

Non-clinical investigations about cytotoxic and anti-platelet activities of gamma-terpinene

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Abstract

Gamma-terpinene (γ -TPN) is a cyclohexane monoterpene, isolated from essential oils of pharmacologically active plant species, such as tea tree (*Melaleuca alternifolia*), oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* Marchand) and eucalyptus (*Eucalyptus* sp.). Terpenes are widely studied for their recognized pharmacological actions on the cardiovascular system, hemostasis and antioxidant actions. The objective of this study was to investigate the cytotoxic and antiplatelet activity of γ -TPN in non-clinical study models. For the in silico evaluation, the PreADMET, SwissADME and SwissTargetPrediction software were used. Molecular docking was performed using the AutoDockVina and BIOVIA Discovery Studio databases. The cytotoxicity of γ -TPN was analyzed by the MTT assay with normal murine endothelial (SVEC4-10) and fibroblast (L929) lines. Platelet aggregation was evaluated with platelet-rich (PRP) and platelet-poor (PPP) plasma from spontaneously hypertensive rats (SHR), in addition to SVEC4-10 cells pre-incubated with γ -TPN (50, 100 and 200 μ M) for 24 h. In in vivo tests, SHR animals were also used, pre-treated by gavage with γ -TPN for 7 days, distributed into four groups (control, 25, 50 and 100 mg/Kg). At the end, blood samples were collected to measure nitrites using the Griess reagent. γ -TPN proved to be quite lipid-soluble (Log P = + 4.50), with a qualified profile of similarity to the drug, good bioavailability, and adequate pharmacokinetics. The monoterpene exhibited affinity mainly for the P2Y₁₂ receptor (6.450 ± 0.232 Kcal/mol), moderate cytotoxicity for L929 (CC₅₀ = 333.3 μ M) and SVEC 4–10 (CC₅₀ = 366.7 μ M). The presence of γ -TPN in SVEC 4–10 cells was also able to reduce platelet aggregation by 51.57 and 44.20%, respectively, at the lowest concentrations (50 and 100 μ M). It was concluded that γ -TPN has a good affinity with purinergic receptors and an effect on the reversal of platelet aggregation and oxidative stress, being promising and safe for therapeutic targets and subsequent studies in the control of thromboembolic diseases.

Introduction

Cardiovascular diseases (CVDs) are a group of chronic non-communicable diseases that affect the heart and blood vessels, being responsible for 17.7 million deaths worldwide, most of them (85%) associated with the occurrence of thromboembolic events as heart attack and stroke (Benjamin et al. 2019; Massa et al. 2019; World Health Organization 2019).

It is widely accepted that platelets play a fundamental role in hemostasis and blood clotting at sites of vascular injuries, representing a link between thrombosis, inflammation and atherogenesis. When blood vessels are injured, platelets are activated by the exposed subcutaneous matrix and aggregate at the site of injury to stop bleeding, causing thrombotic occlusive ischemic events (Papapanagiotou et al. 2016; McFadyen et al. 2018; Rengasamy et al. 2019; Olgasi et al. 2024).

The development of drugs that inhibit platelet aggregation has been one of the main targets for the prevention and treatment of thrombotic brain and cardiovascular diseases (Xiang et al. 2019; Iqbal et al. 2020; Gerhards et al. 2021). Thrombosis therapy involves the use of P2Y₁₂ purinergic receptor inhibitors, such as ticlopidine, clopidogrel, prasugrel, cangrelor and ticagrelor (Mackman 2008; Hamilton 2009). However, limiting adverse effects increase the risk of intracranial hemorrhage and dyspnea (Berger 2018). In this context, the use of medicinal plants can be a complementary alternative (Simonetti et al. 2016; Sales & Alencar 2019; Lima et al. 2019; Mortada 2024).

Medicinal plants contain essential oils (EOs), secondary metabolites of low molecular weight molecules often used as drugs, aromas and fragrances, pharmaceutical products, agrochemicals, dyes and pigments, pesticides, cosmetics, food additives, among others (Alves-Silva et al. 2020; Çakmak et al. 2020; Toledo et al. 2020; Rocha et al. 2022). In the pharmacological point of view, terpenes stand out, namely gamma-terpinene (γ -terpinene, γ -TPN or 1-isopropyl-4-methylcyclohexa-1,4-diene), a monoterpene found in “melaleuca” (*Melaleuca alternifolia*), “orégano” (*Origanum vulgare*), rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* Marchand), tangerine (*Citrus delicious*) and eucalyptus (*Eucalyptus* sp.) (Silva et al. 2015; Ramalho et al. 2016; Baldissera et al. 2016; Khan et al. 2020; Xu et al. 2020; Koyama & Heinbockel 2020; Mota 2022).

Taking into account the biological properties of γ -TPN that have been elucidated so far, such as: antimicrobial activity (Piaru et al. 2012; Ramalho et al. 2016), in vitro antioxidant and preventive potential for chronic and cardiovascular (Choi et al. 2000; Li & Liu 2009); anti-inflammatory (Ramalho et al. 2016), antinociceptive (Passos et al. 2015) and lipid-lowering (Takahashi et al. 2003), the objective of this article was to investigate the cytotoxic and antiplatelet activity of this compound in study models non-clinical.

Materials and methods

Chemicals

Gamma-terpinene (γ -TPN), sodium nitrite, Griess reagent, xylazine, adenosine diphosphate (ADP), sodium phosphate and MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal calf serum, DMEM, DMEM High, trypsin-EDTA, penicillin and streptomycin were purchased from Cultilab (Campinas, Brazil). Tween 80 and dimethylsulfoxide (DMSO) were obtained from Dinâmica Química Contemporânea LTDA (São Paulo, Brazil). Ketamine, ammonium oxalate and sodium thiopental were obtained from Cristalia (São Paulo, Brazil).

Stock solutions were prepared with distilled water or DMSO and diluted to appropriate concentrations. γ -TPN was dissolved in DMSO for *in vitro* and *in vivo* protocols. Tween 80 (0.1% v/v) was used as eluent. All solutions were stored at 0°C.

Cells and animals' facilities

Murine axillary lymph node endothelium-like cell lines (SVEC4-10) and murine fibroblasts (L-929) were maintained in DMEM and DMEM high glucose cell media, 10% fetal serum fetal bovine and 1% (w/v) penicillin/streptomycin. The culture flasks (containing 2×10^6 viable cells) were observed under an inverted microscope (Biosystems, USA), followed by incubation in an oven at 37°C, 95% humidity and a 5% CO₂ atmosphere (Shel Lab CO₂ Incubator, USA).

Spontaneously hypertensive (SHR) female Wistar rats (*Rattus norvegicus*) weighing between 180 and 200 g were obtained from the Central Animal Facility at Universidade Federal do Piauí, Teresina, Brazil. They were kept in well-ventilated cages under standard conditions of light (12 h with alternative day and night cycles) and temperature ($22 \pm 1^\circ\text{C}$) and were housed with access to commercial rodent stock diet (Nutrilabor, Campinas, Brazil) and water *ad libitum*. All procedures were approved by the Committee on Animal Research at UFPI (#572/2019) and followed Brazilian (*Colégio Brasileiro de Experimentação Animal* - COBEA) and International

rules on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes).

In silico evaluation of γ -TPN

The 2D, 3D structures and the chemical representation with normal characters (ASCII), called SMILE (Simplified Molecular Input Line Entry Specification) of γ -TPN are shown in Fig. 1 below. These parameters were used for *in silico* analysis of ADMET (Absorption, distribution, metabolism, excretion/toxicity) and target predictions were extracted from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov>). The molecular optimization of γ -TPN was carried out using ChemSketch software, version 14.0 and the molecular targets were prepared using Discovery Studio 20.1.0.

For the prediction of physicochemical properties (lipophilicity - logP, molecular weight, polar surface area, number of hydrogen bond donors and acceptors, number of rotatable bonds and water solubility), pharmacokinetic parameters (ADMET) and similarity to drugs (Lipinski rules, CMC like, MDDR like, Leadlike and WDI like), the software PreADMET (<https://preadmet.bmdrc.kr/>) and SwissADME (<http://www.swissadme.ch/>) were used. Complementary data were exposed by the Bioavailability Radar and BOILED-Egg. To predict the possible macromolecular targets of γ -TPN, the SwissTargetPrediction program (<http://www.swisstargetprediction.ch/>) was also applied.

Regarding metabolic parameters, it can be checked whether the substance undergoes first pass metabolism and whether it inhibits cytochrome P450 (CYP) enzymes. The mathematical models used detected mutagenicity results, considering the Ames test and strains [*Salmonella typhimurium* (TA98, TA100 and TA1535)] (Ames et al. 1975; Mortelmans & Zeiger 2000). The prediction of carcinogenicity in rodents was carried out based on data from the National Toxicology Program (NTP) and the Food and Drug Administration (FDA) (Dolabela et al. 2018). Regarding acute ecotoxicity, the maximum acceptable concentrations for *Daphnia* sp., *Pimephales promelas*, *Oryzias latipes*, as well as the inhibition of growth in algae were investigated. Additionally, the classification of cardiotoxic risk was investigated based on inhibition of the human gene related to ether-a-go-go (hERG).

Analysis of docking molecular

First, the molecular design and optimization of the three-dimensional structure of γ -TPN were carried out using the ACD/ChemSketch software, version 14.0, based on classical mechanics parameters (bond distance, bond angle and dihedral angle). The human P2Y₁₂ receptor (4NTJ) was obtained from the Protein Data Bank (PDB). Only structures with a resolution below 3.0Å and obtained from *Homo sapiens* were considered for this study. The files obtained were imported into the Discovery Studio Visualizer software, version 21.1.0. For ligand removal, crystallographic water molecules were kept and targets were saved in (.pdb) format. Next, the .pdb files were opened in the AutoDockTools software, version 1.5.7, where polar hydrogens and Gasteiger charges were added and saved in (.pdbqt) format (Panda et al. 2020; Melo et al. 2022; He et al. 2023).

The molecular structures of γ -TPN and conventional antiplatelet agents (Prasugrel and Ticagrelor) were obtained from the PubChem website in (.sdf) format and individually subjected to geometry optimization using the Avogadro software, version 1.2.0, using the field of MMFF94s strength, subsequently being saved in (.pdb) format. Then, the optimized ligands were also imported into the AutoDockTools software, version 1.5.7, to add

polar hydrogens and Gasteiger charges. All ligands were kept flexible before being saved in (.pdbqt) format (Hanwell et al. 2012; Yan et al. 2022; Kintamani et al. 2023).

The dimensions and coordinations in the Cartesian plane of the docking grid were automatically calculated using the AutoGrid module of AutoDock software version 4.2.6 and used to generate the configuration files. The grid volume was defined at 60x60x60 points (dimensions X, Y, Z), with a spacing of 0.375 Å, for the two targets tested. The AutoDock Vina software version 1.2.0 was used to perform the molecular docking of the ligands on each target following the scripts for flexible and hydrated docking made available by the Center of Computational Structural Biology (CCSB) at Scripps Research, with the exhaustivity parameter set at 100 races. The analysis of the bonds between targets and ligands and creation of interaction images was carried out using the BIOVIA Discovery Studio Visualizer version 21.1.0 and PyMOL version 2.5.4 software (Marzaro et al. 2014; Forli et al. 2016; Eberhardt et al. 2021).

MTT assay

Cytotoxic tests used MTT ([3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide]) according to Mosmann (1983). SVEC4-10 and L-929 (5×10^3 cells/well) were incubated and about twenty-four hours later, γ -TPN (0 to 734 μ M) was added to each well and the plates were incubated at 37 °C in a 5% CO₂ atmosphere (Shel Lab CO₂ Incubator, USA) for 72 h. After the incubation period, 20 μ L of the MTT solution (5 mg/mL) was added to each well and the plates were reincubated for 4 h. Next, formazan product was dissolved in DMSO and absorbance was read using a multiple reader (GloMax Explorer Multimode Microplate Reader, USA) at 595 nm.

Design of experimental in vivo protocol

SHR rats were orally treated by gavage with γ -TPN at different doses (25, 50 and 100 mg/kg/day) for seven days. The animals were randomly divided into four groups of 5 animals each as follows: Group I (control group, saline solution), Group II (25 mg/kg/day of γ -TPN), Group III (50 mg/kg/day of γ -TPN), and Group IV (100 mg/kg/day of γ -TPN). Following the treatment, the animals were anesthetized with ketamine (90 mg/kg) plus xylazine (4.5 mg/kg) for blood collection. Afterwards, all mice were euthanized with sodium thiopental (150 mg/kg i.p.).

Nitrite dosage

To determine the nitrite content, the test uses the Griess reaction (Green et al. 1981) in which, a total of 500 μ L of Griess reagent was added to 500 μ L of distilled water. In another test tube, 500 μ L of Griess reagent was added to 500 μ L of 10% erythrocyte homogenates (50 mM sodium phosphate buffer pH 7.4) extracted from γ -TPN-treated mice. Measurements were carried out at 560 nm and results were expressed as μ M/mg of protein.

Antiplatelet activity of γ -TPN

Blood was collected (3–5 mL/animal) from anesthetized SHR rats through the aorta artery and by cardiac puncture (right ventricle) with plastic syringes and stored in tubes with citrate and 3.2% sodium in a ratio of 9:1 (blood: anticoagulant). The blood was centrifuged at 1650 rpm for 10 min to obtain platelet-rich plasma (PRP: 2.5 to 4.5×10^5 platelets/ mm^3) and at 3000 rpm for 15 min to obtain platelet-poor plasma (PPP: $< 2.0 \times 10^4$ platelets/ mm^3). PPP was used as a diluent to adjust the final volume of PRP (Vinholt et al. 2017).

Platelet counting was performed using a Neubauer chamber (Brecher & Cronkite 1950) with plasma diluted in 1% ammonium oxalate in a proportion of 1:200 (Tomasiak et al. 2004). The antiplatelet activity of γ -TPN was determined in citrated PRP by the turbidimetric method described by Born & Cross (1963) and monitored by an aggregometer (Platelet Aggregometer, EasyAgreg software, Benfer). Platelet aggregation was expressed as percentage of aggregation for ADP and as aggregation speed (Cattaneo 2009; Koltai et al. 2017).

SVEC4-10 cells were pretreated with γ -TPN at different concentrations (50, 100 and 200 μ M) for 24 h. After this period, the level of platelet aggregation was determined in a suspension (300 μ L) containing pre-treated platelets (3×10^8 platelets/mL) and SVEC4-10 cells (1.5×10^3 cells/mL). First, the first cuvette was placed in the device containing 300 μ L of PPP. Then, the cuvette with 270 μ L of PRP was placed with 20 μ L of cells pretreated with γ -TPN. After the first five minutes of recording platelet aggregation, 10 μ L of the aggregator ADP (10 μ M) was added (Cho et al. 2017), followed by additional 5 min for final recording.

Statistical analysis

Results were expressed as mean $\pm$ standard error of the mean (S.E.M.). The binding energy values were subjected to normality analysis using the D'Agostino & Pearson test. Nonlinear regression was used to calculate average values of IC_{50} (50% growth inhibition of cell proliferation). In order to determine differences between treatments, data were compared by unpaired T-test and one-way analysis of variance (ANOVA) followed by Dunnet, Tukey or Bonferroni test, considering p values < 0,05 as statistically significant (GraphPad Prism version 6.0).

Results and discussion

In silico parameters

In silico analysis data revealed physicochemical characteristics, pharmacokinetic profile and drug similarity prediction of γ -TPN (Table 1). Regarding physicochemical aspects, γ -TPN has a molecular weight of 136.23 g/mol, and it was found to be quite lipid-soluble (Log P = + 4.50), moderately water-soluble (Log S=-3.45), with 1 rotary bond, without hydrogen donor and acceptor groups, sp^3 carbon fraction of 0.60, molar refractivity of 47.12, and tension in polar surface area (TSPA) of 0 Å².

γ -TPN revealed complete human intestinal absorption (100%), high permeability in Madin-Darby canine kidney cells - MDCK (244.91) and low permeability in human epithelial adenocarcinoma (Caco-2, 23.64 nm/s), besides a high degree of plasma protein binding (100%). It presented a comparative concentration ratio in the brain and plasma of 8.037, resulting in excellent penetration in the blood-brain barrier. The compound exhibited a skin permeability of -0.8857 and was not able to inhibit P-glycoprotein and CYP2C19 and CYP2C9 enzymes. On the other hand, it is a substrate of CYP3A4 (Table 1).

Regarding the prediction of drug similarity, Table 1 showed that γ -TPN was classified as “drug-like” taking into consideration the Lipinski and World Drug Index (WDI like) rules, intermediate by the Modern Drug Data Report rule (MDDR like) and disqualified in this parameter by the Leadlike rule and the Comprehensive Medicinal Chemistry (CMC) database.

Table 1
Physicochemical, pharmacokinetic properties and drug-like prediction of γ -terpinene.

Property	Parameter	Result
Physical chemistry	Molecular weight (g mol ⁻¹)	136.23
	Water solubility (Log S)	-3.45
	Liposubility (Log P; XLog P3)	+ 3.35; +4.50
	Number of rotary connections	1
	Number of hydrogen bond donors	0
	Number of hydrogen bond acceptors	0
	Fraction of sp ³ carbons	0.60
	Molar refractivity	47.12
	Voltage on polar surface area (Å ²)	0.00
Pharmacokinetics	Human Intestinal Absorption (%)	100.00
	Permeability coefficient in Caco-2 (nm s ⁻¹)	23.64
	Permeability coefficient in MDCK (nm s ⁻¹)	244.91
	Binding to Plasma Proteins (%)	100.00
	Penetration of the Blood-Brain Barrier	8.037
	Skin permeability (cm ² h ⁻¹)	-0.8857
	P-glycoprotein inhibition	No
	Inhibition of CYP2C19	Yes
	CYP2C9 inhibition	Yes
	CYP2D6 inhibition	No
	CYP2D6 substrate	No
	CYP3A4 inhibition	No
	CYP3A4 substrate	Yes
Drug-likeness prediction	Lipinski's Rule	Qualified
	CMC Like Rule	Disqualified
	MDDR Like Rule	Intermediary
	LEAD Like Rule	Disqualified
	WDI Like Rule	Qualified

There is an intrinsic correlation between the physicochemical parameters of a compound and its biological performance, which facilitates the interpretation of its pharmacokinetics, pharmacodynamics and toxicity

(Wenlock and Barton 2013). As seen in Table 1, *in silico* ADME predicts 100% human intestinal absorption (HIA) for the drug candidate (Yee 1997). γ -TPN has high intestinal absorption due to the oil-water partition coefficient (Log P), which guarantees elevated lipid solubility and absorption (from 70–100%) crossing membranes by passive diffusion (Hadda et al. 2013). It should be noted that the ideal HIA value of a drug will depend on its pharmaceutical indications and must be produced according to the therapeutical purposes (Dolabela et al. 2018).

Anyway, the best-known rule for linking chemical cores to biological activities is the Lipinski's rule, named "rule of five". It is based on lipid solubility ($\text{LogP} \leq 5$), Molecular weight ($\text{MW} \leq 500$), and number of H acceptors ($\text{HBA} \leq 10$ and H donors ($\text{HBD} \leq 5$) (Lipinski et al. 1997; Lagorce et al. 2017). Thus, outcomes revealed that γ -TPN met all these prerequisites.

Permeability by intestinal Caco-2 cells is used for selecting drug candidates for oral administration, classifying γ -TPN as showing medium permeability through the intestinal epithelium (Yamashita et al. 2000). Meanwhile, γ -TPN revealed to be highly permeable through MDCK cells, a useful tool for rapid screening of cell membrane permeability and pharmacological viability (Vistoli et al. 2008).

γ -TPN also exposed high permeability with regard to penetration of the blood-brain barrier (BBB) (Table 1) and is therefore classified as active on the Central Nervous System (CNS) (Zhao et al. 2001). This biological property is relevant for ADME studies, as it provides data about therapeutic action on the CNS, binding to plasma proteins, their disposition and efficacy, especially in cerebrovascular diseases' conditions (Sekhar et al. 2014; Harika et al. 2017).

Another essential ADME parameter is the assessment of binding to the P-glycoprotein (P-gp). This protein is part of the ATP-dependent efflux pump, acting as a physiological barrier to protect the body against toxins and xenobiotics. Then, P-gp is directly involved to the intestinal absorption, metabolism and penetration of the BBB for the majority of drugs, and its inhibition or induction can significantly alter oral bioavailability and metabolism of a drug (Pereira 2019).

It was also provided a module whose objective is to predict the skin permeability coefficient (K_p) using a linear regression model ($\log K_p$). The skin permeability parameter reveals the ability of γ -TPN to be absorbed through the skin, anticipating possible exposure to toxins or even accidental absorption of the compound during handling. Negative value for this coefficient (Table 1) indicates the compound is impermeable to the skin, with a low possibility of being used intradermally (Souza et al. 2022), but probably without toxic effects upon skin exposure.

Within this myriad of enzymes and transporters, the cytochrome P450 (CYP) superfamily of metabolic isoenzymes plays a crucial role (Testa & Kraemer 2007). Many drugs are targeted by these catalytical proteins, causing high rate of drug individual variability due to different degrees of CYP expression (Hollenberg 2002; Huang et al. 2008). Herein, *in silico* showed γ -TPN inhibits the enzymes CYP2C19 and CYP2C9 (Table 1). This type of activity may result in increased plasma drug concentrations, but it can induce early adverse effects (Teague et al. 1999; Sekhar et al. 2014). On the other hand, γ -TPN did not show inhibitory activity of the CYP2D6 gene, which is responsible for the oxidative metabolism of many drugs and other xenobiotics and toxins in the cellular environment. The cytochrome CYP3A4, one of the most oxidative liver enzymes, an isoform that

metabolizes 50% of all drugs, it is not inhibited by the compound, acting discreetly as a substrate of this enzyme (Oprea 2000).

Regarding genotoxic aspects (Table 2), γ -TPN presented a positive result for the Ames test, a mutagenicity method that uses strains of *Salmonella typhimurium* carrying mutations in histidine synthesis-involved genes (Prival & Zeiger 1998; Araki et al. 2004). Table 2 also displays the acute toxicity profile of γ -TPN upon aquatic organisms, with the maximum tolerable concentrations for *Daphnia* sp., *Pimephales promelas*, and *Oryzias latipes* of 0.23728, 0.04082, and 0.06008 mg/L, respectively.

Table 2
In silico prediction of γ -terpinene toxicity.

Parameter	Result
Ames test	Mutagen
Carcinogenicity test (rats and mice)	Carcinogenic
Acute toxicity to <i>Daphnia</i> sp.	Acceptable C_{max} : 0.23728 mg L ⁻¹
Acute toxicity to <i>Pimephales promelas</i>	Acceptable C_{max} : 0.04082 mg L ⁻¹
Acute toxicity to <i>Oryzias latipes</i>	Acceptable C_{max} : 0.06008 mg L ⁻¹
Inhibition of growth in algae	Acceptable C_{max} : 0.06008 mg L ⁻¹
hERG gene inhibition	0.02505 mg L ⁻¹
	Medium risk

C_{max} = Maximum concentration.

Acute evaluations on aquatic organisms, as well as those with *Artemia salina* and *Danio rerio* (zebrafish, family Cyprinidae), have been widely used to predict ecotoxicity of contaminants in aquatic organisms with compatible enzyme receptors (Chen et al. 2020; Kingcade et al. 2021). According to the European legislation 92/32/EEC on safety of chemical substances (Weyers et al. 2000), γ -TPN has no potential to cause long-term adverse effects on aquatic environments (Solubility < 1mg/L).

γ -TPN presented a moderate risk for inhibition of the human gene related to ether-a-go-go (hERG). It is responsible for encoding the subunit responsible for the formation of fast-type delayed-rectification potassium channels (IKr), important in the cardiac repolarization stage. Inhibition and dysfunction of this gene causes QT prolongation and fatal ventricular arrhythmia (Lamothe et al. 2016; Bjerregaard 2018).

Figure 2 demonstrates a diagram corresponding to the appropriate oral bioavailability profile of γ -TPN. The colored zone reveals itself as the appropriate physicochemical area, configuring an excellent oral bioavailability and meeting parameters of lipophilicity (LIPO), size (SIZE), polarity (POLAR), insolubility (INSOLU), unsaturations (INSATU), and flexibility (FLEX).

Bioavailability Radar (Fig. 2) was displayed for rapid assessment of drug similarity, where six physicochemical properties are taken into consideration. To be considered a drug, the compound line under study must be fully

included in the pink area (Daina et al. 2017). Any deviation, as observed in the result expressed by γ -TPN regarding size, represents a sub-optimal physicochemical property for bioavailability.

Figure 2 also shows the BOILED-Egg analysis and points out that γ -TPN in the yellow region presents good HIA and excellent BBB penetration power. However, it was not shown to be a substrate for P-glycoprotein, due to the indication of the red dot (Pgp-).

The graph called BOILED-Egg displays a correlation of TPSA with lipophilicity (LogP). It is important to emphasize that the ability to cross the BBB is only relevant when the clinical target is located in the CNS, otherwise the drug may cause side effects, such as headache, drowsiness, dizziness, partial loss of vision, among others depending on the area where chemical interaction(s) may occur. Furthermore, it is important that the drug is not a substrate for proteins such as P-gp and therefore remains at an adequate concentration at the site of action to obtain the desired effect (Borges 2018; Pereira 2019).

Figure 2 predicts the possible and best targets of the molecule and cytoplasmic receptors (26.3%), G protein-coupled receptors (20%) and the CYP enzyme (12.7%) were recognized as the majority.

G protein-coupled receptors, as seen in Fig. 4, represent the second largest class of pharmacological targets of γ -TPN, a result that corroborates the proposal of this study, which is to verify the antiaggregation potential of the monoterpene, considering purinergic ADP receptors, which belong to the G_i class of GPCRs (Hollopeter et al. 2001).

Docking molecular of γ -TPN

The values of ΔG lig. of γ -TPN and RP2Y12, comparing them with conventional antiplatelet therapy drugs are also detailed in Fig. 2. The affinity of γ -TPN (-6.450 ± 0.232 Kcal/mol) was statistically higher than that of Prasugrel (-5.793 ± 0.223 Kcal/mol) in RP2Y12. Ticagrelor (-6.883 ± 0.276 Kcal/mol), in turn, showed higher affinity than γ -TPN and Prasugrel. Interaction energy and affinity of the ligand-target complex are inversely proportional quantities, therefore, the lower the binding energy, the greater the stability of the interaction between ligand and protein (Trott & Olson 2010; Zhang et al. 2021; Xiang et al. 2022).

The RP2Y12 exhibits a response to the ADP signal and is involved in the inhibition of adenylyl cyclase, Ca^{2+} -dependent cell migration, regulation of cell morphology, and cell aggregation. RP2Y12 must be activated for ADP-induced platelet aggregation to occur, which can be prevented by a substance that impartially inhibits the receptor. RP2Y12 has a specific tissue distribution, making it a fundamental target for therapeutic intervention (Ahn et al. 2016). Such binding inhibits the activity of P2Y12 on platelet aggregation. Therefore, the best coupling position of the γ -TPN in the 2D and 3D structures can be seen in Fig. 3.

There is similarity in the interaction sites of γ -TPN and Prasugrel in RP2Y12, with repetition of some amino acid residues (A:LYS:80, A:PHE:104, A:TYR:105). Ticagrelor, on the other hand, interacted with RP2Y12 in a region very close, but wider to the active site, probably due to its greater molecular weight. The amino acids that interacted with both γ -TPN and Ticagrelor were A:THR:76, A:LYS:80, A:SER:101, A:PHE:104, A:TYR:105 and A:LEU:284.

It is known that γ -TPN had a lower average interaction energy with RP2Y12 than Prasugrel, and is therefore capable of forming a complex with greater stability (Zhang et al. 2021; Xiang et al. 2022). The justification for greater affinity of γ -TPN with the receptor arises from higher number of hydrophobic bonds created in relation to

Prasugrel. On the other hand, still according to Fig. 3E, even though the Ticagrelor- RP2Y12 complex formed an unfavorable bond (represented by the red color), which compromises the stability of the complex, the overall number of bonds formed was higher than the other two molecules, which may justify target greater affinities (Dhorajiwala et al. 2019; Bender et al. 2021).

Similarly, Nikitina et al. (2022) evaluated the molecular fit of newly synthesized myrtenol-derived monoterpenes carrying different heteroatoms (sulfur, oxygen or nitrogen) as possible antiplatelet agents, comparing their affinities to P2Y12 with of ticagrelor. It was evident that molecular anchoring confirmed the interaction of all tested compounds with RP2Y12, suggesting that their antiaggregation properties are implemented by blocking P2Y12 function. The results of the study also show that the activity of the monoterpene is linked to the selective inhibition of platelet aggregation.

In vitro cytotoxicity of γ -TPN by colorimetric assays

It was essential to carry out a cytotoxic screening of a compound in order to know at what concentration it is capable of causing damage to normal cells and, thus, proceed with other experiments with concentrations that are not toxic. Gama-terpinene at concentrations of 367 and 734 μ M (50 and 100 μ g/mL) significantly interfered with the cell viability of SVEC4-10 and L-929, compared to the control with untreated cells (Fig. 4). It was not detected statistically significant viability differences on both cell lines tested at concentrations $\leq$ 183.50 μ M (25 μ g/mL). Furthermore, it was found that γ -TPN presented a CC₅₀ of 366.70 and 333.33 μ M (49.96 and 45.41 μ g/mL), respectively, for SVEC4-10 and L-929 cells.

The US National Cancer Institute (NCI) classifies a compound as having high cytotoxic activity when i) CC₅₀ < 20 μ g/mL; ii) moderate cytotoxic activity if CC₅₀ ranges between 21–200 μ g/mL; iii) weak cytotoxic activity if CC₅₀ is between 201–500 μ g/mL, and iv) no cytotoxic activity if CC₅₀ > 500 μ g/mL (Thienthiti et al. 2017). According to these categorization, γ -TPN has moderate cytotoxicity on normal cells. Previous studies with γ -TPN-treated HaCaT cell lines (long-lived human keratinocytes) showed increase in cell viability after 2h of exposure (Casalle 2016).

At any rate, terpenes, specifically, act on cells causing damage to lipids and proteins, breaking down cell walls and membranes, resulting in cell lysis. In eukaryotic cells, they also destabilize the mitochondrial membrane and damage plasma membrane proteins (Bakkali et al. 2008). Consequently, the number of metabolically active cells should be followed to understand cell toxic effects. Knowing that γ -TPN is a monoterpene mostly found in essential oils from plants whose anticoagulant and antithrombotic properties have already been duly proven, we seek to identify its antiplatelet action (Lamponi 2021).

Antiplatelet effect and nitrite dosage of γ -TPN

The antiplatelet activity of PRP from rats was evaluated against 10 μ M ADP. Firstly, it was observed that in the presence of ADP, there was platelet aggregation, a percentage extrapolated to 100% (Fig. 5A). In the presence of SVEC4-10, there was a significant 39.59% of reduction in platelet aggregation when compared to the negative control (ADP alone) (Fig. 5B and 5F). In the presence of γ -TPN 50 and 100 μ M (Fig. 5C and 5D), there was a greater reduction in platelet aggregation promoted by ADP (51.57 and 44.27%, respectively). Meanwhile, γ -TPN 200 μ M promoted a reversal of platelet antiaggregation significantly compared to the control with ADP and SVEC4-10 (Fig. 5E and 5F).

Under *in vitro* conditions, the aggregation stimulated ADP to PRP triggers to its G protein-coupled receptors - P2Y1 and P2Y12 - initiating the process. ADP signaling through the P2Y1 coupled to the Gq protein, promotes a rapid short-lasting response. It activates phospholipase C (PLC) and converts phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol (1,4,5)-triphosphate (IP₃) and diacylglycerol (DAG). DAG mobilizes intracellular Ca²⁺ and, after activation of protein kinase C (PKC), these both secondary messengers induces increase of free Ca²⁺ levels in the cytoplasm environment, resulting in reversible platelet aggregation (Gachet 2012; Fayaz & Rajanikant 2012; Aslam et al. 2013).

Endothelial SVEC4-10 cells exposed to ADP (Fig. 5B and 5F) promoted a significant reduction in platelet aggregation, possibly justified by healthy endothelial cells expressing antiplatelet agents. Thus, when platelets encounter endothelial cells, platelet-endothelium interactions can occur for stimulating endothelial surface to produce and secrete mediators such as prostacyclin (PGI₂) and nitric oxide (NO) (D'amico & Villaça 2006; Xie et al. 2024).

Regarding γ -TPN-pre-treated SVEC4-10 cells, it was found that the compound alter platelet anti-aggregation, since under lower concentrations (Fig. 5C, 5D and 5F), it stimulates endothelial cells, probably through endothelial nitric oxide synthases (eNOS or NOS3) to produce nitric oxide (NO). It was confirmed by *in vivo* studies, in which the dose of 100 mg/kg ($91.86 \pm 12.31 \mu\text{M}$) markedly increased indirect levels of nitric oxide quantified as nitrite content, which could be one of the factors responsible for inhibiting platelet aggregation (Radomski et al. 1990; Tran et al. 2022). This method was established using a calibration curve ($R^2 = 0.9942$) (Fig. 6).

With NO formed, the enzyme soluble guanylyl cyclase (GCs) was activated and the second messenger cyclic guanosine monophosphate (cGMP) was produced (Förstermann & Sessa 2012; Wittenborn & Marletta 2021). After activation, the platelets themselves generated NO (Radomski et al. 1990; Malinski et al. 1993), inhibiting adhesion (Radomski et al. 1987a; Radomski et al. 1987b) and aggregation (Freedman et al. 1997). cGMP, in turn, is the agent that determines the inhibitory actions of NO on platelets and this occurs through the decrease in the concentration of intracellular Ca²⁺ ([Ca²⁺]_i) and modulation of the expression of surface receptors. The limitation of [Ca²⁺]_i occurs due to the inhibition of the release of Ca²⁺ via the receptor of the dense tubular system, an increase in the rate of Ca²⁺ extrusion, lower than its entry, through the extracellular environment, an increase in the activity of the Ca²⁺-ATPase of the endoplasmic reticulum, resulting in a lower amount of Ca²⁺ available for platelet activation and aggregation mechanisms. Furthermore, cGMP also promotes the reduction of conformationally active GPIIb/IIIa receptors on the platelet surface, generating greater dissociation between fibrinogen and the GPIIb/IIIa receptor, producing platelet antiaggregation (Jin & Loscalzo 2010; Ren et al. 2024).

Regarding purinergic receptors, γ -TPN may also be strongly involved in the inhibition of P2Y12, due to the results found at lower concentrations (Fig. 5C, 5D and 5F) and the high molecular affinity of the compound with this receptor, the which was compared with prasugrel and ticagrelor. Additionally, it is important to clarify that higher concentration of γ -TPN caused a nearly complete reversal of antiplatelet effect, suggesting γ -TPN 200 μM likely has cytotoxic action, promoting death of endothelial cells and decline in the production of antiaggregation mediators.

Zhou et al. (2019) also evaluated *in vitro* antiplatelet activity of monoterpenes and found that such molecules showed moderate inhibitory activities on ADP-induced blood platelet aggregation. Aragão et al. (2006) describe a mixture of triterpenes called α - and β -amyrin, isolated from *Protium heptaphyllum* (Aubl) March (Burseraceae) with antiplatelet activity in a concentration-dependent manner which probably acts in a common biochemical pathway to all established agonists (ADP, collagen, arachidonic acid and AAS).

Conclusions

The compound γ -TPN has promising potentialities as to anti-platelet prototype, since it promoted platelet aggregation in the presence of ADP, while lower doses inhibited aggregation and weak cytotoxicity on endothelial cells and fibroblasts. These biological activities are linked to drug-likeness properties, including good bioavailability, high lipophilicity and intestinal absorption and capacity for crossing the blood-brain barrier. Moreover, molecular docking analysis revealed γ -TPN as a molecule with affinity for the active site of the P2Y₁₂ receptor (Fig. 7).

Declarations

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Authors' contribution statement

All authors participated in the study. RPS wrote the article. RPS and RWRS performed cytotoxicity assays with MTT and nitrite dosing protocols. RPS and VDP carried out *in silico* and molecular docking studies. RPS, EPS and ACAS were responsible for the *in vitro* and *in vivo* antiplatelet aggregation. DD and PMPF supervised and scientifically supported the experiments. APO planned the research, managed scientific and financial support, supervised all stages and reviewed the final article. The authors further stated that all data was generated internally and that no paper mills were used.

Compliance with ethical standards

All procedures were approved by the Committee on Animal Research at UFPI (#572/2019) and followed Brazilian (*Colégio Brasileiro de Experimentação Animal* - COBEA) and International rules on the care and use of experimental animals (Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes).

Data availability statement

We declare that, in case of acceptance of the manuscript for publication, we agree to transfer the right of first publication to Naunyn-Schmiedeberg's Archives of Pharmacology and the open access policy, therefore, the texts are available for anyone to read, download, copy, print, share, reuse and distribute, with due citation of the source and authorship. The datasets generated and/or analyzed during the current study are not yet publicly available, but are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this manuscript.

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Figures

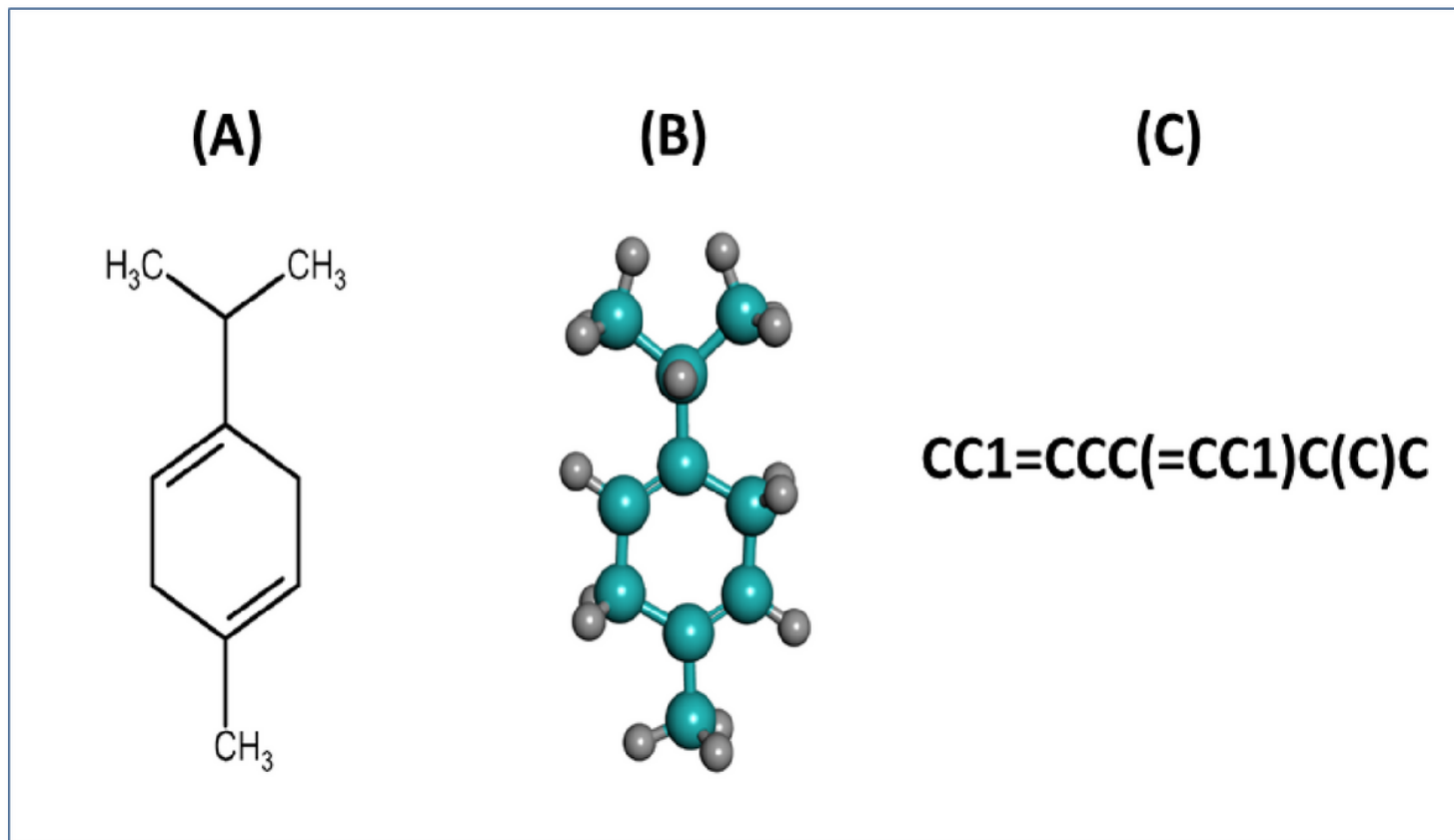


Figure 1

2D, 3D and SMILE molecular structures of γ -terpinene

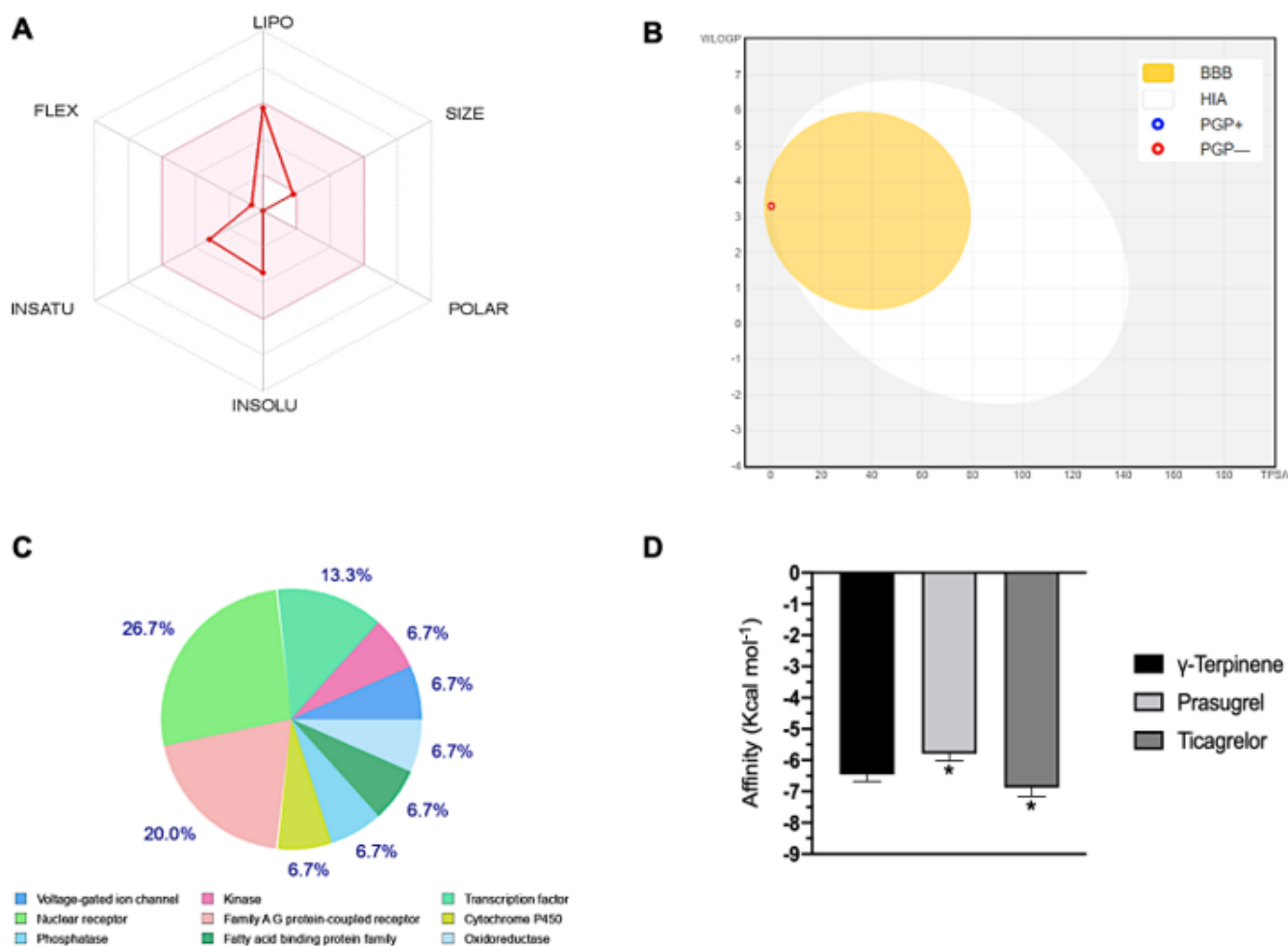


Figure 2

Physicochemical *in silico* properties of γ-terpinene according to: A) Bioavailability radar; B) BOILED-Egg for γ-terpinene (BBB: Blood-Brain Barrier; HIA: Human Intestinal Absorption; PGP: P-glycoprotein); C) Main classes of pharmacological targets; D) Binding energy (ΔG) of γ-Terpinene, Prasugrel and Ticagrelor in RP2Y12. Analysis of variance ANOVA followed by Tukey's test for unpaired samples. Values were expressed as mean $\pm$ standard deviation (SD). (*) represents statistical difference with $p < 0.05$.

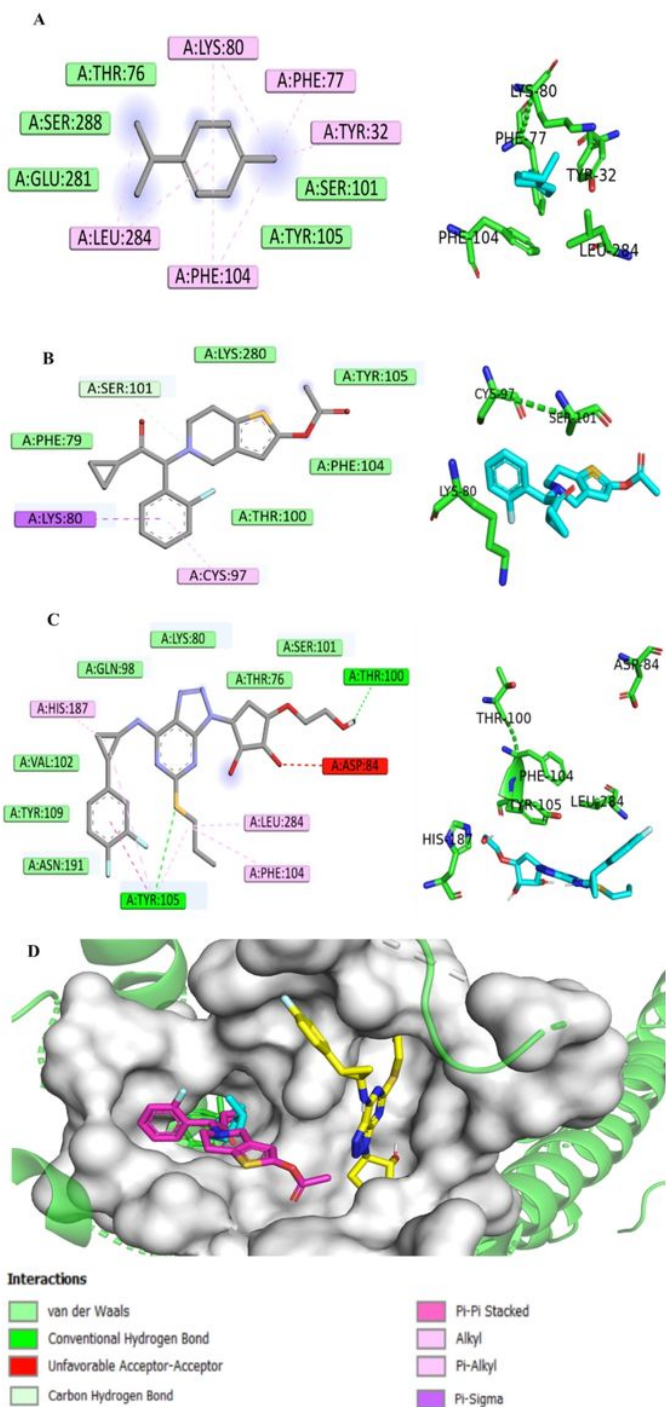


Figure 3

Intermolecular interactions of γ -Terpinene (A), Prasugrel (B), and Ticagrelor (C) with RP2Y12 alone and in combination (D).

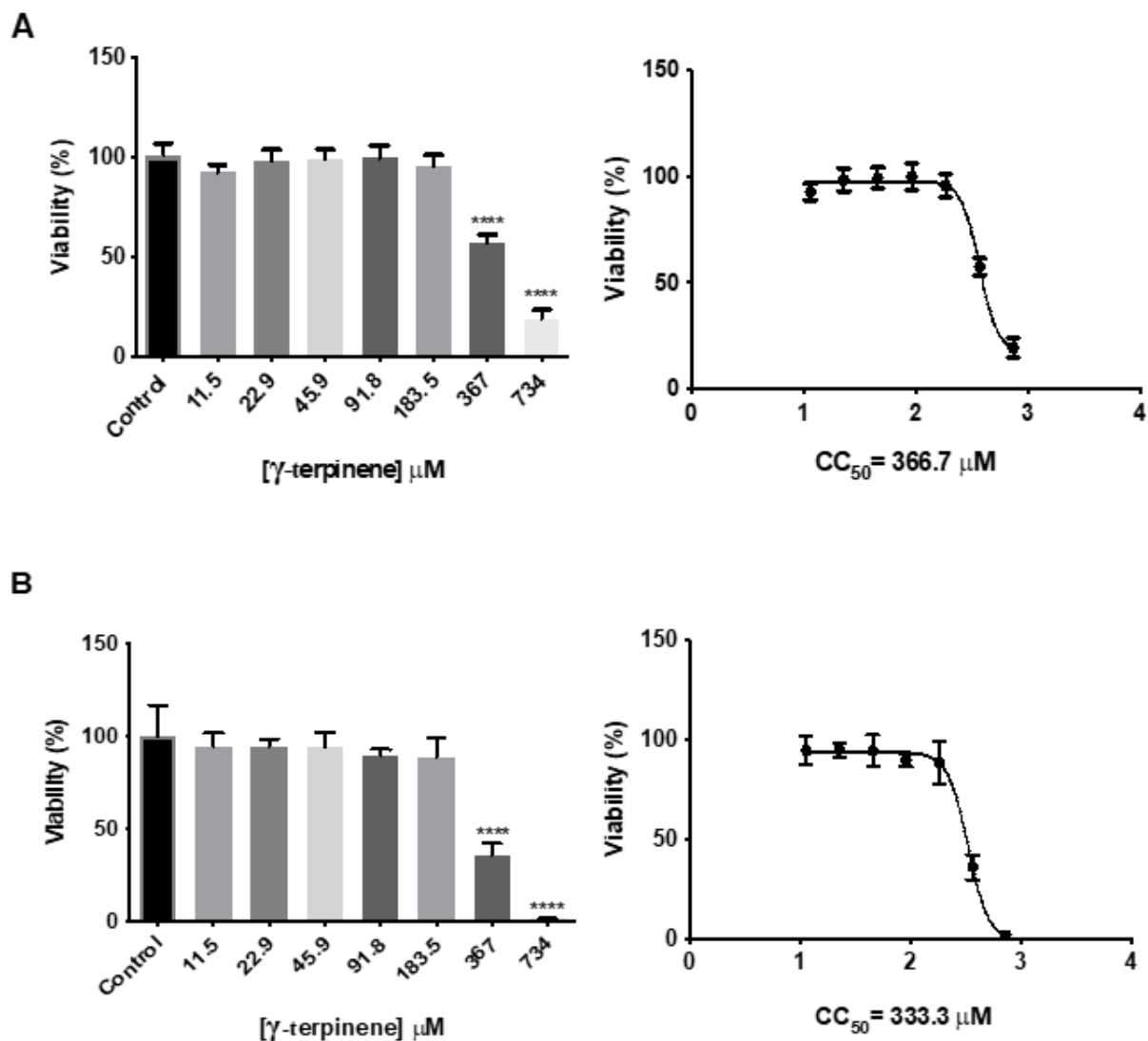


Figure 4

Cell viability and cytotoxic concentration assessed by MTT assay in SVEC4-10 (A) and L929 (B) cells after 72 h of exposure to γ -terpinene. Values represent the mean $\pm$ standard deviation (SD) of three independent experiments, with **** p < 0.001 when comparing control and γ -terpinene concentrations (11.50 to 734 μ M). CC₅₀ = cytotoxic concentration, which indicates 50% inhibition; CI = confidence interval; R² = coefficient of determination. ANOVA followed by Tukey (multiple comparisons) one-way post-test.

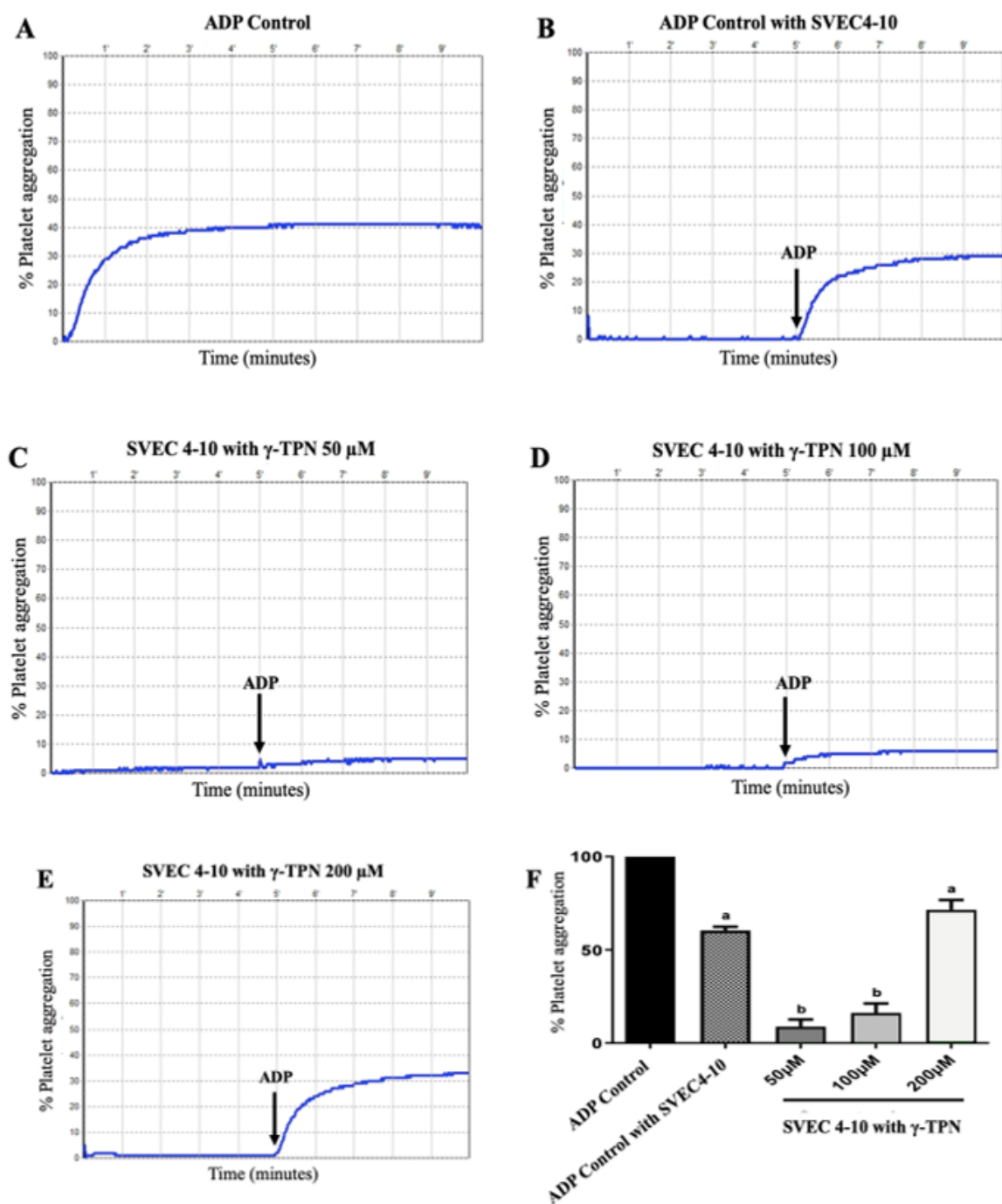


Figure 5

Platelet aggregation in the presence of SVEC4-10 cells treated with γ -TPN (50, 100 and 200 μ M) for 24h and incubated with aggregated platelets under ADP stimulation (10 μ M). The percentage of platelet aggregation as a function of time was determined using an aggregometer (A-E) and expressed as the average of maximum aggregation between different concentrations (F). *** $p < 0.001$ (one-way ANOVA, Tukey post-test).

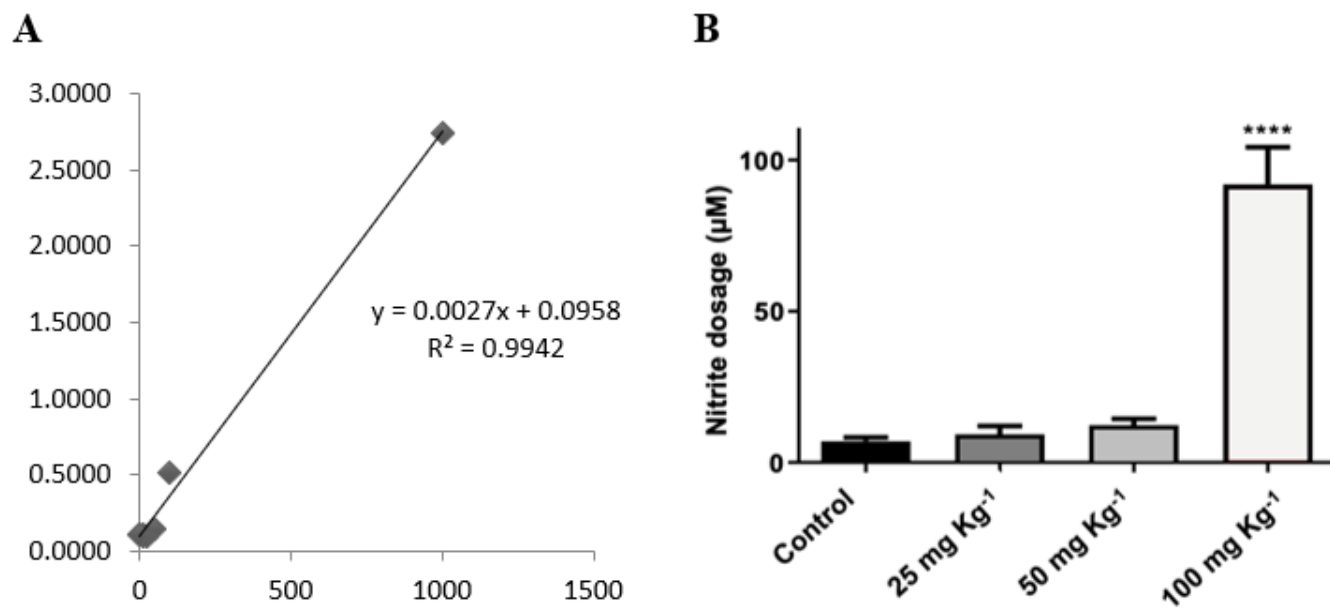


Figure 6

Calibration curve (A) and nitrite dosage (B) in erythrocyte concentrate from rats treated with different doses of γ -terpinene (25, 50 and 100 mg Kg^{-1}). ^a compared to the negative control. **** $p < 0.0001$ (one-way ANOVA, Dunnet post-test).

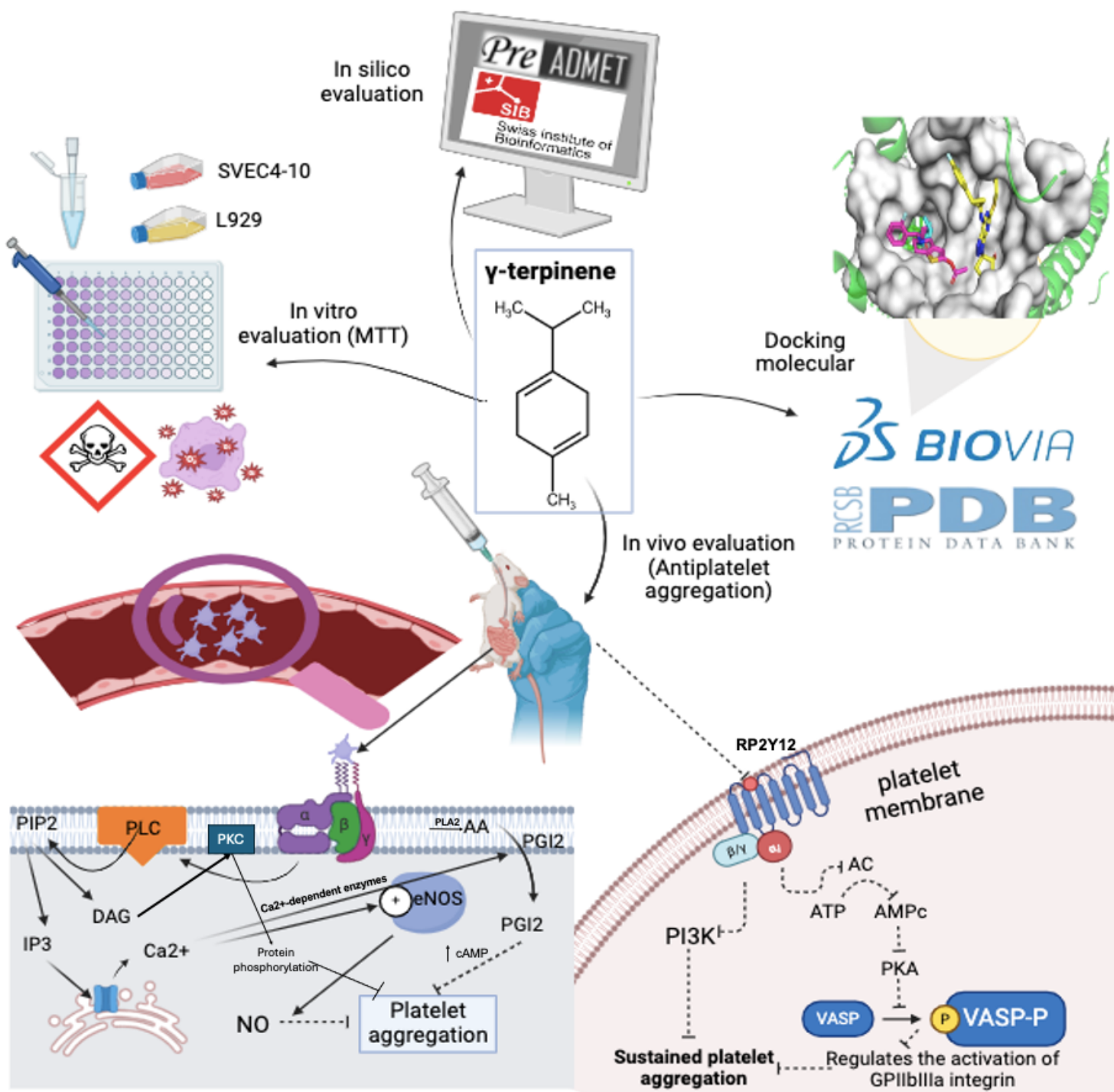


Figure 7

Summary of *in vitro* and *in vivo* biological actions of γ -terpinene.

Legend: AA – arachidonic acid; AC: adenylyl cyclase; cAMP – cyclic adenosine monophosphate; ATP – adenosine triphosphate; Ca²⁺ - calcium ions; COX1 – Cyclooxygenase 1; DAG – diacylglycerol; eNOS – endothelial nitric oxide synthase; IP3 – inositol 1,2,4-trisphosphate; NO – Nitric oxide; PLA2 - Phospholipase A2; PLC – Phospholipase C; PI3K – phosphoinositide 3-kinase; PKA – protein kinase A; PKC - protein kinase C; PIP2: phosphatidylinositol 4,5-bisphosphate; PGI2 – prostacyclin; VASP – vasodilator-stimulated phosphoprotein; VASP-P – phosphorylated vasodilator-stimulated phosphoprotein.