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Virtual screening and in vitro experiments highlight cannabidiol as a drug-like phosphodiesterase 9 inhibitor

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Abstract

The growing interest on the therapeutic potential against neurodegeneration of *Cannabis sativa* extracts, and of phytocannabinoids in particular, is paralleled by a limited understanding of the undergoing biochemical pathways in which these natural compounds may be involved. Computational tools are nowadays commonly enrolled in the drug discovery workflow and can guide the investigation of macromolecular targets for such molecules. In this contribution, in silico techniques have been applied to the study of *C. sativa* constituents at various extents, and a total of seven phytocannabinoids and four terpenes were considered. On the side of ligand-based virtual screening, physico-chemical descriptors were computed and evaluated, highlighting the phytocannabinoids possessing suitable drug-like properties to potentially target the central nervous system. Our previous findings and literature data prompted us to investigate the interaction of these molecules with phosphodiesterases (PDEs), a family of enzymes being studied for the development of therapeutic agents against neurodegeneration. Among the compounds, structure-based techniques such as docking and molecular dynamics (MD), highlighted cannabidiol (CBD) as a potential and selective PDE9 ligand, since a promising calculated binding energy value (−9.1 kcal/mol) and a stable interaction in the MD simulation timeframe were predicted.

Additionally, PDE9 inhibition assay confirmed the computational results, and showed that CBD inhibits the enzyme in the nanomolar range in vitro, paving the way for further development of this phytocannabinoid as a therapeutic option against neurodegeneration.

KEYWORDS
CBD, docking, molecular dynamics, neurodegeneration, PDE9

Giovanni Ribaudo and Elisa Landucci contributed equally.

1 | INTRODUCTION

Neurodegenerative diseases result from neuronal disfunction and cell death and are categorized as amyloidosis, which include Alzheimer's disease (AD), tauopathies, α-synucleinopathies and TDP-43 proteinopathies (Dickson, 2009). Importantly, such disorders are predicted to become the second most prevalent cause of death by 2040 according to World Health Organization (Gammon, 2014; Stone et al., 2020). Current treatments of these neurological conditions are mostly limited to symptoms management rather than providing a direct effect on the underlying biochemical mechanisms that trigger disease onset and progression. More specifically, pharmacological options for the treatment of AD, which is the most common form of dementia affecting elderly people, include acetylcholinesterase (AChE) inhibitors (donepezil, rivastigmine and galantamine) and compounds targeting N-methyl-D-aspartate (NMDA) receptors, such as memantine (Marcos-Rabal et al., 2021; Zanforlin et al., 2017b). Moreover, gantenerumab, a monoclonal antibody that promotes amyloid plaque reductions, was recently approved for the treatment of AD (Salloway et al., 2021; Selkoe, 2021). Nevertheless, as the average age of the population increases, there is an urgent need to identify new targets and to develop new therapies in order to overcome the limitations in terms of efficacy and side effects of currently available drugs (Prickaerts et al., 2017).

While the hypothesis that *Cannabis* or its extracts could ameliorate neurodegenerative conditions dates back to at least two decades ago (Jackson et al., 2004), the investigation of the underlying molecular mechanisms is still undergoing. Over 400 chemical species, including more than 100 terpeno-phenol compounds, termed phytocannabinoids, have been identified in *Cannabis sativa* (Cassano et al., 2020). The current definition of 'cannabinoids' is rather broad and encompasses endocannabinoids, phytocannabinoids acting on the cannabinoid receptors and synthetic cannabinoids. More traditionally, cannabinoids are defined as oxygenated aromatic hydrocarbon bearing 21 carbon atoms derived from marijuana, which are classified into 10 subclasses (dos Reis Rosa Franco et al., 2021). Both psychotropic and non-psychoactive phytocannabinoids have been isolated from *C. sativa*, and while Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the most abundant and widely studied, literature recently started focusing also on 'minor phytocannabinoids' in light of their promising neuroprotective potential (Stone et al., 2020).

In general, phytocannabinoids are endowed with a promiscuous activity on a variety of targets, and they are reported to modulate several receptors. In particular, they interact at different extents with cannabinoid receptors CB1R and CB2R (Grotenhermen, 2003; Howlett & Abood, 2017). Through the activity on the endocannabinoid system, such compounds can increase anandamide, enhancing dopamine concentration and transmission. Moreover, direct or indirect effects on transient receptor potential vanilloid (TRPV) channels could represent the mechanism leading to antipsychotic and antidepressant effects observed in animal models. In this connection, we recently proposed that CBD preferentially binds TRPV2 over TRPV1 (Landucci et al., 2022). Interaction with serotoninergic receptor (5-HT_{1A}) and peroxisome proliferator-activated receptor gamma (PPARγ) appears to be involved in their reported neuroprotective activity, together with effects on adenosine signalling and G-protein-coupled receptors (dos Reis Rosa Franco et al., 2021; Landucci et al., 2021; Mori et al., 2021). Literature on the potential effects of *Cannabis* components on neurodegeneration is very prolific, as testified by recently emerging contributions, that outline the putative role of CBD against neurological damage induced by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) (Sarkar et al., 2021). It must be also considered that the chemical structure of phytocannabinoids, bearing phenolic moieties, hydroxyl groups and aliphatic residues, allows at the same time antioxidant capability and a good blood–brain barrier (BBB) permeability (Stone et al., 2020). Moreover, anti-inflammatory properties have been reported (Cassano et al., 2020). Thus, considered that brain is extremely sensitive to damage produced by oxidative stress and inflammation, these features, together with the polypharmacological profile of *Cannabis* components, prompt the investigation of phytocannabinoids as potential therapeutic agents for the treatment of central nervous system (CNS)-related diseases.

Additionally, it must be considered that other molecules may contribute to the effects of *Cannabis* extracts. The so-called 'entourage effect' relates to the synergistic action of other components of the phytocomplex, such as terpenes. Terpenes are the most abundant class of naturally occurring small molecules and are constituted by isoprene subunits forming cyclic or acyclic structures, and recent reports are focusing on their potential role in neuroprotection (Weston-Green et al., 2021).

The aim of this study is to investigate at the molecular level, with the aid of computational tools derived from the drug discovery workflow, the potential of representative chemical constituents of *C. sativa* against neurodegeneration and to provide a preliminary

proof of concept of an underlying, previously undisclosed, mechanism of action.

2 | MATERIALS AND METHODS

2.1 | Prediction of physicochemical descriptors

Physicochemical descriptors relevant for drug-likeness, absorption, distribution, metabolism, and excretion (ADME) parameters and pharmacokinetic properties were calculated using the SwissADME tool (www.swissadme.ch, Molecular Modeling Group–Swiss Institute of Bioinformatics, Lausanne, CH) (Daina et al., 2017; Daina & Zoete, 2016). Topological polar surface area, logP, volume, molecular weight, number of rotatable bonds, number of hydrogen bond donors and acceptors were computed and analysed, together with other parameters relevant for the prediction of absorption rate. Combined results were plotted as radar graphs, highlighting the ideal chemical space for oral bioavailability.

2.2 | Molecular docking

The files containing the atomic coordinates for the considered proteins were obtained from the RCSB Protein Data Bank (PDB, www.rcsb.org). The blind docking experiments of the studied ligands towards the target proteins were performed using Autodock Vina (Molecular Graphics Laboratory, Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, CA, USA) (Trott & Olson, 2009). The number of docking poses was set to 10, with other Vina parameters set as default. Grid parameters for PDE enzymes are reported in the following. For PDE4, 1XOT model was selected (organism: *Homo sapiens*, expression system: *Escherichia coli*). Grid centre: $x = 2.282$, $y = 2.337$, $z = 53.001$, size: $52.620 \times 49.938 \times 59.490$. For PDE5, 2H42 model was selected (organism: *H. sapiens*, expression system: *E. coli* BL21). Grid centre: $x = 21.158$, $y = 125.010$, $z = 16.968$, size: $61.818 \times 53.522 \times 56.831$. For PDE9, 4Y86 model was selected (organism: *H. sapiens*, expression system: *E. coli* BL21). Grid centre: $x = 84.750$, $y = 45.462$, $z = 45.118$, size $57.343 \times 65.975 \times 59.104$ Å. Residue numbering used in the PDB files was adopted, and no further modifications were operated on the protein models. Output data, such as calculated binding energy and interaction patterns, were analysed and scored using Chimera molecular viewer (UCSF, San Francisco, CA, USA) (Pettersen et al., 2004), which was also used to produce the artworks.

2.3 | Molecular dynamics simulations

MD simulations were carried out using PlayMolecule (Accelera, Middlesex, UK) starting from the output model of docking experiment. More in detail, ligand was prepared by running the Parametrize function, based on GAFF2 force field (Galvelis et al., 2019). The complex was prepared for the MD simulation using ProteinPrepare and SystemBuilder functions, setting pH = 7.4, AMBER force field and default experiment parameters (Martínez-Rosell et al., 2017). Simulation runs of 25 ns in presence of water in a globular system were carried out using SimpleRun tool with default settings. Plotting of root-mean-square deviation (RMSD) values over time was performed using Excel 15.31 (Microsoft Corporation, Redmond, WA, USA).

2.4 | Assessment of PDE9 inhibitory activity

To assess the inhibitory activity of CBD and PF-04447943 against full-length recombinant human PDE9 (PDE9A) (SignalChem, Richmond, Canada), PDE-Glo Phosphodiesterase Assay (Promega Corp., Madison, WI, USA) was used. The compounds were dissolved in DMSO and mixed with the PDE-Glo Reaction Buffer at a v/v ratio of 1:5. Aliquots of PDE-Glo Reaction Buffer with appropriate amounts of human recombinant PDE9A were placed in separate wells of a 96-well plate (PerkinElmer, Milan, Italy). Subsequently, the solution (5 µl) containing a PDE inhibitor and 12.5 µl of cGMP solutions as substrate were added to each well. Each time after addition of the solutions, the plates were gently shaken on a 3D Rotator to ensure the content was evenly distributed over the bottom of each well (PS-M3D Variable Speed/Angle, Multi-Function 3D Rotator, Kisker Biotech GmbH & Co. KG, Steinfurt, Germany). The samples were incubated for 90 min at room temperature. Then, PDE-Glo Termination Buffer and PDE-Glo Detection Solution (12.5 µl) were added to each well. After incubation (20 min, room temperature) and an addition of Kinase-Glo Reagent (50 µl) to each well, the luminescence of each sample was measured by a microplate scintillation and luminescence counter reader TopCountNXT (Packard, Ramsey, MN, USA). IC_{50} values were estimated using nonlinear regression using Prism v.8 for Windows (GraphPad Software, San Diego, CA, USA). Experiments were performed in triplicates.

3 | RESULTS

3.1 | Prediction of physicochemical descriptors

The phytocannabinoids included in this study are represented by CBD, cannabidiolic acid (CBDA), THC, Δ^9-tetrahydrocannabinolic acid (THCA), cannabinol (CBN), cannabitriol (CBT) and cannabigerolic acid (CBGA). Moreover, terpenes β-myrcene, α-pinene, trans-β-ocimene and α-terpinolene were considered. The chemical structures of such compounds are reported in Figure 1.

The cited terpenes were chosen because of their high concentration in *indica* and *sativa* strains of *Cannabis* (Casano et al., 2011). Similarly, the phytocannabinoids were selected because of their relevant concentrations in these strains and since some of them were considered in previous studies as representative of different chemical categories (Citti et al., 2019; Dei Cas et al., 2020; Stefkov et al., 2022).

SwissADME software was used to predict the physicochemical descriptor relevant for the drug-likeness of orally bioavailable compounds, and the results are resumed in Figure 1. Overall, phytocannabinoids possess more drug-like features when compared to terpenes, even if their lipophilicity was highlighted as a possible pitfall by this screening. It must be also noted that, according to SwissADME calculations, terpenes are not predicted to be absorbed through the gastrointestinal (GI) tract, and very limited bioavailability is expected. In a similar manner, acidic forms CBDA, THCA and CBGA are not predicted to cross BBB. Thus, according to this ligand-based screening, CBD, THC, CBN and CBT were highlighted as the most appropriate drug candidates and were carried to the next step of investigation, while the other compounds were discarded.

3.2 | Molecular docking

A blind docking approach was enrolled to investigate the interaction motif of CBD, THC, CBN and CBT, selected according to the ligand-based screening described above, towards the following phosphodiesterase (PDE) isoforms: PDE4, PDE5 and PDE9. The results, in terms of calculated binding energy values and predicted interaction pattern, are resumed in Figure 2. For all the screened compounds, docking provided calculated binding energy values below −7.5 kcal/mol, which is considered a cut-off value for the identification of ligands (Proto et al., 2017). Nevertheless, a peculiar behaviour was observed for CBD, that showed a preferential interaction with PDE9. Thus, the interaction of the compound with this isoform was investigated more in detail. In Figure 2, the predicted binding motif of CBD with PDE9 is also depicted. THC also showed a high predicted affinity for PDE9 in this simulation, but this compound was discarded in light of its known psychotropic properties and lack of selectivity among isoforms.

3.3 | Molecular dynamics simulations

Following ligand-based screening and docking experiments, that outlined the selectivity of CBD for PDE9, the stability of the complex formed by the phytocannabinoid with this isoform was investigated by means of MD simulations. The complex reached stabilization within few nanoseconds and low RMSD fluctuations were observed for both the protein and the ligand. Importantly, CBD was retained within the binding site throughout simulation time (Figure 3).

3.4 | Assessment of PDE9 inhibitory activity

CBD and the selective PDE9 inhibitor PF-04447943 as reference compound (Hutson et al., 2011; Lee et al., 2015) were tested as to their ability to inhibit PDE9 in vitro. Experimentally, PDE9 activity was assayed in the presence of increasing concentrations of the single compounds, and the results were converted into percentages to calculate the IC_{50} values (Figure 4). An IC_{50} value of 110 nM was calculated for CBD, and of 14 nM for PF-04447943.

4 | DISCUSSION

With the current study, we aimed at dissecting one of the possible molecular mechanisms underlying the potential of *C. sativa* components in the context of CNS-related diseases, and against neurodegeneration in particular, given the multifaceted neuroprotective activity showed by phytocannabinoids and other *C. sativa* constituents.

A total of seven phytocannabinoids and four terpenes were included in the workflow, and the compounds were chosen also considering previous studies reported in literature as representative of different chemical categories of *Cannabis* constituents (Casano et al., 2011; Citti et al., 2019; Dei Cas et al., 2020; Sommano et al., 2020; Stefkov et al., 2022; Stone et al., 2020). In this connection, it must be considered that recent literature reporting bioactivity data of *Cannabis* extracts against

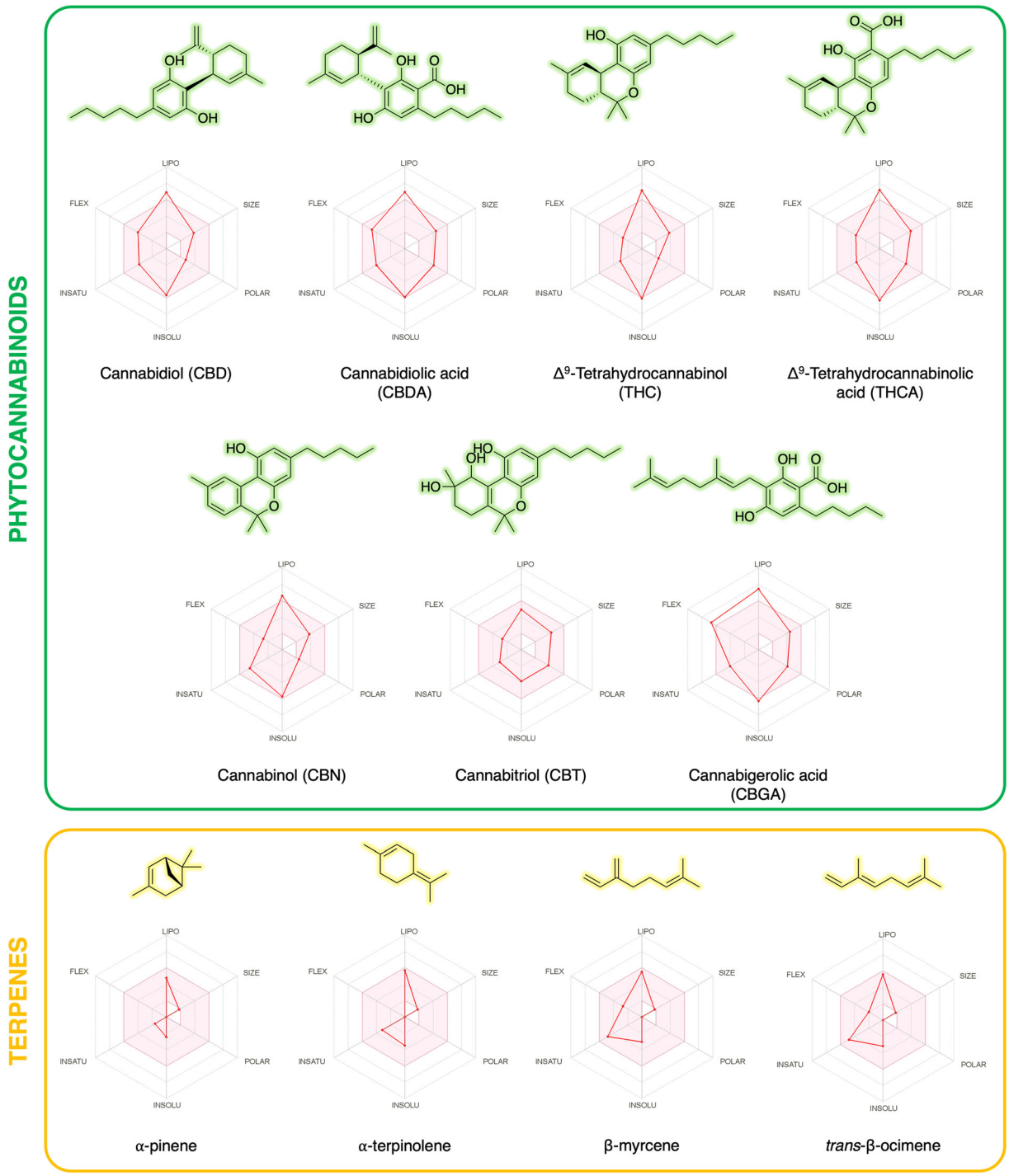

FIGURE 1 Chemical structures of the studied phytocannabinoids and terpenes and overview of the predicted physicochemical descriptors. The red area in the radar graphs represents the suitable physicochemical space for oral bioavailability according to lipophilicity, size, polarity, solubility, insaturation and flexibility scores of the molecules.

neurodegeneration does not focus anymore on phytocannabinoids only, but also on the role of the phytocomplex, which includes other constituents, such as, indeed, terpenes (Weston-Green et al., 2021).

Initially, the small pool of 11 natural compounds (Figure 1) underwent a computational drug discovery workflow, since ligand-based and structure-based in silico techniques were enrolled to screen the molecules. First, physicochemical features relevant for the drug-likeness of orally bioavailable, CNS targeting compounds, were computed. The widely accepted 'Lipinski's rule' defines the reference values for such

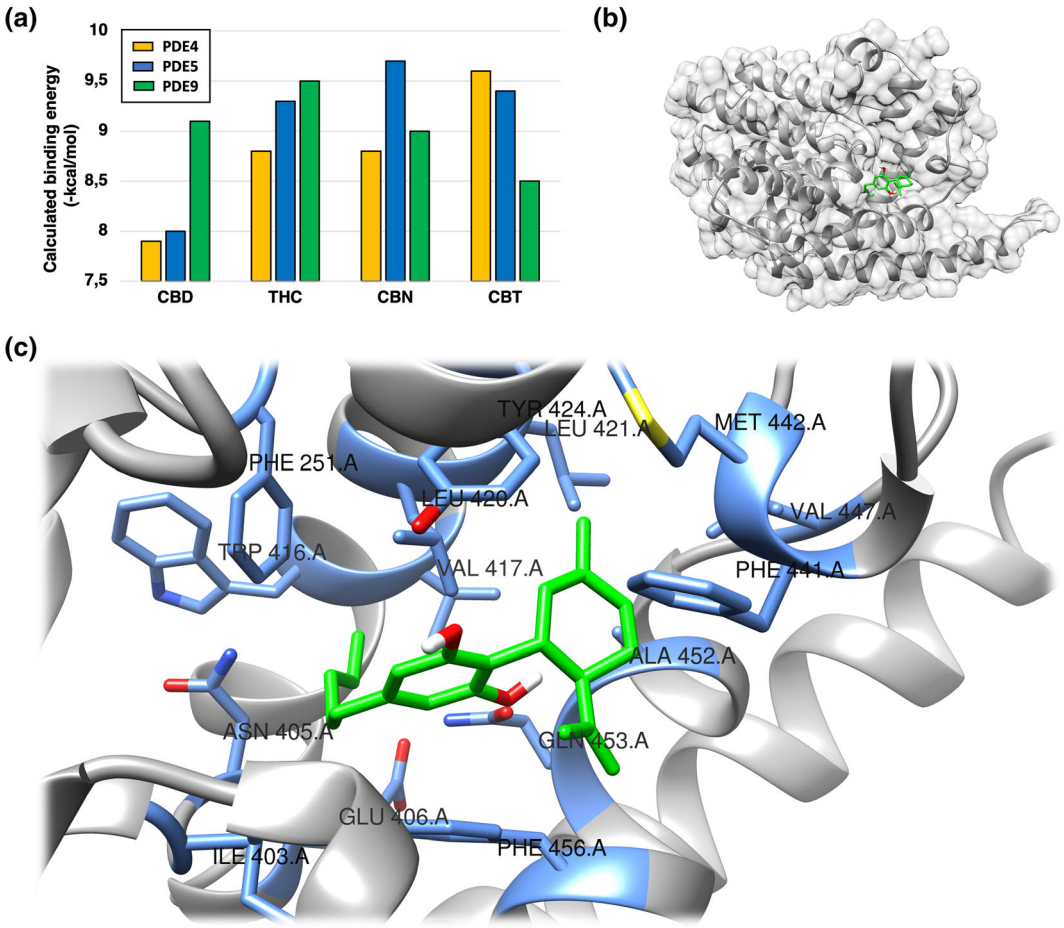

FIGURE 2 Calculated binding energy values for the interaction of the compounds with PDE4, PDE5 and PDE9; the value predicted for the interaction of the positive control PF-0447943 with PDE9 is reported for reference (a). Predicted complex for the interaction of cannabidiol (CBD) with PDE9 (b). Detailed view of the residues of PDE9 interacting with CBD (<5 Å) within the binding pocket (c)

descriptors, including number of hydrogen bond donors and acceptors, molecular weight and octanol–water partition coefficient for orally bioavailable drug candidates (Lipinski et al., 2001). In general, these parameters influence membrane diffusion, transport properties such as BBB crossing, bioavailability and the percentage of absorption. Additionally, Pajouhesh and Lenz outlined the chemical and structural features of a successful CNS-targeting compound, for which some of the parameters are stricter (Pajouhesh & Lenz, 2005). More in detail, lower topological polar surface area values, molecular weight and number of hydrogen bond donors and acceptors are tolerated. Moreover, it must be considered that molecules bearing ionizable groups (e.g., acidic functions), are in general not predicted to efficiently cross BBB.

Concerning the tested compounds, terpenes showed several violations of the drug-likeness parameters, as could also have been envisioned considering their low molecular weight, rigid and hydrophobic structures. Accordingly, low GI absorption was predicted for them. Two violations in the drug-likeness radar graph were recorded for CBGA (lipofilicity and flexibility), one for CBD, CBDA, THC, THCA and CBN (lipophilicity), while CBT falls within the ideal predicted chemical space for drug-like compounds (Figure 1). CBD, THC, CBN and CBT were also predicted to efficiently cross BBB and were thus carried to the next step of investigation.

In a previous study, through a ligand-based literature-refined target fishing approach, we highlighted esterase enzymes, and acetylcholinesterase (AChE) in particular, as putative macromolecular targets for phytocannabinoids (Mastinu et al., 2020). Considering also the growing attention received lately by PDEs as potential targets for developing new therapeutic options against neurodegeneration (Ribaudo et al., 2021a, 2021b; Xi et al., 2022), we aimed at assessing the involvement of selected phytocannabinoids in this pathway.

In general, PDEs influence intracellular signal transduction by regulating 3′,5′-cyclic adenosine

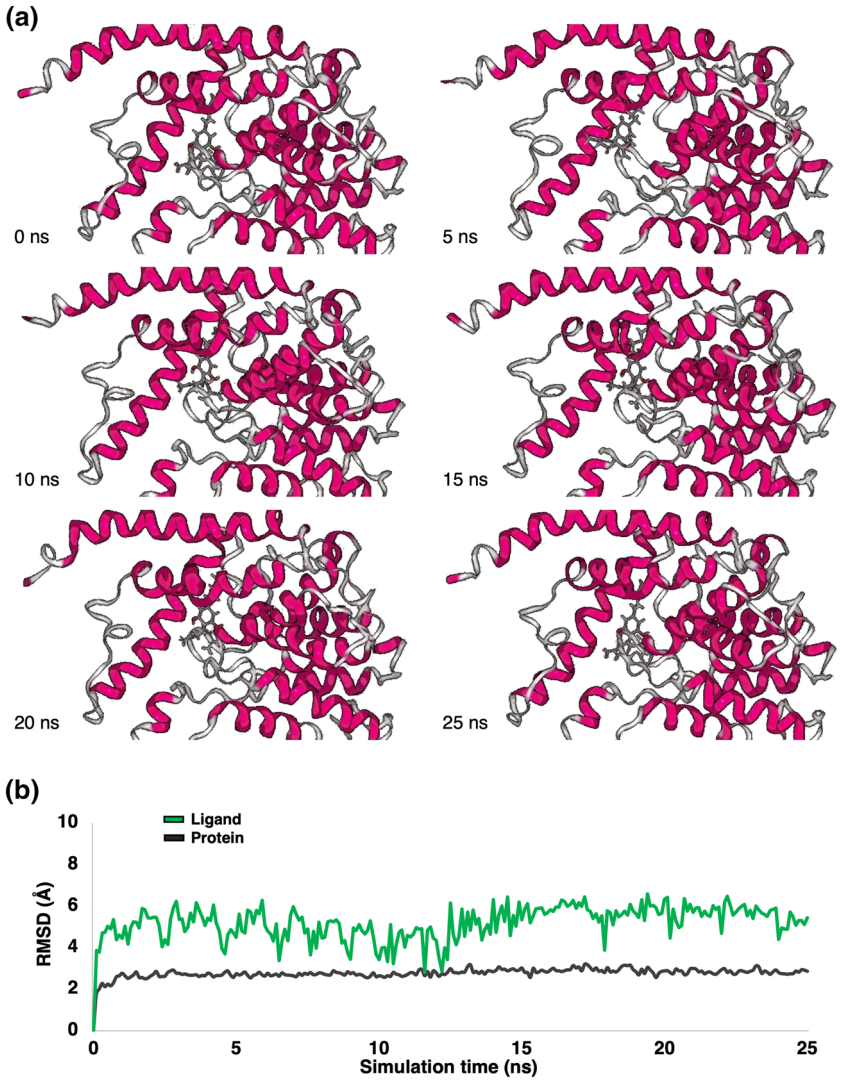

FIGURE 3 Representative frames of the molecular dynamics (MD) simulation after 0, 5, 10, 15, 20 and 25 ns (a). Root-mean-square deviation (RMSD) trajectories of the ligand and of the C_{α}s of the protein (b)

monophosphate (cAMP) and/or 3′,5′-cyclic guanosine monophosphate (cGMP) levels, that they hydrolyze to their linear analogues. PDE4, PDE7 and PDE8 are cAMP-selective, while PDE5, PDE6 and PDE9 are cGMP-selective. PDE1, PDE2, PDE3, PDE10 and PDE11 are referred to as 'dual substrate' enzymes (Bender & Beavo, 2006). PDEs are present in several tissues, and except for PDE11 and for PDE6, all PDE families are expressed in the CNS, to different extents (Argyrousi et al., 2020). In the context of neuroprotection and cognitive enhancement, increased cAMP and cGMP levels mediated by PDE inhibition promote neurotransmitter release, amelioration of microvascular dysfunction and neuronal plasticity (Kraus & Prast, 2001; Nakagawa et al., 2002). The involved biochemical mechanisms include stimulation of cAMP response element binding protein (CREB) and brain derived neurotropic factor (BDNF), as well as improved release of nitric oxide (NO) (Kumar et al., 2015). Besides applications connected with their peripherical role, in light of their localization also in brain, PDE4, PDE5 and PDE9 are among the most studied isoform in the context of drug discovery against neurodegeneration (Cameron et al., 2017; Ongaro et al., 2021; Ribaudo et al., 2015, 2016, 2020, 2021a). In particular, PDE9 outstands since it is present in amygdala, cerebellum, cortex, hippocampus, hypothalamus, midbrain, olfactory bulb and striatum (Lakics et al., 2010), and it has been found to be highly expressed in the brain of AD patients (Kumar et al., 2015). Moreover, PDE9 inhibitors are already being developed with the aim of ameliorating dementia conditions (Rosenbrock et al., 2019).

While literature on natural compounds acting as neuroprotective agents through different mechanisms is prolific (Basha et al., 2021; Panda & Jhanji, 2020; Ribaudo et al., 2018, 2019; Uddin et al., 2021; Zanforlin et al., 2017a), only few, quite outdated reports focusing on the activity of phytocannabinoids on PDEs at the central level are present: the topic is controversial and such

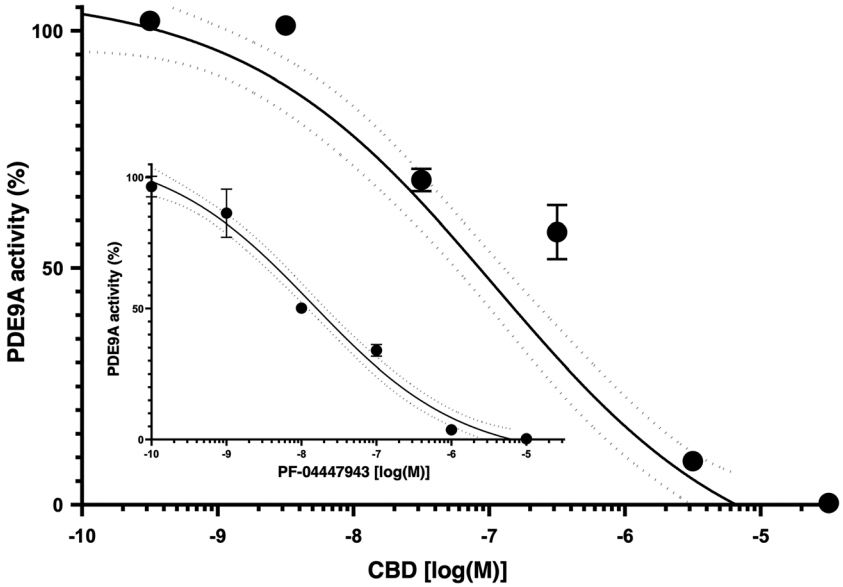

FIGURE 4 Concentration-dependent inhibitory activity of cannabidiol (CBD) on PDE9 in vitro. The concentration-dependent inhibition curve of the reference compound PF-04447943 is reported in the inset of the figure. Results are shown as mean of triplicates ± standard deviation.

reports lack of insights on the molecular interaction of the compounds with the target isoforms (Askew & Ho, 1974; Deadwyler et al., 1995; Dolby & Kleinsmith, 1977).

In the current study, molecular docking was enrolled as the first structure-based screening step, and PDE4, PDE5 and PDE9 were included in the workflow. Blind docking experiments were performed to gain insights on the interaction mode of the most promising compounds, highlighted by the abovementioned ligand-based screening. Overall, considering that the value of −7.5 kcal/mol is generally accepted as a reliable cut-off to highlight promising interactors (Proto et al., 2017), these phytocannabinoids showed good calculated binding energy values towards considered PDE isoforms. Nevertheless, the peculiar behaviour of CBD emerged, as a preferential binding towards PDE9 was highlighted (Figure 2). This aspect is extremely relevant for the medicinal chemist, since among other isoforms, PDE9 is particularly attractive in light of some specific features as anticipated. In particular, PDE9 is characterized by the presence within its structure of a peculiar accessory pocket, which is not found in other isoforms, and by the asymmetry between the two subunits composing the protein dimer. Moreover, a selectivity towards chiral species was reported (Cheng et al., 2014). Taken together, these features make PDE9 a suitable target to rationally develop or identify specific inhibitors (Ribaudo et al., 2021b). Thus, a more focused study was carried out on the interaction of CBD with PDE9. As can be observed in Figure 2, CBD interacts with the catalytic pocket of PDE9. In particular, the ligand mostly binds to non-polar amino acids (highlighted in cyan in the artwork), providing hydrophobic interaction. Notably, a better calculated binding energy value was computed for THC towards PDE9, but this compound comes with the unavoidable limitations connected to its psychotropic effects due to its affinity with CBRs, representing a drawback for its development as a PDE9 inhibitor. Moreover, THC was not predicted as selective for PDE9 isoform according to the simulation. The investigational compound PF-04447943, a nanomolar PDE9-selective inhibitor, was used as positive control in this study and a binding energy value of −9.7 kcal/mol was computed (Figure 2). Thus, the computed value for CBD (−9.1 kcal/mol) is in line with that of the positive control.

Furthermore, 25-ns MD simulations were performed to provide a preliminary insight on the stability over time of the complex formed by the interaction of CBD with PDE9 as predicted by docking. The complex reached stabilization within the first nanoseconds of simulation, and as can be observed from the representative frames reported in Figure 3, CBD was retained within the binding site throughout simulation time with minimal rearrangement.

Overall, the abovementioned computational evidences support the potential of CBD as a CNS-targeting PDE9 inhibitor. Moreover, the effect of CBD on PDE9 was investigated in vitro, and such studies showed that CBD inhibits the enzyme with an IC_{50} value of 110 nM (Figure 4). In this experiment, the same PDE9 inhibitor used as reference compound for docking simulation, namely, PF-04447943, was used as positive control. This observation agrees with computational data and further suggests that CBD may be a promising therapeutic option acting through this mechanism.

5 | CONCLUSION

In this study, we reported the application of a computer-aided drug discovery workflow aimed at the investigation of a possible mechanism involved in the activity of *Cannabis* constituents against neurodegeneration. Ligand-based and structure-based computational tools were enrolled and combined, and the results of drug-likeness studies, docking and MD simulations highlighted CBD as a potential PDE9 inhibitor. Subsequently, in vitro analysis confirmed this evidence, as CBD inhibited the enzyme in the nanomolar range.

In conclusion, the results of this work prompt the development of CBD as a non-psychotropic, drug-like, CNS-targeting and selective PDE9 inhibitor, paving the way for the rational identification of novel therapeutic applications for this compound against neurodegeneration.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

G. R., E. L. and A. G. conceived and designed the project; C. M., G. M. and M. G. acquired the data; G. R., A. M., S. A. B. and A. G. analysed and interpreted the data; G. R. and E. L. wrote the paper; M. M., D. E. P. G. and A. G. revised the paper.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data are available via request from the authors.

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