / INACTIVE (unlikely) [49].

Rimonabant presented alerts for carcinogenicity (EQUIVOCAL) and HERG channel inhibition (PLAUSIBLE). When analyzing phytocannabinoids no alerts were fired about their genotoxicity, neurotoxicity or reproductive effects endpoints. Molecule 24 presented human toxicity alerts regarding nephrotoxicity (equivocal) and hepatotoxicity (equivocal), referring to the styrene or derivative toxicophore. Molecule 32 showed alert for hepatotoxicity (plausible for the p-alkylphenol or derivative toxicophore). Such plausible and equivocal results do not indicate strong evidence of these toxicities and can only be confirmed by experimental evaluation [47, 49].

Given the results obtained for the toxicological properties of phytocannabinoids, molecules 29 and 35 presented the best safety profile (no toxicity alerts) but did not present satisfactory results for activity and ADME predictions. Thus, considering the predictions of biological activity and physicochemical properties, molecules that allow an additional *in silico* evaluation to elucidate their mode of interaction

concerning our study objective, phytocannabinoids 01, 06, 07, 15 and 37 were considered, which present results for the prediction of toxicity even safer than those of Rimonabant.

The 78 molecules that were selected from the ZINC database were also evaluated in the DEREK software. All alerts have been fired as PLAUSIBLE, and their evidence of uncertainty can be confirmed or ruled out by experimental evaluation [47, 49]. The results are summarized below:

- *Human hepatotoxicity*: PLAUSIBLE for molecules 01, 09, 12, 14, 15, 19, 21, 22, 24, 27, 41-43, 45, 46 and 52, having as a toxicophore the benzimidazole or derivative radical, except for molecule 01, which its toxicophore was the furan radical;
- *Human carcinogenicity*: PLAUSIBLE alert for molecule 50, referring to the aromatic group amine or amide;
- *Human in vivo mutagenicity*: PLAUSIBLE for molecule 25, referring to the quinoline group;
- *Cyanidine-type effect alert*: for molecule 05, referring to the nitrile group, considered PLAUSIBLE;
- *Chromosome Damage Alert*: PLAUSIBLE for molecule 78 concerning the toxicophore group indole or benzotriazole;

The best profile Zinc molecules, considering the predictions of biological activity (anti-obesity activity potential >0.4) and physicochemical, pharmacokinetic and toxicological properties (safer than rimonabant), which were selected for *in silico* evaluation to better comprehend their interaction modes towards our target of interest are molecules 06, 17, 20, 38, 67, 69 and 70.

3.7 Molecular Docking Analysis

Molecular docking analysis was based on the results of Shao and collaborators [48] which conducted CB1 receptor molecular anchoring experiments in inactive conformation with PDB ID: 5U09. Validation by redocking [49] has been performed with a RMSD of 1.269 Å, as shown in Figure 4, when superimposing the originally complexed ligand taranabant from protein 5U09 (in green), and the best pose obtained from molecular docking (in blue). This result shows greater reliability when compared to the RMSD of Loo and collaborators [50], which was 2.74 Å for the best pose of antagonist AM115424 on the same protein.

The rCB1 structure has seven transmembrane domains (TM) connected through three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3). The N-terminal domain has a V-shaped loop formed by residues 99-111 that acts as a lid and is, therefore, responsible for favoring or restricting access of a binder to the pouch from the extracellular side [51].

In rCB1 structure (PDB ID: 5U09), the complexed antagonist molecule was taranabant, and the main interactions that occur between these molecules and rCB1, favorable to antagonism, are related to the following amino acids: Phe102, Met103, Asp104, Ile105, Phe108, Ile119, Ser123, Ile169, Phe170, Phe174, Leu193, Val196, Phe268, Trp279, Trp356, Leu359, Met363, Phe379, Ala380, Met384, Cys386 e Leu387 [52].

Filling the orthosteric pouch at the opening of TM1 and TM7 acts by blocking the entry of endocannabinoids, contributing to this blocking the amino acids Ile119 (TM1), Phe381 and Met384 (TM7). Interactions with amino acid residues of these two helices should favor the inactive conformation of the receptor. Leu387 belongs to the membrane access channel and favors the closing of the entrance channel, along with the amino acids mentioned above. TM1 is formed by residues Asn112 to Ser144, while TM7 is formed by residues Asn372 to Arg400. Arg214 and Asp338 favor canonical ion blockade. Lys192, Phe200 and Tyr275 indirectly contribute to conformational equilibrium, even without interacting with the antagonist. THC agonist binding is favored by Phe174, Leu193 and Ser383 [48].

Met103, Ile105, Phe108, Ile119, Phe170, Phe268, Trp279, Trp356, Phe379 and Leu387 are characterized by involvement in taranabant and rimonabant binding affinity but are part of the binding pool of agonists and antagonists [52]. ECL2 (Trp255-Ile271) has 4 residues (Phe268-Ile271) next to the TM5 which when projected into the binding bag mediated interactions that decrease agonist binding. ECL3 is associated with decreased flexibility of this loop region [51].

The interactions that occur with the residues Phe170 and Phe174, from TM2, favor the inclination in relation to TM1 (Arg150-Val179), while the interaction with Lys192 (from TM3) decreases the affinity of agonists and stabilizes the conformation of ECL1, from the domain N-terminal and extracellular part of TM2 and TM3,[51] in addition to moving the receptor to its inactive state [41] .

Murineddu and collaborators [52], when docking antagonist AM6538 into the rCB1 structure (PDB ID: 5TGZ), obtained a new hydrogen bond, now with Ser383, also important in antagonist binding. This docking study showed relevant amino acids from the active site within 10 Å: Phe102, Met103, Ile105, Ile119, Ser123, Ile169, Phe170, Phe174, His178, Leu193, Val196, Thr197, Phe268, Leu276, Val279, Val356, Leu359, Met363, Ala380, Ser383, Met384, C386 e Leu387.

Finally, proceeding as described in materials and methods, we present our docking results, revealing potential interactions of Rimonabant (Fig 5a) and the five selected phytocannabinoids that showed best physicochemical, pharmacokinetic, toxicological profile, among those with Pa >0.4 in the PASS prediction, which were: 01 (Fig 5b), 06 (Fig 5c), 07 (Fig 5d), 15 (Fig 5e) and 37 (Fig 5f). Although they did not present a pharmacokinetic profile favorable to peripheral action, knowing their way of binding to rCB1 allows obtaining additional parameters for analyzing the interaction of the virtual screening molecules on the ZINC server.

a) The rimonabant presented GoldScore of 86.20, performing a total of 12 interactions with the active site, with 10 amino acid residues belonging to the N-terminal domains (Met103 and Ile105), TM2 (Gly166), TM3 (Val196), ECL2 (Phe268), TM5 (Trp279) and TM7 (Phe379, Met384, Cys386 and Leu387). **b)** Molecule 01 (Δ^9 -THC) had a GoldScore of 73.44 and was able to perform ten interactions with the binding site within the 5.2 Å radius, mostly hydrophobic (pink strokes). Their interactions occurred in the N-terminal domain (Met103, Ile105 and Phe108), TM3 (Val196), TM6 (Trp356) and TM7 (Ala380, Met384 and Cys386). **c)** Molecule 06 (Δ^9 -tetrahydrocannabinol) had a GoldScore of 55.79, the smallest of the six

molecules presented and was able to perform fourteen interactions with the binding site within a 5.4Å radius. Their interactions occurred in the N-terminal (Phe102, Met103 and Phe108), ECL2 (Phe268), TM2 (Phe170), and TM7 (Phe379, Ala380, Met384, Cys386 and Leu387) domains. **d)** Molecule 07 (Δ^9 -tetrahydrocannabinol- C_4) had a GoldScore of 69.11 and was able to perform ten interactions with the binding site within the 5.6Å radius. Their interactions occurred in the N-terminal domain (Phe102, Ile105 and Phe108), TM3 (Val196) and TM7 (Ala380, Met384 and Cys386). **e)** Molecule 15 (Δ^8 -THC) had a GoldScore of 69.63 and performed ten interactions with the binding site within a radius of 4.9Å (the smallest interaction radius). Interactions occurred at N-terminal (Met103, Ile105 and Phe108), TM3 (Val196) and TM7 (Ala380, Met384 and Cys386). **f)** Molecule 37 (cis- Δ^9 -THC) presented GoldScore of 75.46, the largest of the phytocannabinoids evaluated, and performed thirteen interactions with the binding site, within a 5Å radius. Interactions occurred at N-terminal (Met103, Ile105 and Phe108), ECL2 (Phe268) and TM7 (Phe379, Ala380, Ser383 and Met384).

The rCB1 orthosteric pocket shows hydrophobic characteristics since this receptor is activated by lipids [30], which explains the fact that the vast majority of occurring interactions are hydrophobic. All phytocannabinoids interacted with the N-terminal domain (mostly hydrophobic bonds), which is related to the restriction of membrane access by ligands from the extracellular side [51]. All had a radius of interaction with the active site below 10 Å, none showed interaction with Lys192, but molecule 37 (Fig. 5f) made an important hydrogen bond with Ser383 [52].

The molecule with the smallest radius of interaction with active site residues was 15 (Fig. 5e), which hinders for an agonist to enter by reducing the access channel, although it has interacted only with seven amino acid residues of three transmembrane domains (including Met384). Rimonabant (Fig. 5a) performed hydrogen bonding between Met103 and the HD region of the rimonabant. Its GoldScore was not surpassed by phytocannabinoids, the closest result being that of molecule 37 (Fig. 5f). The molecule that presented the greatest interaction with the transmembrane domains was molecule 06 (Fig. 5c), displaying fourteen interactions with eleven amino acid residues (including Phe170, Met384 and Leu387). However, it had the lowest GoldScore of the ranking (55.79).

Molecule 01 (Fig. 5b) is known for its CNS psychotropic action on the CB1 receptor as an agonist. It presented a high GoldScore (73.44) and interacted with eight amino acid residues from four domains (including Met384) without showing hydrogen bonds. Molecule 07 (Fig. 5d) interacted with only seven amino acid residues from three transmembrane domains (including Met384), also without presenting hydrogen interactions in the antagonist interaction. Molecule 37 (Fig. 5f) interacted with eleven (four domains) and eight (three domains) amino acid residues, respectively, showing hydrogen bonds with Ser383, and other interactions to residues Phe268 and Met384.

Regarding the docking of Zinc molecules, the following results reveal the anchoring of molecules 06 (Fig. 6a), 17 (Fig. 6b), 20 (Fig. 6c), 38 (Fig. 6d), 67 (Fig. 6e), 69 (Fig. 6f) and 70 (Fig. 6g). These were the ones with better physicochemical, pharmacokinetic and toxicological profile among those that had, in PASS prediction, rCB1 antagonist activity and anti-obesity activity, with $Pa > 0.4$. Docking results showing

specific interactions occurring between these Zinc molecules and the active site are shown in the figure are shown below.

a) The Zinc 06 had a GoldScore of 76.67 and performed eight interactions with the binding site within a radius of 5.3Å. Interactions occurred at N-terminal (Met103, Ile105 and Phe108), TM1 (Ile119), TM2 (Phe170 and Phe174) and TM7 (Ala380 and Leu387). **b)** The Zinc 17 had a GoldScore of 78.19 and performed twelve interactions with the binding site within a radius of 5.9Å. Interactions occurred at N-terminal (Asn101, Phe102, Ile105 and Phe108), TM2 (Phe170), TM3 (Val196), ECL2 (Pro269) and TM7 (Phe379, Ala380, Ser383, Cys386 and Leu387). **c)** The Zinc 20 had a GoldScore of 82.97 and performed fourteen interactions with the binding site within a radius of 5.9Å. Interactions occurred at N-terminal (Met103, Asp104, Ile105 and Phe108), TM2 (Phe174 and His178), TM3 (Val196), ECL2 (Phe268), TM6 (Leu359) and TM7 (Phe379 and Ala380). **d)** The Zinc 38 had a GoldScore of 74.79 and performed eleven interactions with the binding site within a 5.4Å radius. Interactions occurred at N-terminal (Phe102, Met103, Ile105 and Phe108), TM3 (Val196), ECL2 (Phe268) and TM7 (Ser383). **e)** The Zinc 67 had a GoldScore of 79.83 and performed thirteen interactions with the binding site within a radius of 5.67Å. Interactions occurred at N-terminal (Phe102 and Met103), TM2 (Phe170), TM3 (Leu193 and Val196), ECL2 (Phe268), TM5 (Trp279) and TM7 (Ser383 and Cys386). **f)** The Zinc 69 had a GoldScore of 80.28 and performed eighteen interactions with the binding site within a radius of 5.44Å. Interactions occurred at N-terminal (Phe102, Met103 and Asp104), TM3 (Leu193 and Val196), TM5 (Trp279) ECL2 (Ile267, Phe268 and Pro269) and TM7 (Ser383 and Cys386). **g)** The Zinc 70 had a GoldScore of 87.29 and performed eleven interactions with the binding site within a radius of 5.7Å. Interactions occurred at N-terminal (Phe102, Met103 and Phe108), TM3 (Val196), ECL2 (Pro269) and TM7 (Ala380 and Ser383).

The Zinc molecule 06 (Fig. 6a) did not show a hydrogen bond with Ser383 but showed a high value of GoldScore, which was 76.67, higher than phytocannabinoid 37 (Fig. 5f) (75.46), however less than that of rimonabanto (Fig. 5a). It interacted with eight amino acids from four domains, in a radius of 5.3 Å, having as key residues Ile119, Phe170, Phe174 and Leu387, which favor the blockade of the anandamide endocannabinoid entrance by closing the entrance channel by tilting towards TM1.

The Zinc molecule 17 (Fig. 6b) interacted with five transmembrane domains, including N-terminal, ECL2 and TM7, its GoldScore was close to 80, performing interactions with more amino acid residues from the active site (12) than rimonabant (10), performing interactions of hydrogen between Ser383 and hydrogen donor region, and between Pro269 hydrogen acceptor region. The Zinc molecule 20 (Fig. 6c) had a GoldScore of 82.97, performing 14 interactions with the active site in six transmembrane domains. It performed Hydrogen bonds between Met103 and His178 and the molecule's hydrogen donor region.

The Zinc molecule 38 (Fig. 6d) also interacted with eight active site amino acid residues, showing a greater number of favorable interactions, totaling eleven, in four domains within a radius of 5.3 Å. Its GoldScore of 74.79 was slightly lower than that of phytocannabinoid 37 (Fig. 5f), and it presented two key interactions with Phe268 and a hydrogen bond with Ser383, which reduces endocannabinoid agonist binding and favors greater interaction strength and stability.

The Zinc molecule 67 (Fig. 6e) had a higher GoldScore than phytocannabinoid 37 (Fig. 5f), but no higher than rimonabant (Fig. 5a). It performed 13 interactions with the active site in six transmembrane domains, with two hydrogen bonds between Ser383 and the hydrogen donor and acceptor region.

The Zinc molecule 69 (Fig. 6f) had the highest number of interactions with the active site (18) in five transmembrane domains, with GoldScore greater than 80. It performed hydrogen bonds between Ser383 and the hydrogen acceptor region, and between Pro269 and region hydrogen donor. The Zinc molecule 70 (Fig. 6g) was the one that had the best result in docking. Its GoldScore of 87.29, surpassing that of rimonabanto (Fig. 5a) (86.20), performing eleven interactions with four transmembrane domains, including three hydrogen bonds with Ser383 and hydrogen acceptor regions.

Evaluating the results, it can be seen that the phytocannabinoid molecules did not exceed the docking parameters of the rimonabant molecule. Now the Zinc molecules present better quality docking, surpassing rimonabant in GoldScore (zinc molecule 70), and in the number of interactions with the active site (Zinc molecules 17, 20, 67 and 69), where all interacted with the domains transmembrane-related antagonist activity in rCB1. The two best molecules were Zinc 69 and 70 that had a prediction of anti-obesity activity > 0.5 and with GoldScores values 80.29 and 87.29, respectively, the latter being superior to the reference ligand rimonabant, showing that these are molecules potential for future studies of anti-obesity activity.

3.8 Molecular Dynamics Analysis

The docking poses of the Zinc 69 and Zinc 70 compounds were selected as the initial conformations for the MD simulations. For the systems evaluated (Fig. 7 the RMSD values of each system tend to equilibrium after 10 ns simulation time and the average values are less than $2.81 \pm 0.55 \text{ \AA}$, suggesting that the structures were stable throughout the MD simulation

As show in Figure 8, the B-factor plot shows the loop region formed by the Val367-Lys373 residues (represented in red), that presented greater fluctuation to the Zinc 69 compound. In addition, there is another flexible region in the protein at the N-terminal domain.

The results of the binding free energy calculations of the two analyzed compounds with the MM-PBSA/GBSA methods are shown in Table 7. Table 7 shows that the formation of the two complexes formed by the enzyme and the ligands is favorable. Furthermore, comparing the binding free energy values (MM-GB(PB)SA) shows that the Zinc 69 ligand presented the lowest energy values when compared to the Zinc 70.

Table 7 - Free energy values with the respective standard deviation values calculated by MM-GB(PB)SA method

Complex	ΔG_{MMGBSA} (kcal/mol)	ΔG_{MMPBSA} (kcal/mol)
Enzyme-Zinc 69	-58.91 $\pm$ 2.70	-44.06 $\pm$ 3.13
Enzyme – Zinc 70	- 48.82 $\pm$ 3.19	-37.36 $\pm$ 3.93

The energy contribution of each residue for the complexes formed by the Zinc 69 and Zinc 70 obtained by the MM-GBSA method are shown in Figure 9. In this figure on the right side is shown the residue contributions to binding energies using the plugin CHEWD [53] with Chimera software. From this, favorable interactions are at the blue of the color scale, that is, those that contribute to the stabilization of ligand into complex, while the red color represents interaction by residue with positive values and corresponds to unfavorable interactions values.

Figure 9 (A and B) was used to explore the relative energy contribution to binding free energy decomposition of the complexes. Figure 9A revealed the residues that most significantly contribute to the total binding energy and, therefore, to the stabilization of the complex are Phe102, Met103, Phe170, Val196, Phe200, Phe268, Trp279, Trp356, Leu359 and Cys386. Figure 9B shows some of the residues that contribute to the stabilization of the enzyme-Zinc 70 complex. For this system, we can highlight the residues Phe102, Met103, Ile105, Phe108, Phe170, Val196, Phe268, Trp356, Phe379 and Ser383. It is worth highlighting that residues Phe102, Met103, Phe170, Val196, Phe268 and Trp356 were important for both ligands. Besides, the interactions with residuals Phe102, Met103, Phe108, Val196, Trp279, Phe268, Ser383 and Cys386 had already been observed in the molecular docking results.

4. Conclusion

Molecular modeling studies performed in this work allowed us to disclose that the selected phytocannabinoids evaluated here, have shown to be potential rCB1 antagonists, according to their predicted biological activities by the PASS server. Moreover, docking results showed a broad possibility of establishing key interactions between these molecules and the receptor, considering GoldScores values greater than the original ligand taranabant from the crystallographic structure. It is worth to note that the phytocannabinoid that would most likely present a strong tendency to act as a rCB1 antagonist, with interest in the anti-obesity activity, is molecule 37 since it presented a relevant hydrogen bond with Ser383 that is essential for antagonists' activity. However, all have a prediction of action in the CNS, profile that is related to the triggering of adverse reactions inherent to the pharmacological target studied.

When comparing to another virtual screening campaigns previously found in the literature, our virtual screening performance showed promising results, because it was able to achieve potential molecules with positive predictions for rCB1 antagonism and also indicated their potential to possess anti-obesity activity, with $\text{Pa} > 0.4$. Their corresponding docking results were also significant since it was observed similar interactions to the reference and GoldScores values greater than 80.00, with the highest result for the Zinc 70 molecule surpassing rimonabant, and this may suggest binding affinities as intense as those observed for phytocannabinoids. To sum up, the best profile screened Zinc molecules to act as rCB1

antagonist in peripheral antiobesity activity were 69 e 70 (for presenting key binding for the blockade of anandamide endocannabinoid agonist and the high GoldScores). MD simulations indicate that the selected ligands are stable, with little variation of the RMSD values along the trajectory, and the prediction of the binding energy with the MM-GB(PB)SA methods showed that the formation of the complexes are favorable. Besides, the decomposition of the binding free energy revealed that the complexes formed between the Zinc 69 and 70 form interactions with residues Phe102, Met103, Phe170, Val196, Phe268 and Trp356, which are important for catalytic activity.

In addition, these molecules have a possible factor favorable to the safety of the use of a peripheral level cannabinoid antagonist, due to its pharmacokinetic profile, which may overcome the barriers of adverse effects inherent in this pharmacological class.

Declarations

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Figures

Figure 1

Representation of chemical structures of 41 phytocannabinoids present in *C. Sativa* with rCB1 antagonism activity

Figure 2

A pharmacophoric pattern containing five points obtained by alignment 32 molecules with the best potential antagonist over rCB1

Figure 3

Representation of chemical structures of 78 Zinc molecules obtained as hits

Figure 4

Docking Validation showing original ligand taranabant from complex 5U09 in green, and the best pose obtained from molecular docking in blue

Figure 5

Molecular docking of Rimonabant and selected phytocannabinoids

Figure 7

Graphical representation of the RMSD values versus simulation time for the two studied complexes

Figure 8

Graphical representation of the structural fluctuations taking into account the enzyme's residues from the MD simulations

Figure 9

Interaction graph by residues for the (A) Zinc 69 and (B) and Zinc 70 complexes obtained in Amber 18 software and on the right, the residues obtained with the CHEWD plugin in the Chimera program, the residues that more contributed the are shown in blue