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Cardiovascular effects of farnesol and its β-cyclodextrin complex in normotensive and hypertensive rats

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ABSTRACT

Farnesol (FAR) is a sesquiterpene alcohol with a range of reported biological effects including cardioprotective, antioxidant and antiarrhythmic properties. However, due to its volatility, the use of drug incorporation systems, such as cyclodextrins, have been proposed to improve its pharmacological properties. Thus, the aim of this study was to evaluate and characterize the cardiovascular effects of FAR alone, and to investigate the antihypertensive effects of FAR complexed with β-cyclodextrin (βCD) in rats. Mean arterial pressure (MAP) and heart rate (HR) were measured before and after intravenous administration of FAR (0,5; 2,5; 5 and 7,5 mg/kg) in normotensive rats, and after oral acute administration (200 mg/kg) of FAR and FAR/βCD complex in NG-nitro-L-arginine-methyl-ester (L-NAME) hypertensive rats. In normotensive animals, FAR induced dose-dependent hypotension associated with bradycardia. These effects were not affected by pre-treatment with L-NAME or indomethacin (INDO), but were partially attenuated by atropine. Pre-treatment with hexamethonium (HEXA) only affected hypotension. In the hypertensive rats, FAR/βCD potentialized the antihypertensive effect when compared to FAR alone. Molecular docking experiments demonstrated for the first time that FAR has affinity to bind to the M_3 and M_2 muscarinic, and nicotinic receptors through hydrogen bonds in the same residues as known ligands. In conclusion, our results demonstrated that FAR induced hypotension associated with bradycardia, possibly through the muscarinic and nicotinic receptors. The inclusion complex with βCD improved the antihypertensive effects of FAR, which can be relevant to improve current cardiovascular therapy using volatile natural components.

1. Introduction

Arterial hypertension is a multifactorial chronic disease that is considered the most common risk factor for cardiovascular disease (CVD) development, which currently affects more than 1 billion people worldwide (Mendis et al., 2011; Mills et al., 2016). Thus, controlling high blood pressure is crucial to avoiding cardiovascular complications (Sociedade Brasileira de Cardiologia, 2016). Worldwide, less than 40% of people with hypertension are currently in treatment, and only around 14% have their systolic blood pressure controlled (Mills et al., 2016). Hence, the effective treatment of hypertension remains a global challenge, and the development of effective, cheaper drugs with minimal side effects could help to reduce the burden on public health.

In this context, essential oils have in recent decades been attracting attention due to their therapeutic effects (Bakkali et al., 2008; de Andrade et al., 2017; Menezes et al., 2010; Moreira et al., 2010). They are a volatile liquid comprising a complex mixture of different substances that occur naturally in aromatic plants, with terpenes being the

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main component (Bakkali et al., 2008; Dewick, 2009). Farnesol (FAR), (3,7,11-trimethyl-2,6,10-dodecatriene-1-ol), is an alcoholic sesquiterpene, with the chemical formula $C_{15}H_{26}O$ (Duke, 1981) (Fig. 1). It is widely employed in industry in cleaning products, cosmetics and perfumes. Moreover, FAR is generally recognized as safe (GRAS) as a flavor ingredient for human consumption by the U.S. Food and Drug Administration (FDA) (Lapczynski et al., 2008).

Pharmacological effects of FAR have been demonstrated in respect of: growth suppression of pancreatic carcinoma (Burke et al., 1997), antioxidant potential (Khan and Sultana, 2011), blockade of type-L calcium voltage-gated channels in vascular smooth muscle cells (Luft et al., 1999; Roullet et al., 1997), antiarrhythmic effects (Souza et al., 2019), and cardioprotective activity against ischemia/reperfusion injury (Szűcs et al., 2013). However, such properties can be compromised by the high volatility, poor solubility in aqueous systems and short half-life of most terpenes (Carneiro et al., 2019; Siqueira-Lima et al., 2014), especially when oral administration is used. To overcome these limitations, encapsulation in drug-delivery systems has been used, mainly in cyclodextrins (Camargo et al., 2018; Carneiro et al., 2019; de Oliveira-Filho et al., 2018; Moreira et al., 2016; Pinho et al., 2014).

Cyclodextrins (CDs) are cyclic oligosaccharides composed by six, seven or eight glucopyranose units (α, β and γ cyclodextrins, respectively) united together by α-1,4-glycosidic bonds (Stella and He, 2008). They are characterized by a hydrophilic exterior surface and a lipophilic central cavity (Loftsson et al., 2007). CDs have been widely used to form complexes with natural compounds, such as terpenes, and it has been shown that they improve their solubility and stability, while possibly reducing any toxic effects (Marques, 2010; Moreira et al., 2016; Pinho et al., 2014; Quintans-Júnior et al., 2013). Moreover, CDs are also recognized by the FDA as being safe and are already present in several products, including drugs (Loftsson and Brewster, 2010; Uekama et al., 1998; Zafar et al., 2014).

In recent years, the use of CDs has increased, mostly in complexing essential oils, such as mono and sesquiterpenes (Carneiro et al., 2019). When complexed with CDs, such compounds become an easy-to-handle powder, show better therapeutic effects, bioavailability and allow dose reduction (Marques, 2010; Pinho et al., 2014). β-cyclodextrin (βCD) is one of the most commonly used CDs, due to its low price, versatility, efficiency and low toxicity (Ciobanu et al., 2013; de Oliveira-Filho et al., 2018; Pinho et al., 2014).

Therefore, we aimed to evaluate the cardiovascular effects of farnesol alone, and possible improvements produced by complexing it with βCD to use in the treatment of arterial hypertension.

2. Material and methods

2.1. Drugs and reagents

Farnesol (FAR, ≥ 95% purity), β-cyclodextrin (βCD, 98% purity), hexamethonium bromide (HEXA), atropine sulphate, indomethacin (INDO), nifedipine and NG-nitro-L-arginine-methyl-ester (L-NAME) were all purchased from Sigma-Aldrich (St. Louis, MO, USA); Tween 80 from Oxiteno, Brazil; and ketamine chloride and xylazine from SESPO, SP, Brazil. For vehicle solutions, tween 80 was solubilized in saline solution (0.15% v/v). FAR was solubilized in saline/tween 80 (0.15% v/v), for intravenous administrations, or distilled water/tween 80 (0.15% v/v), for oral administrations.

2.2. Preparation of inclusion complex

The inclusion complex between FAR and βCD was made by slurry complexation (SC) process, as described by Silva et al., (2017) (Silva et al., 2017). Briefly, 222.37 mg of FAR was manually mixed in a porcelain crucible containing βCD (1135 mg), in a 1:1 M ratio. For SC, 20 ml of distilled water was added to the above mixture in a magnetic agitator for 36 h. Thereafter, the material was dried in a desiccator at room temperature to form a film, which was removed by manual trituration and then stored in airtight glass containers (Menezes et al., 2013; Silva et al., 2017).

2.3. Animals

Adult male Wistar normotensive rats (250–350 g) were used for all the experiments. The animals were randomly housed in appropriate cages at a controlled temperature (25 ± 1 °C) on a 12-h light/dark cycle (6:00 a.m. to 6:00 p.m.) with free access to food (Purina®, Sao Paulo, Brazil) and tap water.

All procedures described in the present study were approved by the Animal Research Ethics Committee of the Federal University of Sergipe (protocol 56/17). Animal handling was in compliance with the Principles of Laboratory Animal Care (NIH publication 86–23, revised 1985; nih.gov/regs/index.htm). In addition, all efforts were made to minimize the number of animals used and any discomfort.

2.4. Hemodynamic evaluation of FAR by intravenous administration in normotensive rats

The measurements of mean arterial pressure (MAP) and heart rate (HR) were performed as described by Nascimento et al. (2019) (Nascimento et al., 2019). Briefly, the rats were anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Polyethylene catheters filled with heparinized saline were inserted into the abdominal aorta artery and the lower vena cava via the left femoral artery and vein, respectively. The vein access was used for FAR and pharmacological tool administration. Thereafter, following subcutaneous insertion and fixation, the catheters were exteriorized through a skin incision between the scapulae. The animals were put in individual cages and allowed a 24-h post-surgery recovery period before the experiments.

For the measurements, the arterial catheter was connected to a pressure transducer (Edwards Lifesciences, Irvine, CA, USA) coupled to an amplifier (FE221, Bridge Amp; ADInstruments, Bella Vista, NSW, Australia). The data were recorded using a computer with an analog-digital interface, and were processed using the software LabChart Pro v.7 (ADInstruments, USA). After the recovery period (24h), MAP and HR were measured before (baseline values) and after the intravenous administration of FAR (0.5, 2.5, 5 and 7.5 mg/kg, n = 8) or vehicle (n = 8) in order to obtain dose-response curves. The injections were made randomly in each animal subject, with time interval between doses enough to allow full recovery of the baseline hemodynamic values.

To verify the role of the muscarinic receptors; nitric oxide (NO); cyclooxygenase (COX) metabolites, mainly prostacyclin (PGI_2); and nicotinic receptors, a second series of experiments were performed in which animals (n = 6, each group) were pre-treated separately and randomly with either atropine, (2 mg/kg, i.v., 15 min), a muscarinic cholinergic antagonist (Mitchelson, 1984); L-NAME, (20 mg/kg, i.v., 30

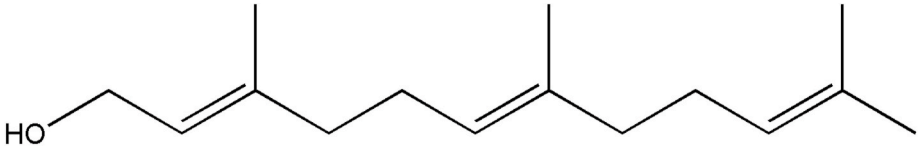

Fig. 1. Chemical structure of farnesol.

min), a nitric oxide (NO) synthase inhibitor (Ribeiro et al., 1992); indomethacin (INDO), (5 mg/kg, i.v., 30 min), a potent cyclooxygenase inhibitor (Clark and Fuchs, 1997); or hexamethonium (HEXA), (20 mg/kg, i.v., 30 min), a ganglionic blocker (Takahashi and Owyang, 1997), respectively. All doses were chosen based on previous studies (Bastos et al., 2010; Moon et al., 2013; Moreira et al., 2010; Santos et al., 2015). After the pre-treatments, the same doses of FAR was administrated to obtain new dose-response curves. The changes of MAP and HR for each dose was expressed as a percentage of baseline values in awake normotensive animals by the formula:

$$\%\ Response = \left[\frac{\left(MAP_{final} - MAP_{initial}\right) \times 100}{MAP_{initial}}\right]$$

2.5. Hemodynamic evaluation of FAR and the complex FAR/βCD by oral administration in hypertensive rats

To identify the effects of FAR and its complex FAR/βCD administrated orally, another set of experiments were performed. Hypertensive animals were obtained through administration of L-NAME (20 mg/kg) by oral route (gavage) for seven days (Biancardi et al., 2007; Moreira et al., 2016). The animals were divided into four groups (n = 5, each) as follows: farnesol (200 mg/kg) (FAR), complex form FAR/βCD (200 mg/kg) (FAR/βCD), vehicle group (distilled water and βCD) (VG) and group treated with nifedipine (10 mg/kg) (NG), a reference antihypertensive drug used as a positive control (Beevers et al., 2015). All treatments were administered by gavage in a single dose. MAP and HR were recorded at the time 0 (before treatments) and 0.5, 1, 2, 3, 4, 8, 24, 30 and 48 h after treatments. The hemodynamic measurements were made as described by Nascimento et al. (2019).

2.6. Molecular docking

Molecular docking simulations were performed in order to study the interactions between FAR and three possible targets associated with its cardiovascular activity. The selected targets were: i) Nicotinic acetylcholine receptor α3 subunit (Nicotinic receptor), ii) Muscarinic acetylcholine receptor M_2 (M_2 receptor) and iii) Muscarinic acetylcholine receptor M_3 (M_3 receptor), these subtypes were chosen according to previous studies (Bény et al., 2008; De Biasi, 2002; Harvey, 2012; Hoover et al., 1994). Of the three defined targets, only the M_3 receptor structure has been experimentally determined for *Rattus norvegicus* (PDB code: 5ZHP). In the case of M_2 and nicotinic receptor, structural models for the rat proteins were generated using Modeller 9.19 (https://salilab.org/modeller/) (Sali and Blundell, 1993).

This homology modelling technique is based on the observation that similarity between proteins at the sequence level is reflected at the structural level. Therefore, if the 3D structure of a given protein is available, it is possible to generate models of proteins homologous to it. Generally, when the sequence identity is above ~ 30%, homology is assumed (Hillisch et al., 2004). In addition, there is a consensus that 3D models that share a sequence identity with their templates greater than ~ 50% are frequently accurate enough for drug discovery studies (Cavasotto and Phatak, 2009; Hillisch et al., 2004). Thus, the M2 and nicotinic receptors from rat have 67.56% and 77.19% sequence identity, respectively, with experimentally solved structures.

To perform homology modelling, the amino acid sequences obtained from Uniprot (https://www.uniprot.org/) and the templates with the Protein Data Bank (PDB) codes 3UON and 6PV7 were used, which correspond to the human M_2 and nicotinic receptor, respectively. Ten models were generated for each receptor, then a validation of the models was made using Procheck (Laskowski et al., 1993). Procheck is a physic-based method that assess the quality of a structure based on several stereochemical parameters, including the distribution of backbone torsion angles Φ and Ψ (Ramachandran plot). The different regions of the plot are core, allowed, generously allowed and disallowed. Structures with more than 90% of residues in the core region indicate good quality. The best model of M_2 receptor presented 95% of the residues in this region, and the best model of the nicotinic receptor presented 91.5%.

For molecular docking, a comparative approach was used in which the predicted interactions for the target-FAR complexes were compared with interactions between the targets and known antagonists or agonists. The structure of farnesol was obtained from the PubChem database (Compound id: 3327) (https://pubchem.ncbi.nlm.nih.gov/). In addition to FAR, the compounds (1R, 2R, 4S, 5S, and 7S) −7 - ({[4-fluoro-2-(thiophen-2-yl) phenyl] carbamoyl} oxy) −9,9- dimethyl-3-oxa-9-azatricyclo [3.3.1.0–2.4 ~] nonan-9-ium) [abbreviated to 9 EC: M_3 receptor antagonist]; (3R) -1-azabicyclo [2.2.2] oct-3-yl hydroxy (diphenyl) acetate [QNB: M_2 receptor antagonist]; and Nicotine [NCT: nicotinic receptor agonist] were also used as ligands. Ligand structures were obtained from the PDB with the above-mentioned codes. Ligand and receptor structures were prepared using the programs Open Babel (O'Boyle et al., 2011) and VMD 1.9.3 (Humphrey et al., 1996).

Docking calculations were then performed using the AutoDock Vina method (Trott and Olson, 2009), implemented in PyRx 0.8 (Dallakyan and Olson, 2015). AutoDock Vina measures the binding energy through a hybrid scoring function that uses aspects of knowledge-based potentials and empirical scoring functions. The scoring function has five terms: three correspond to steric interactions, one to hydrophobic interactions and one to hydrogen interactions. The energy calculation is based on the weighted sum of these terms (Trott and Olson, 2009). For each receptor, the grid center was positioned at the allosteric or orthosteric binding pockets, with the cube dimensions being 25 × 25 × 25 Å. All other parameters were defined as default. To analyze the results of the molecular docking, the Discovery Studio v16 program was used (BIOVIA).

2.7. Statistical analysis

Values were expressed as mean ± standard error of the mean (S.E.M). To evaluate differences between means, Student's t-test or one- or two-way analysis of variance (ANOVA) followed by Bonferroni's post-test were used, and a $p < 0.05$ was considered significant. All statistical analyses were done using Graph Pad Prism 6.01™ (Graph-Pad Prism Software Inc., San Diego, CA, USA).

3. Results

3.1. Effect of intravenous administration of FAR on hemodynamic parameters in normotensive rats

In order to obtain the first dose-response curves of the effect of FAR in normotensive rats, intravenous administration in awaken rats were performed. The baseline MAP value in the non-anaesthetized normotensive rats was 118 ± 8 mmHg, and HR was 342 ± 9 bpm (n = 8). In these animals, intravenous bolus injections of FAR (n = 8) induced a dose-dependent and transitory hypotension from 0.5, 2.5 and 5 mg/kg doses (105.2 ± 3.9, 78.4 ± 6.4, 71.4 ± 7.5 mmHg, respectively), and at dose of 7.5 mg/kg, a less intense hypotension (82.1 ± 12.1 mmHg). This effect was associated with a significant bradycardia at all doses (279.9 ± 34, 167.4 ± 31, 152.1 ± 35 and 171.8 ± 45 bpm, respectively) (Fig. 2).

3.1.1. Participation of muscarinic and nicotinic receptors, COX metabolites and nitric oxide in FAR-induced responses in normotensive rats

Regarding pre-treatment with atropine, the HR baseline increased from 342 ± 9 to 535 ± 12 bpm, with no change in MAP; L-NAME increased MAP from 118 ± 8 to 162 ± 4 mmHg, and decreased HR from 342 ± 9 to 277 ± 9 bpm; HEXA decreased MAP from 118 ± 8 to 104 ± 3 mmHg, and increased HR from 342 ± 9 to 396 ± 14 bpm. However, there was no change in any parameter following pre-treatment with INDO.

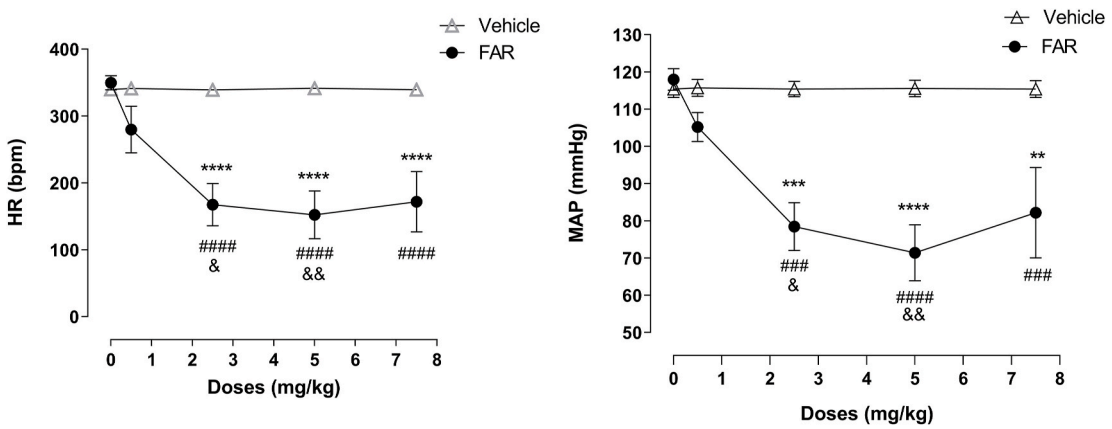

Fig. 2. Original traces showing the effect of FAR (0.5, 2.5, 5 and 7.5 mg/kg, i.v.) on pulsatile arterial blood pressure of normotensive rats. The arrows represent the time of injection.

The hypotension and bradycardia induced by FAR in rats pre-treated with L-NAME and INDO did not significantly change. However, pre-treatment with HEXA was able to attenuate the hypotension induced by the doses of 0.5 and 2.5 mg/kg ($P < 0.01$). In the presence of atropine, hypotension was attenuated only by the 2.5 mg/kg dose ($P < 0.01$), in addition, the bradycardia was fully abolished by the 2.5, 5 ($P < 0.0001$), and 7.5 mg/kg ($P < 0.001$) doses (Fig. 3A and B).

3.2. Inclusion complex containing FAR in β-cyclodextrin (βCD) enhanced the antihypertensive effect of sesquiterpene via oral route

After confirming the cardiovascular effects of FAR administered by the intravenous route in normotensive rats, we aimed to test the administration of both FAR and its complexed form with βCD by the oral route in hypertensive rats. The animals were pre-treated with L-NAME (20 mg/kg/day) for seven days via oral route (gavage) and showed an increase in MAP from 122 ± 4 to 156 ± 7 mmHg ($P < 0.01$; $n = 5$), confirming the hypertensive condition (Fig. 4A). On the other hand, mean HR before (377 ± 7 bpm) and after L-NAME treatment (397 ± 7 bpm) did not change significantly (Fig. 4B).

L-NAME hypertensive animals treated with vehicle (VG) did not present any significative change in MAP. However, treatment with nifedipine (NG) was able to significantly reduce MAP from 0.5 until 8 h after treatment, compared to the complex (FAR/βCD), farnesol (FAR) and vehicle group (VG). FAR only induced a significant decrease in MAP at 48 h; however, FAR/βCD significantly reduced MAP at 30 and 48 h when compared to the VG (Fig. 4A). Regarding heart rate (HR), the NG group presented a significant increase between 0.5 and 3 h, when compared to FAR/βCD and FAR groups (Fig. 4B). HR was reduced significantly in the FAR/βCD group at 0.5, 1 and 2 h compared to VG, and additionally between 1 and 3 h, compared to the FAR group. Neither VG or FAR presented any significant change in HR.

3.3. Molecular docking

The molecular docking procedure was validated based on the redocking of ligands 9 EC, QNB, and NCT, in their respective targets obtained from PDB (5ZHP, 3UON and 6PV7, respectively). The poses of the ligands with the best redocking scores were very close to the experimentally determined conformations (Fig. 5). Regarding the docking analysis, although the volume of the defined grid is considerably greater than the volume of the allosteric or orthosteric binding pockets, and the ligands can bind in other regions of the proteins, for all the studied targets the conformations of the ligands with better scores

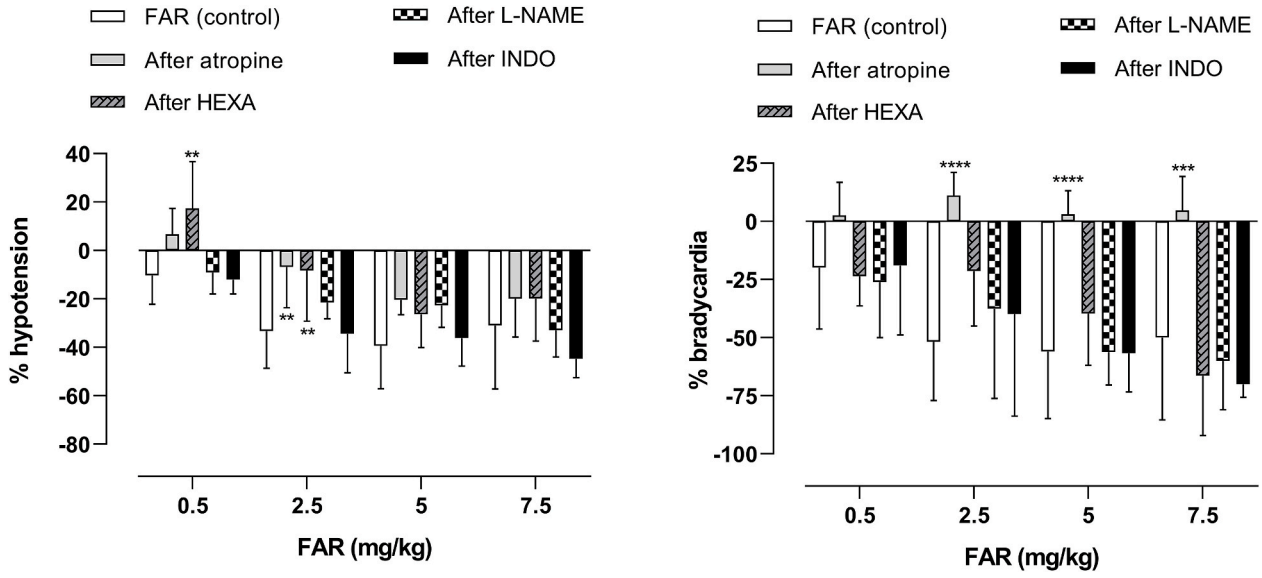

Fig. 3. Effect of FAR (0.5; 2.5; 5, and 7.5 mg/kg, i.v.) on MAP (A) and HR (B) in normotensive rats. Values are mean ± S.E.M. of eight experiments. $^{**}P < 0,01$; $^{***}P < 0,001$ and $^{****}P < 0,0001$ vs vehicle; $^{\#\#\#}P < 0,001$ and $^{\#\#\#\#}P < 0,0001$ *vs* baseline; $^{\&}P < 0,05$ and $^{\&\&}P < 0,01$ FAR *vs* FAR (0.5 mg/kg).

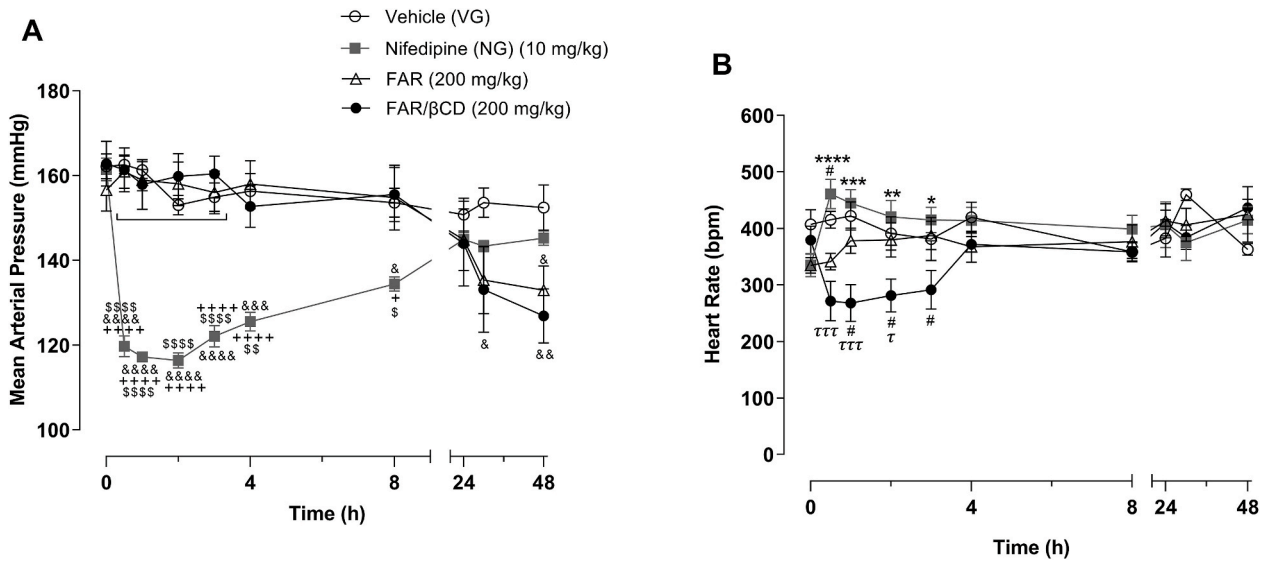

Fig. 4. Hypotensive and bradycardic response induced by FAR in normotensive rats before (FAR control) and after acute administration of atropine (2 mg/kg, i.v.), HEXA (20 mg/kg, i.v.), L-NAME (20 mg/kg, i.v.) or INDO (5 mg/kg, i.v.). Values are mean ± S.E.M. of 6 experiments. **P < 0.01, ***P < 0.001 and ****P < 0.0001 *vs* FAR (control).

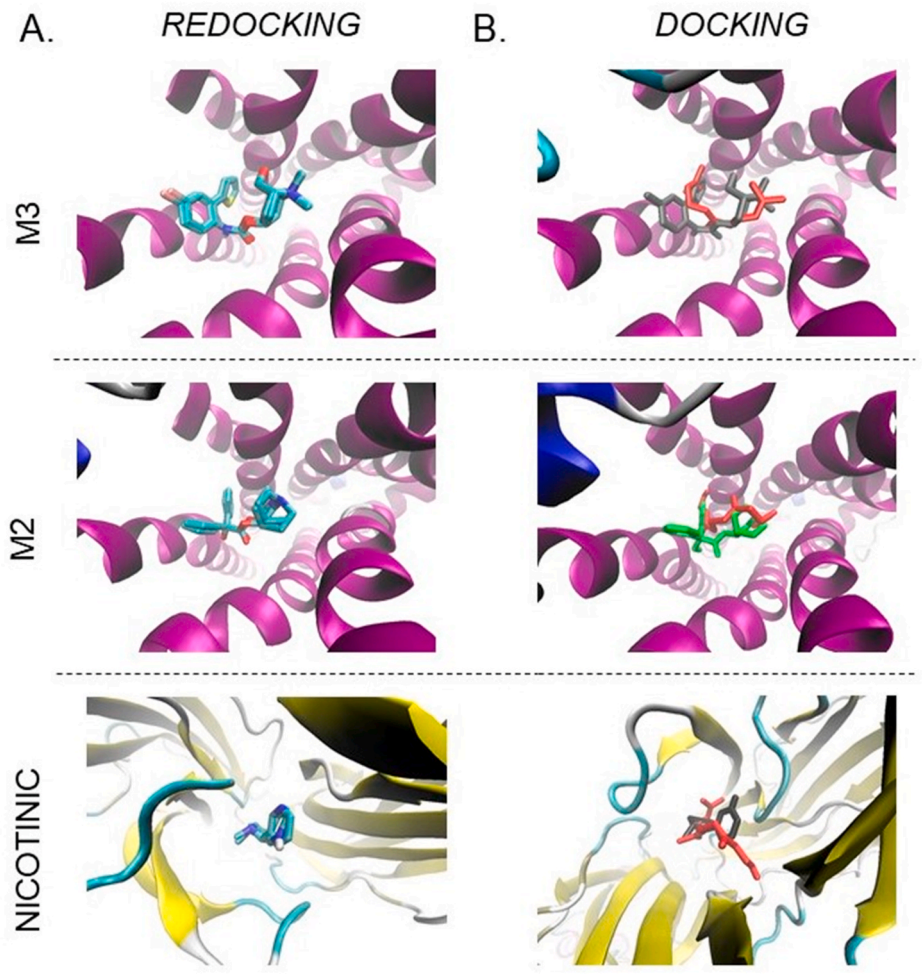

Fig. 5. Mean Arterial Pressure (A) and Heart Rate (B) of L-NAME hypertensive rats before (time 0) and after 0.5, 1, 2, 3, 4, 8, 24, 30 and 48 h of the administration of vehicle (VG), nifedipine (NG), farnesol (FAR) or the complex (FAR/βCD) by oral route. Values are mean ± S.E.M. of 5 experiments. $^{\$}$ P < 0,05, $^{\$\$}$ P < 0,01, $^{\$\$\$\$}$ P < 0,0001, *P < 0,05, **P < 0,01, ***P < 0.001, ****P < 0.0001 *vs* FAR/βCD; $^{\&}$ P < 0,05, $^{\&\&}$ P < 0,01, $^{\&\&\&}$ P < 0.001, $^{\&\&\&\&}$ P < 0.0001, $^{\tau}$ P < 0,05, $^{\tau\tau\tau}$ P < 0.001 *vs* VG;$^{+}$P < 0.01, $^{++++}$ P < 0.0001, $^{\#}$P < 0,05 *vs* FAR.

were restricted to these sites (Fig. 5). For the following analyzes, the best pose of ligands for each receptor was selected.

The predicted binding energies for the complexes formed with FAR were −7.6 kcal/mol, −7.3 kcal/mol, and −7.2 kcal/mol for the M_3, M_2, and nicotinic receptors, respectively. This indicates that FAR has a slightly higher affinity for the M_3 receptor compared to the others. The

2D representation of the complexes, the types of interactions, and participatory residues can be seen in Fig. 6. The main interactions that occur between the M_3 receptor and the 9 EC antagonist are hydrogen bonds through the residues SER151 and ASN507, electrostatic interactions with ASP147 and TYR529, and hydrophobic interactions. Similarly, according to the docking analysis, the M_3-FAR complex interacts with ASN152 and ALA238 residues through hydrogen bonds, in addition to hydrophobic interactions with seven other residues, including TYR529, which also interacts with 9 EC.

The QNB antagonist binds to its target, human-M_2, through hydrogen bonds with ASN404, in parallel with seven other residues that favor the formation of the complex through hydrophobic interactions, mainly TYR104 and TYR403. Our results indicated that FAR also interacts with the TYR104 residue from rat-M_2, but through a hydrogen bond, in addition to another h-bond formed with ASN108. Moreover, hydrophobic interactions with other residues of the allosteric site also contributed to stabilization.

Finally, it was predicted that FAR can bind precisely at the orthosteric site of the nicotinic receptor. Interestingly, the binding energy of FAR to the rat-nicotinic receptor was higher than that of nicotine, being −7.2 and −6.9 kcal/mol, respectively. These results indicate that FAR has an affinity for the nicotinic receptor comparable to its agonist. The predictions suggest that FAR interacts with this receptor through a hydrogen bond with residue LYS132, that is homologous to LYS113 from human nicotinic receptor, and located at β4 subunit region that constitutes the binding pocket. Hydrophobic interactions mediated by residues located at the interface also occurred.

4. Discussion

The search for new pharmacological therapies has been increasing in recent years, especially in respect of CVDs, for which there is an urgent need to find new treatment options. For this reason, and given the few pharmacological studies regarding the cardiovascular effects of FAR, the present study aimed to investigate its therapeutic effects, and to evaluate whether the FAR/βCD inclusion complex would be able to improve the pharmacological properties of this sesquiterpene. Our main results showed that when administrated intravenously, FAR alone was able to reduce MAP and HR in normotensive rats. This hypotensive effect seems to involve the muscarinic and/or nicotinic receptors, while the bradycardia induced by FAR is probably through the muscarinic receptors. This indication was later confirmed by a docking analysis. Furthermore, oral treatment with FAR and the FAR/βCD complex showed an antihypertensive effect in an L-NAME-induced animal model, and, more interestingly, the pharmacological activity of the βCD complex had a better and longer lasting effect than the FAR alone, which seems to be as a result of an improvement in the bioavailability of FAR when protected in the βCD complex.

In order to test the hypothesis whether FAR would induce cardiovascular effects, we injected FAR in normotensive rats. In these animals,

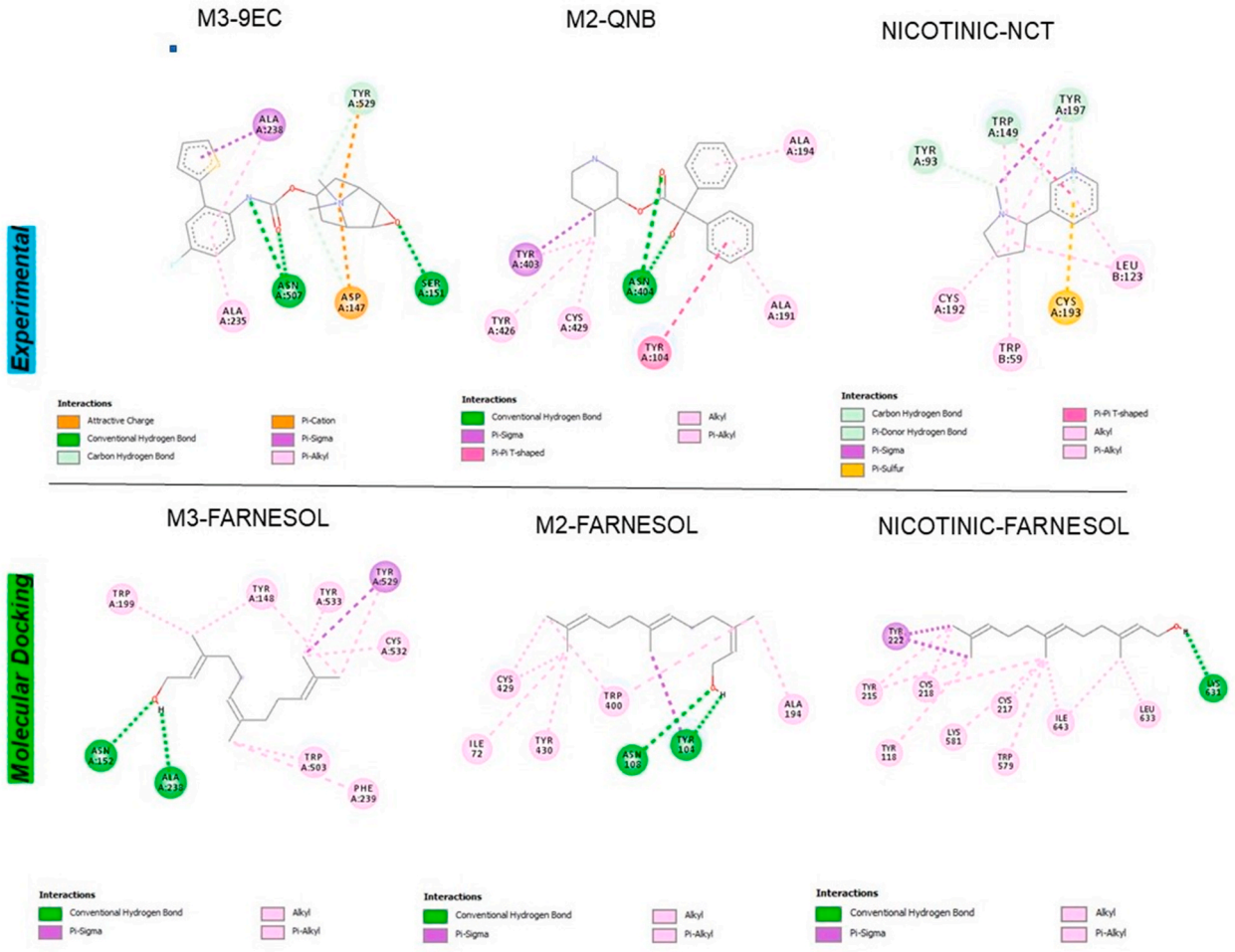

Fig. 6. Comparison of the conformation of ligands in allosteric (M3 and M2) and orthosteric (nicotinic) receptor binding sites. (A) represents the redocking procedure for each experimental structure of receptor utilized, the ligand conformation at experimental and redocking fit are superimposed. (B) Tridimensional representation of molecular docking results, all three proteins are from *Rattus norvegicus*. The conformation of farnesol (red) was compared with the respective antagonist (9 EC for M_3, QNB for M_2) or agonist (NCT: nicotinic).

the baseline MAP and HR values were the same as described in previous studies (Anjos et al., 2013; Cunha et al., 2004; de Siqueira et al., 2006; Menezes et al., 2010). Intravenous bolus doses of FAR was able to induce a dose-dependent and transitory hypotension at the initial doses (0.5, 2.5 and 5 mg/kg), and a less intense but significant hypotension at 7.5 mg/kg dose. Such effect was associated with bradycardia. To the best of our knowledge, this is the first time that such effects of FAR have been reported in rats. Also, it is worth mentioning that even at such small doses FAR could induce these cardiovascular effects, proving its effectiveness. Cardiovascular studies with other terpenes, such as (−)-α-bisabolol (Menezes et al., 2010), geranial (Moreira et al., 2010), anetole and estragole (de Siqueira et al., 2006), d-limonene (Nascimento et al., 2019), and carvacrol (Dantas et al., 2015) have also demonstrated the same effect of hypotension followed by bradycardia. Recently, Souza et al. (2019) also demonstrated that FAR had bradycardic and antiarrhythmic properties on isolated rat heart.

The first studies indicating the pharmacological importance of FAR suggested it acted through controlling vascular tone by inhibiting vasoconstriction in both rat and human arteries (Roullet et al., 1996). Indeed, in vascular smooth muscle cells (VSMCs) it was demonstrated that 10 μM of FAR inhibited Ca^{+2} signaling (Roullet et al., 1997), specifically in respect of the α(1C) subunit in the L-type Ca^{+2} channel (Luft et al., 1999). As is well known, VSMCs play an essential role in modulating vascular tone in blood vessels, and, consequently, in the control of blood pressure (Somlyo and Somlyo, 1994). The relaxation or contraction of these cells are mediated mainly by hormones, neurotransmitters and endothelium derived factors (Furchgott and Zawadzki, 1980; Jackson, 2000). In contraction, the $[Ca^{+2}]_i$ is increased either by external influx from the Ca^{+2} channel opening, or by release from the sarcoplasmic reticulum (Devine et al., 1972; Horowitz et al., 1996; Webb, 2003).

Furthermore, it is also known that activation of endothelial muscarinic receptors results in vasorelaxation due to the release of endothelium-derived relaxing factors, such as NO and PGI_2 (Moncada and Higgs, 1993; Schulz and Triggle, 1994), which decrease peripheral vascular resistance, ultimately leading to hypotension (Furchgott and Zawadzki, 1980). Hence, to verify the involvement of these receptors in the effects induced by FAR, we performed experiments where rats were pre-treated with atropine, a non-selective antagonist of these receptors (Mitchelson, 1984). In these animals, atropine was able to significantly attenuate the hypotension induced by FAR only at a dose of 2.5 mg/kg (Fig. 3). Thus, endothelial muscarinic receptors seems to be involved in this effect, at least at this dose.

Moreover, physiological responses can also be elicited direct in the heart through cardiac muscarinic receptors, mostly the M_2 type (Hoover et al., 1994; Irisawa et al., 1993), which by vagal activation in the sinoatrial node cause intense bradycardia, followed or not followed by hypotension (Harvey, 2012; Irisawa et al., 1993; Peterson et al., 1984). Thus, our results showed that the bradycardia was fully abolished by the 2.5, 5 and 7.5 mg/kg doses in rats pre-treated with atropine (Fig. 3). This suggests that bradycardia seems to specifically involve the cardiac muscarinic receptors, hence contributing to a reduction in blood pressure. Cunha et al. (2004) found similar results, with the sesquiterpene trans-caryophyllene, the major compound of *Ocotea duckei* essential oil with bradycardia being eliminated by atropine pre-treatment.

On the other hand, the lack of effect on blood pressure after muscarinic blockade suggest that other mechanisms, such as ganglionic or vascular factors may be involved in this effect. Thus, to check if FAR acts via cholinergic activation through nicotinic receptors, animals were pre-treated with HEXA, a ganglionic blocker (Takahashi and Owyang, 1997). Under these conditions, FAR-induced hypotension was abolished only by the 0.5 and 2.5 mg/kg doses, while the bradycardia was not significantly changed at any doses (Fig. 3). Hence, hypotension caused by FAR may involve, at least in part, participation of the nicotinic receptors.

It is well stablished that hypotension may be caused by endothelium derived factors that promote vascular tonus regulation, mainly NO and PGI_2 (Moncada and Higgs, 1991; Schulz and Triggle, 1994). Thus, it is possible that the decrease in blood pressure induced by FAR may be caused by these factors. To investigate this possible pathway, we performed experiments with animals pre-treated with L-NAME or INDO. As shown in Fig. 3, hypotension and bradycardia were not changed by L-NAME pre-treatment. This suggests that NO does not appear to be involved in the hypotension produced by FAR, neither through an independent route of muscarinic activation or by direct activation of these receptors, since pre-treatment with atropine did not change MAP significantly either. These results are in agreement with the results of studies of the sesquiterpene trans–caryophyllene (Cunha et al., 2004), and the monoterpenes citronellal (Andrade et al., 2012) and geranial (Moreira et al., 2010). Likewise, INDO did not change any of the parameters either (Fig. 3), suggesting that PGI_2 is not involved in the effects of FAR.

Hypertension is a chronic condition characterized by sustained high blood pressure which can lead to many other cardiovascular diseases (Kearney et al., 2005; Whelton et al., 2018), thus increasing the burden on the health systems (Vasan et al., 2001). Recent data has shown that, despite the advances in recent decades in drug development research, the U.S. Food and Drug Administration (FDA) approved 33% less new cardiovascular drugs (Kaitin and DiMasi, 2011) compared to other classes of drugs such as those for the treatment of cancer and neurological disorders (Batta et al., 2020). Therefore, the need for new, less expensive molecular entities for CVD treatment is needed (de Andrade et al., 2017; Fabricant and Farnsworth, 2001; Prashant, 2017).

For this reason, the number of studies of the pharmaceutical effects of natural products such as terpenes, has increased in recent years (Alves-Silva et al., 2016; Li et al., 2015; Newman and Cragg, 2016; Prashant, 2017; Silva et al., 2019). However, natural components, especially those from essential oils, often have some limitations that prevent their broadest use, including low aqueous solubility and bioavailability, and high instability (Siqueira-Lima et al., 2014; Zaheer et al., 2010). To overcome these issues, drug delivery systems using inclusion complexes with cyclodextrins (CDs) have been widely used (Ciobanu et al., 2013; de Oliveira-Filho et al., 2018; Lima et al., 2016; Miyake et al., 2000). Additionally, CD complexation transforms oily substances into easy-to-handle powder, which can also improve their volatility (Menezes et al., 2012; Pinho et al., 2014). Recently, a review by Carneiro et al. (2019) (Carneiro et al., 2019) demonstrated the biological benefits of CDs complexes in both *in vivo* and *in vitro* studies. In this regard, β-cyclodextrin (βCD) has been the most commonly used cyclodextrin to form inclusion complexes with volatile compounds, such as sesquiterpenes, for oral administration (Challa et al., 2005; Marreto et al., 2008; Serafini et al., 2012), and βCD-complexation applied in hypertension therapy seems to have a promising future (Alves et al., 2019).

Therefore, given the evidence for the pharmacological effects through the intravenous route, we next aimed to evaluate the effects of FAR, and its inclusion complex with βCD, in hypertensive rats via the oral route. As known, the oral route it is the most used for pharmacological treatment for hypertension (Burnier et al., 2020; Camargo et al., 2018). The L-NAME hypertensive model is well stablished in the literature, based in the non-specific inhibition of nitric oxide sintase (NOS) (Biancardi et al., 2007; Ribeiro et al., 1992) when given orally for 7 days consecutively, and have been used elsewhere (Biancardi et al., 2007; Moreira et al., 2016).

In regard to the complex FAR/βCD, a very detailed physicochemical characterization was made previously by our group (Silva et al., 2017), which shows that SC method had a 8.3% of mass loss with FAR being 100% volatilized (100–209 °C). The water percentage found was 7.81 ± 0.54%, proving great substitution of water molecules from βCD cavity with molecules of FAR. Moreover, the complex featured small and misshapen crystals, which also ensure the quality and effectiveness of the complexation process (Silva et al., 2017).

In our results, the group treated with nifedipine (NG), a well-known Ca^{+2} channel blocking antihypertensive drug (Burnier et al., 2020; Cohan and Checcio, 1985) characterized by a rapid onset of action (Ishii et al., 1980), reduced MAP only from 0.5 to 8 h, and increased HR from 0.5 to 3 h after administration. In the group treated with farnesol (FAR), MAP only decreased significantly at 48 h, compared to vehicle group (VG), with no significant change in HR at any time. Meanwhile, the complexed form (FAR/βCD) was able to reduce MAP at 30 and 48 h, compared to the VG. Further, FAR/βCD reduced HR from 0.5 to 2 h, compared to VG, and from 1 to 3 h compared to FAR. Vehicle treated animals did not present any changes in MAP or HR.

Hence, by making a FAR/βCD inclusion complex it was possible to improve the antihypertensive effects of this sesquiterpene in hypertensive rats in a long-lasting manner, which can be used as complement to existing drugs, such as nifedipine. It is worth mentioning that the complexed form contained only 32.7 mg of FAR, with the most of the complex comprising βCD (Silva et al., 2017), thus it is safe to say that complexation improved the cardiovascular properties of FAR.

Accordingly, studies have shown significant improvements in the pharmacological properties of terpenes when complexed with cyclodextrins, including in relation to the duration of the effects (Camargo et al., 2018; Moreira et al., 2016; Quintans-Júnior et al., 2013; Silva et al., 2017). Higher doses of FAR than the 200 mg/kg used in this study have been used previously by Luft et al. (1999), which indicates its safety. Therefore, it is possible that a higher dose in the complex with βCD may be even more effective in an animal model of hypertension, and should be further investigated.

Finally, through the use of docking technique we aimed to predict the interaction of FAR and the receptors that may be relevant for its mechanism of action. According to our results, two receptors seems to be involved in FAR effects, the muscarinic and nicotinic receptors. It has been suggested that physiological responses in the heart are predominantly mediated by the M_2 subtype muscarinic receptor (Harvey and Belevych, 2003; Hoover et al., 1994), and by the M_3 subtype in blood vessels (Bény et al., 2008; Harvey, 2012; Khurana et al., 2004). Similarly, autonomic control of the heart (Poth et al., 1997; Rust et al., 1994), and some arteries (Brüggmann et al., 2002) is intermediated mainly by the α-3 subtype nicotinic receptor (De Biasi, 2002), and, therefore, were chosen as models.

Molecular docking is a computer-based technique that analyzes chemical interactions between two or more structures, indicating the most favorable conformation, and predicting their behavior (Attique et al., 2019; Kitchen et al., 2004). It is a widely known approach used to help and accelerate drug discovery, since testing different molecules in respect of the binding site of a biological target can increase the drug's effectiveness by improving the physiological response (Gohlke and Klebe, 2002; Kitchen et al., 2004). This technique is useful when investigating the pharmacological properties of novel natural compounds, such as terpenes, and has been extensively used (Attique et al., 2019; Camargo et al., 2020; Moreira et al., 2016; Sampurna et al., 2019; Silva et al., 2017; Zheng et al., 2013).

In our results, we demonstrated, for the first time, that FAR binds in the same pockets as known antagonists do, and interacts with some critical residues at these sites, suggesting that FAR may act as an antagonist for the targets of interest here. Therefore, it is possible to propose that the observed effects (i.e. bradycardia and hypotension) in the studied model may be the result of the action of FAR on the M_3, M_2 and nicotinic receptors.

As known, the minimization of energy spent in protein-ligand bonds is a key factor for better interactions in docking analyses (Kitchen et al., 2004; Pinzi and Rastelli, 2019). An interaction between a ligand and a receptor is mediated by both structural affinity and energy through covalent or noncovalent bonds (Böhm and Klebe, 1996; Gohlke and Klebe, 2002). Among the noncovalent bonds, hydrogen bonds are suggested to play determinant role in protein-ligand complexes (Böhm and Klebe, 1996; Zhou et al., 2012), including in respect of greater structural stability (Scotti et al., 2018; Zhou et al., 2012). Thus, our data showed that FAR had a higher affinity for the muscarinic acetylcholine receptor M_3 (−7.6 kcal/mol) than the muscarinic acetylcholine receptor M_2 (−7.3 kcal/mol) and nicotinic (−7.2 kcal/mol) receptors. Considering that this difference is relatively small, more detailed studies are needed to investigate these interactions.

Interestingly, the M_3 receptor-FAR complex occurred through hydrogen bonds with ASN152 and ALA238, and a hydrophobic interaction with TYR529, with the latter also interacting with the known ligand 9 EC. The interaction between FAR and the M_2 receptor was via a hydrogen bond to TYR104, the same residue shown for its antagonist QNB. In addition, bonding site similarities may help in the identification of potential novel molecules, such as natural products (Ma et al., 2011). The only previous study using a docking approach with FAR was undertaken by Silva et al. (2017), who found that FAR formed a stable inclusion complex with βCD with a low binding energy through hydrogen bonds, which is in accordance with our results.

Finally, it is known that the interaction of the nicotinic receptor with its agonist (NCT) occurs at α-β interfaces, more specifically through interactions with the aromatic residues TYR93, TRP149, TYR190 and TYR197 (Gharpure et al., 2019). Our data showed that FAR binds at the orthosteric site of the nicotinic receptor with a binding energy slightly higher than the nicotine (NCT). These results indicate that farnesol has an affinity for the nicotinic receptor comparable to its agonist, also through a hydrogen bond with LYS132 residue.

5. Conclusions

Taken together, our results indicate that FAR induces hypotension and bradycardia in normotensive rats, probably through interaction with cholinergic receptors - data that was further supported by *in silico* docking experiments. Furthermore, oral treatment with the FAR/βCD complex potentialized the antihypertensive effect of FAR compared to its alone form. Thus, we hope our results will point the direction for further pharmacological studies with farnesol regarding antihypertensive therapy, biochemical analysis and its possible clinical use for cardiovascular diseases.

Author agreement

I herewith state in my function as corresponding author that all of my co-authors –Eric Aian P. Silva, Jéssica S. Carvalho, Danillo M. Santos, Ana Maria S. Oliveira, Adriano A. de Souza Araújo, Mairim R. Serafini, Lucas A. B. Oliveira Santos, Marcus V. de A. Batista, Márcio R.Viana Santos, Jullyana de S. Siqueira Quintans, Lucindo J. Quintans-Júnior, André S. Barreto – support the resubmission of our manuscript in its revised form for publication in European Journal of Pharmacology.

CRediT authorship contribution statement

Eric Aian P. Silva: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Jéssica S. Carvalho:** Methodology, Formal analysis, Investigation. **Danillo M. dos Santos:** Resources, Writing - Review. **Ana Maria S. Oliveira:** Methodology, Resources. **Adriano A. de Souza Araújo:** Resources, Supervision. **Mairim R. Serafini:** Resources, Supervision, Methodology, Resources, Supervision, Project administration, Funding acquisition. **Lucas A.B. Oliveira Santos:** Methodology, Formal analysis. **Marcus V. de A. Batista:** Methodology, Formal analysis, Investigation. **Jullyana de S. Siqueira Quintans:** Resources, Supervision, Funding acquisition. **Lucindo J. Quintans-Júnior:** Resources, Supervision, Project administration. **André S. Barreto:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors report no conflicts of interest.

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