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RESEARCH ARTICLE

Synthesis and Biological Activity of a Bis-steroid-methanocyclobuta-naphthalene-dione Derivative against Ischemia/Reperfusion Injury *via* Calcium Channel Activation

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Abstract: ***Background*:** There is some experimental data on the effect exerted by some steroid derivatives against ischemia/reperfusion injury; however, the molecular mechanism is very confusing, perhaps this phenomenon could be due to the protocols used and/or differences in the chemical structure of each one of the steroid derivatives.

***Objectives*:** The aim of this study was to synthesize a new bis-steroid-methanocyclobuta-naphthalene-dione derivative using some tools chemical.

***Methodology*:** The biological activity exerted by the bis-steroid-methanocyclobuta-naphthalene-dione derivative against ischemia/reperfusion injury was evaluated in an isolated heart model using noradrenaline, milrinone, dobutamine, levosimendan, and Bay-K-8644 as controls. In addition, other alternative experiments were carried out to evaluate the biological activity induced by the bis-steroid-methanocyclobuta-naphthalene-dione derivative against left ventricular pressure in the absence or presence of nifedipine.

***Results*:** The results showed that 1) the bis-steroid-methanocyclobuta-naphthalene-dione derivative significantly decreases the ischemia-reperfusion injury translated as a decrease in the the infarct area in a similar manner to levosimendan drug; 2) both bis-steroid-methanocyclobuta-naphthalene-dione and Bay-K-8644 increase the left ventricular pressure and 3) the biological activity exerted by bis-steroid-methanocyclobuta-naphthalene-dione derivative against left ventricular pressure is inhibited by nifedipine.

***Conclusion*:** In conclusion, the bis-steroid-methanocyclobuta-naphthalene-dione derivative decreases the area of infarction and increases left ventricle pressure *via* calcium channels activation; this phenomenon could constitute a new therapy for ischemia/reperfusion injury.

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1. INTRODUCTION

Myocardial infarction is a major cause of death worldwide; this clinical pathology can be conditioned by the cardiac myocyte cell death caused by prolonged myocardial ischemia [1, 2]. It is important to mention that acute myocardial infarction can produce alterations in the topography of both the infarcted and non-infarcted regions of the ventricles [3]. There are some studies [4] which indicate that the most effective method of limiting necrosis is through restoration of the blood flow; however, the effects of reperfusion may be associated with tissue injury resulting in heart failure. Here, it is important to mention that several drugs such as digoxin (ATP-ase inhibitor] [5], levosimendan (Ca^{++}-sensitizing) [6], dobutamine (β_1-agonist) [7], milrinone (Phosphodiesterase-III inhibitor) [8], captopril [angiotensin-converting enzyme inhibitor] [9], and spironolactone aldosterone antagonist [10] have been used for treatment of heart failure. Nevertheless, some of these drugs could produce diverse secondary effects such as ischemia, arrhythmia, hyperkalemia, hypopotassemia, and others [11]. In the search for new therapeutic alternatives, several compounds have been prepared for the treatment of heart failure and ischemia/reperfusion injury; for example, a study showed that benzofuranone-derivative (MK-7145) decreases blood pressure *via* guanylate cyclase activation in heart failure patients [12]. Another report showed that a pyridazinone analog can inhibit the activity of phosphodiesterase III using a model animal [13] In addition, a study showed that a benzoylguanidine derivative can exert protection against ischemia/reperfusion injury as an inhibitor of Na+/H+ exchanger [14]. Other studies suggest that magnolol can exert an anti-apoptotic effect and protects against ischemia/reperfusion injury through the upregulation of the anti-apoptotic Bcl-XL gene [15]. Additionally, there are several studies that suggest that steroids could have an influence on ischemia/reperfusion injury; for example, a report showed that 17β-estradiol, but not 17α-estradiol, reduces myocardial necrosis in rabbits after ischemia and reperfusion [16]. Other studies indicate that treatment with 17β-estradiol can decrease the dysfunction of coronary endothelial after an ischemia/reperfusion injury using an animal model [17]. In addition, progesterone has been used in conjunction with estrogen in an ischemia/reperfusion model, resulting in a significantly less severe myocardial injury; the protective effect could be mediated by attenuation of inflammation and its possible interaction with endogenous estrogen [18]. Other studies showed that a progestin (norethindrone acetate) can also reduce ischemia-reperfusion injury in ovariectomized monkeys, received estrogen therapy previously [19]. Nevertheless, there are reports which indicate that the administration of another progesterone derivative (medroxyprogesterone acetate) can inhibit the effects of estradiol which lead to protection of the myocardium from reperfusion injury and that this involves both neutrophil-dependent and neutrophil-independent mechanisms [20]. All these data showed that some steroid derivatives have been used for the treatment of heart failure and ischemia/reperfusion injury; however, the molecular mechanism involved in their biological activity is very confusing. These phenomena could be due to: i) the different protocols or biological models used and ii) the differences in the chemical structure of each steroid derivative. Therefore, in this study, a bis-steroid-methanocyclobuta-naphthalene-dione derivative was prepared in order to evaluate its biological activity against ischemia/reperfusion injury using an isolated rat heart.

2. MATERIALS AND METHODS

2.1. General Methods

The 2-nitro estradiol derivative was prepared using a previously method reported [21]. In addition, compound **1** (1,4,4a,8a-tetrahydro-1,4-methanonaphthalene-5,8-dione) and all the reagents used in this study were purchased from Sigma-Aldrich Sigma-Aldrich Co., Ltd. The melting point for compounds was evaluated on an Electrothermal (900 model). Infrared spectra (IR) were determined using KBr pellets on a Perkin Elmer Lambda 40 spectrometer.^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were recorded on a Varian VXR300/5 FT NMR spectrometer at 300 and 75.4 MHz (megahertz) in $CDCl_3$ (deuterated chloroform) using TMS (tetramethylsilane) as an internal standard. EIMS (electron impact mass spectroscopy) spectra were determined using a Finnigan Trace Gas Chromatography Polaris Q-Spectrometer. Elementary analysis data were de-

termined from a Perkin Elmer Ser. II CHNS/02400 elemental analyzer.

2.2. Chemical Synthesis

2.2.1. Preparation of (2)

2.2.1.1. (3R,3aS,7aR,8S,8aS)-1,5-bis(hydroxy(phenyl)methyl)-2a,3,3a,4a,6a,7a,8,8a-octahydro-3,8-methanodicyclobuta[b,g]naphthalene-4,7-dione (2)

In a round bottom flask (10 ml), compound **1** (1,4,4a,8a-tetrahydro-1,4-methano-naphthalene-5,8-dione (200 mg, 1.15 mmol), 1-Phenyl-2-propynol (100 µl 0.82 mmol), cupric chloride anhydrous (105 mg 0.78 mmol) and 5 ml of methanol were stirred to reflux for 12 h. The solution was obtained at reduced pressure and purified through a crystallization using the methanol:water(3:1) system.

*2.2.1.2. Synthesis of (3aS,7aR)-1-((((13R)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16, 17-decahydro-6H-cyclopenta[a]phenanthren-2-yl) oxy)(phenyl)methyl)-5-((((13S)-3,17-dihydroxy-13-methyl-7,8,9,11,12,13,14,15,16,17-decahydro-6H-cyclopenta[a]phenan- thren-2-yl)oxy)(phenyl) methyl)-2a,3a,4,7,7a,8a-hexahydro-4,7-methano-cyclobuta[b] naphthalene-3,8-dione (3)**

*2.2.1.2.1. *(bis-steroid-methanocyclobuta-naphthalene-dione Derivative)*

In a round bottom flask (10 ml), compound **2** (200 mg, 0.46 mmol), 2-nitroestrone (290 mg, 0.91 mmol), potassium carbonate (70 mg 0.50 mmol) and 5 ml of dimethyl sulfoxide were stirred to room temperature for 12 h. The solution was obtained at reduced pressure and purified through a crystallization using the methanol:benzene (4:1) system.

2.2.2. Physicochemical Properties of Both Compounds 2 and 3

Some theoretical electronic properties, such as HOMO (highest occupied molecular orbital), LUMO (lowest unoccupied molecular orbital) energy, orbital coefficients distribution, molecular dipole moment and HBD (hydrogen bond donor groups) and HBA (hydrogen bond acceptor groups) and TPSA (topological polar surface area) were evaluated using SPARTAN'06. In addition, other chemical factors such as logP (logKowin), molecular refractivity (MR), and volume reactivity (VR) were determined using both chem sketch and Avogadro programs [22-24].

2.2.3. Pharmacophore Evaluation

The 3D pharmacophore model for compounds **2** and **3** was designed using the Ligand Scout 4.08 program [25, 26].

3. EVALUATION OF BIOLOGICAL ACTIVITY

3.1. Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the University Autonomous of Campeche (No. PI-420/12) and were in accordance with the guide for the care and use of laboratory animals [11]. Male Wistar rats, weighing 200-250 g were obtained from the University Autonomous of Campeche.

3.2. Reagents

All the reagents were obtained from Sigma-Aldrich Chemical Co Ltd. In addition, the drugs used in this study were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution (≤ 0.01%, v/v) [27, 28].

3.3. Langendorff Method

Briefly, the male rats (200-250 g) were anesthetized by injecting them with pentobarbital at a dose rate of 50 mg/Kg body weight. Then the chest was opened, and a loose ligature passed through the ascending aorta. The heart was then rapidly removed and immersed in an ice-cold physiologic saline solution. The heart was trimmed of noncardiac tissue and underwent retrograde perfusion *via* a non-circulating perfusion system at a constant flow rate. It is important to mention that perfusion medium was the Krebs-Henseleit solution (pH 7.4, 37°C) composed of (mM); 117.8 NaCl; 6 KCl; 1.75 $CaCl_2$; 1.2 NaH_2PO_4; 1.2 $MgSO_4$; 24.2 $NaHCO_3$; 5 glucose, and 5 sodium pyruvate. The solution was actively bubbled with a mixture of O_2/CO_2 (95:5). The coronary flow was adjusted with a variable-speed peristaltic pump. An initial perfusion rate of 15 ml/min for 5 min was followed by a 25 min equilibration period at a

Table 1. Values of both ^{1}H NMR and ^{13}C NMR (500 MHz, Chloroform-*d*) spectra of compound 2.

δ_H: 1.54-3.45 (m, 10H), 5.15 (broad, 2H), 5.29 (d, 1H, J = 0.80 Hz), 5.60 (m, 1H), 6.16 (d, 1H, J = 1.82 Hz), 7.50-7.69 (m, 10 H) ppm.
δ_C: 32.82, 39.23, 41.94, 43.72, 44.74, 50.82, 52.68, 52.71, 57.23, 75.56, 78.64, 127.31, 127.92, 128.43, 128.45, 128.71, 134.22, 134.88, 140.18, 140.22, 149.36, 150.02, 193.01, 207.62 ppm.

perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

3.4. Evaluation of Left Ventricle Pressure

To evaluate the biological activity of drugs involved in this study against left ventricle pressure, a latex balloon filled with saline solution (0.01 mm, diameter) was inserted into the left ventricle through the left atrium. It is important to mention that the latex balloon was bound to a pressure transducer which was connected to a computerized data capture system (MP-100). After this, the inotropic effect produced by compounds involved in this study was evaluated by determining left ventricular developed pressure (LV/dP) [27].

3.5. Experimental Design

3.5.1. First Stage

3.5.1.1. Effect Exerted by 2-nitroestrone and the Compounds 1-3 using an Ischemia/Reperfusion Model

After 15 minutes of equilibration time, the hearts were subjected to ischemia for 40 minutes by turning off the perfusion system [27]. Then, the system was restarted, and the hearts were reperfused by 40 minutes with Krebs-Henseleit solution. The hearts were randomly divided into 5 major treatment groups that involved the conditions control (without treatment), 2-nitroestrone and compounds 1-3 (Table **1**) with n = 9 as follows:

Group I. Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).

Group II. Hearts were subjected to ischemia/reperfusion and treated with 2-nitroestrone (0.001 nM).

Group III. Hearts were subjected to ischemia/reperfusion and treated with compound 1 (0.01 nM).

Group IV. Hearts were subjected to ischemia/reperfusion and treated with compound 2 (0.01 nM).

Group V. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (0.01 nM).

It is important to mention that the areas of the normal left ventricle no-risk region, area at risk, and infarct region were determined using a previous method reported [28]. The total area at risk was expressed as the percentage of the left ventricle. It is noteworthy that at the end of each experiment, the perfusion pump was stopped, and 0.5 ml of fluorescein solution (0.10%) was injected slowly through a sidearm port connected to the aortic cannula. The dye was passed through the heart for 10 sec to ensure its uniform tissue distribution. The presence of fluorescein was used to demarcate the tissue that was not subjected to regional ischemia, as opposed to the risk region. Then, the heart was removed from the perfusion apparatus and cut into two transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. The areas of the normal left ventricle region not at risk, area at risk, and infarct region were determined using methods previously reported [28]. The total area at risk was expressed as the percentage of the left ventricle.

3.5.2. Second Stage

3.5.2.1. Biological Activity of the Compound 3 on Infarct Area

The hearts perfused with the Krebs-Henseleith solution were subjected to ischemia for 40 minutes by turning off the perfusion system; then, the system was restarted. After that, the hearts were randomly divided into 6 major treatment groups that involved the conditions-control (without treatment; Group VI), and the compounds to doses of 0.001 to 100 nM (n = 9) as follows:

Group VI. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (0.001 nM).

Group VII. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (0.01 nM).

Group VIII. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (0.1 nM).

Group IX. Hearts were subjected to ischemia/ reperfusion and treated with compound 3 (1 nM).

Group X. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (10 nM).

Group XI. Hearts were subjected to ischemia/reperfusion and treated with compound 3 (100 nM).

Then, the areas of the normal left ventricle no-risk region, area at risk, and infarct region were determined as mentioned above.

3.5.3. Third Stage

3.5.3.1. Biological Activity Induced by the Noradrenaline, Milrinone, Dobutamine, Levosimendan and Compound 3 against Infarct Area

The hearts were randomly divided into 6 major treatment groups with n = 9, as follows:

Group XII. Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).

Group XIII. Hearts were subjected to ischemia/reperfusion and treated with noradrenaline (0.001 nM).

Group XIV. Hearts were subjected to ischemia/reperfusion and treated with milrinone (0.001 nM).

Group XV. Hearts were subjected to ischemia/reperfusion and treated with dobutamine (0.001 nM).

Group XVI. Hearts were subjected to ischemia/reperfusion and treated with levosimendan (0.001 nM).

Group XVII. Hearts were subjected to ischemia/reperfusion and treated with Compound 3 (0.001 nM).

Following this, the areas of the normal left ventricle no-risk region, area at risk, and infarct region were determined. Here, it is important to mention that the doses administered of the drugs noradrenaline, milrinone, dobutamine, and levosimendan were based on some previously reported methods [29]. Therefore, in this study, the biological activity exerted by these drugs on ischemia/reperfusion injury was used as a control to compare it with the effect exerted by compound **3** (bis-steroid-tetracyclodione derivative) against ischemia/reperfusion injury.

3.5.4. Fourth Stage

3.5.4.1. Effects Exerted by Compound 3 or the Bay-k-8644 Drug on Left Ventricular Pressure

Intracoronary boluses (50 µL) of the compound **3** or Bay-k-8644 drug at a dose of 0.001 to 100 nM were administered and the corresponding effect on the left ventricular pressure was evaluated.

3.5.5. Fifth Stage

3.5.5.1. Effects Induced by Compound 3 on Left Ventricular Pressure in the Absence or Presence of Nifedipine

Intracoronary boluses (50 µL) of compound 3 [0.001 to 100 nM] were administered and the corresponding effect on the left ventricular pressure was evaluated. The dose-response curve (control) was repeated in the presence of nifedipine* at a concentration of 1 nM (duration of the preincubation with nifedipine was for a period of 10 min). * The dose of nifedipine has been reported in a previously reported study [11].

3.6. Statistical Analysis

The obtained values are expressed as average ± SE, using each heart as its own control. The data obtained were put under an analysis of variance (ANOVA) with the Bonferroni correction factor using the SPSS 12.0 software [30]. The differences were considered significant when p was equal to or smaller than 0.05.

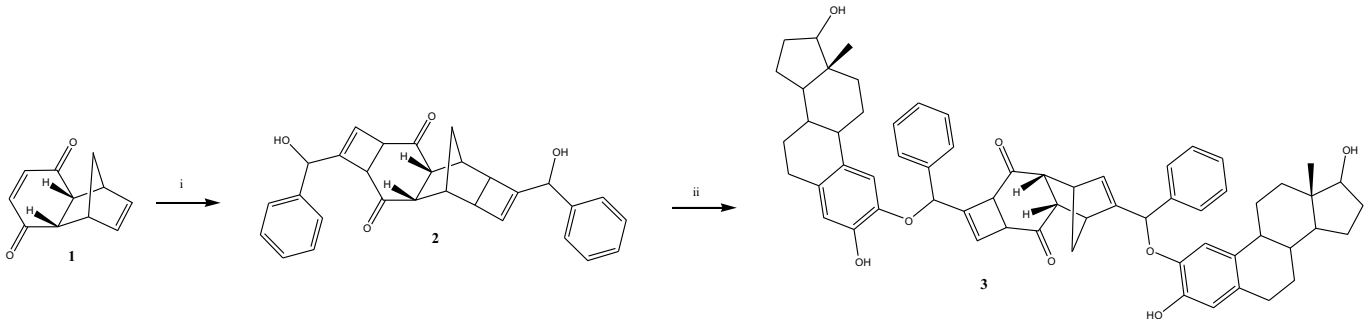

Fig. (1). Preparation of a bis-steroid-methanocyclobuta-naphthalene-dione derivative (**3**). Reaction of 1,4,4a,8a-tetrahydro-1,4-methano-naphthalene-5,8-dione (**1**) with 1-Phenyl-2-propyn-ol (i) to form the 5,12-bis[hydroxyl (phenyl)dione analog (**2**). Then, **2** reacted with 2-nitroestrone (ii) for synthesis of **3**.

3.7. Docking Evaluation

Interaction of both compounds **2** and **3** (bis-steroid-methanocyclobuta-naphthalene-dione) with calcium channel was determined using 4DEX protein from the protein data bank (Young) and DockingServer software [31]. The distance in the interaction between compound **3** with the amino acid residues of 4DEX protein surface was calculated using SeeSAR 8.0 program [21].

4. RESULTS AND DISCUSSION

There are several studies which indicate that some steroid derivatives exert effects against ischemia/reperfusion injury [32, 33]; however, the biological activity is very confusing, this phenomenon could be due to different molecular mechanisms involved, through activation of an estrogen-receptor [34], β_1-adrenergic receptor [35], potassium channels [36] or connexin43 protein [37] and others. Therefore, the aim of this study was to synthesize a new bis-steroid-methanocyclobuta-naphthalene-dione derivative (compound **3**) to evaluate its biological activity on ischemia/reperfusion injury. The first stage was achieved by the preparation of a 1,5-bis(hydroxy(phenyl)methyl)-methanodicyclobuta[b,g]naph- thalene-4,7-dione derivative (compound **2**). It is important to mention that several dione-derivatives have been prepared using some reagents such as glycol [38], nickel [39], Jones reagent [40] and others; however, some of these reagents are expensive and require special conditions. Therefore, in this study, compound **2** (Fig. **1**) was prepared from 1,4,4a,8a-tetrahydro-1,4-methano-naphthalene-5,8-dione and 1-phenyl-2-propyn-ol using cupric chloride as a catalyst with a yielding of 67 % and a melting point of 122-124°C The results of the spectroscopic analyses show signals for IR (V_{max}, cm^{-1}) 3400, 3360, 3322 and 1712. In addition, the ^1H NMR spectrum showed a characteristic signal at 5.15 ppm for hydroxyl group involved in their chemical structure (Fig. **2**, Table **1**). Additionally, the ^{13}C NMR spectrum showed 207.62 ppm for both ketone groups (Table **1**). Finally, the results of mass spectroscopy (MS) (70 eV) showed an ion mass (*m*/*z*) of 438.18 and the elementary analysis data for the estradiol derivative ($C_{29}H_{26}O_4$) was found to be (C, 79.43; H, 5.98; O, 14.59) and (C, 79.40; H, 5.92).

On the other hand, the bis-steroid-methanocyclobuta-naphthalene-dione derivative (compound **3**) was synthesized *via* etherification of compound **2**. It is noteworthy that some reports have shown the preparation of several ether derivatives *via* displacement of nitro group using methoxide as a dipolar aprotic solvent [41]. In this study, compound **3** was synthesized by the reaction of **2** with 2-nitroestrone in the presence of dimethyl sulfoxide at mild conditions (Fig. **1**) with a yield of 44% and a melting point of 138-140 °C. The ^1H NMR spectrum showed a characteristic signal at 6.28 ppm for both hydroxyl groups bound to steroid nucleus (Fig. **3**, Table **2**). In addition, the ^{13}C NMR spectrum showed 207.64 ppm for both ketone groups (Table **2**). Finally, the results of mass spectroscopy (MS) (70 eV) showed an ion mass (*m*/*z*) of 953.20 and the elementary analysis data for the estradiol derivative ($C_{63}H_{68}O_8$) was calculated to be (C, 79.38; H, 7.19; O, 13.43) and (C, 79.34; H, 7.15).

4.1. Electronic Parameters Evaluation

Both the molecular orbitals (HOMO and LUMO) of compounds **2** and **3** were theoretically

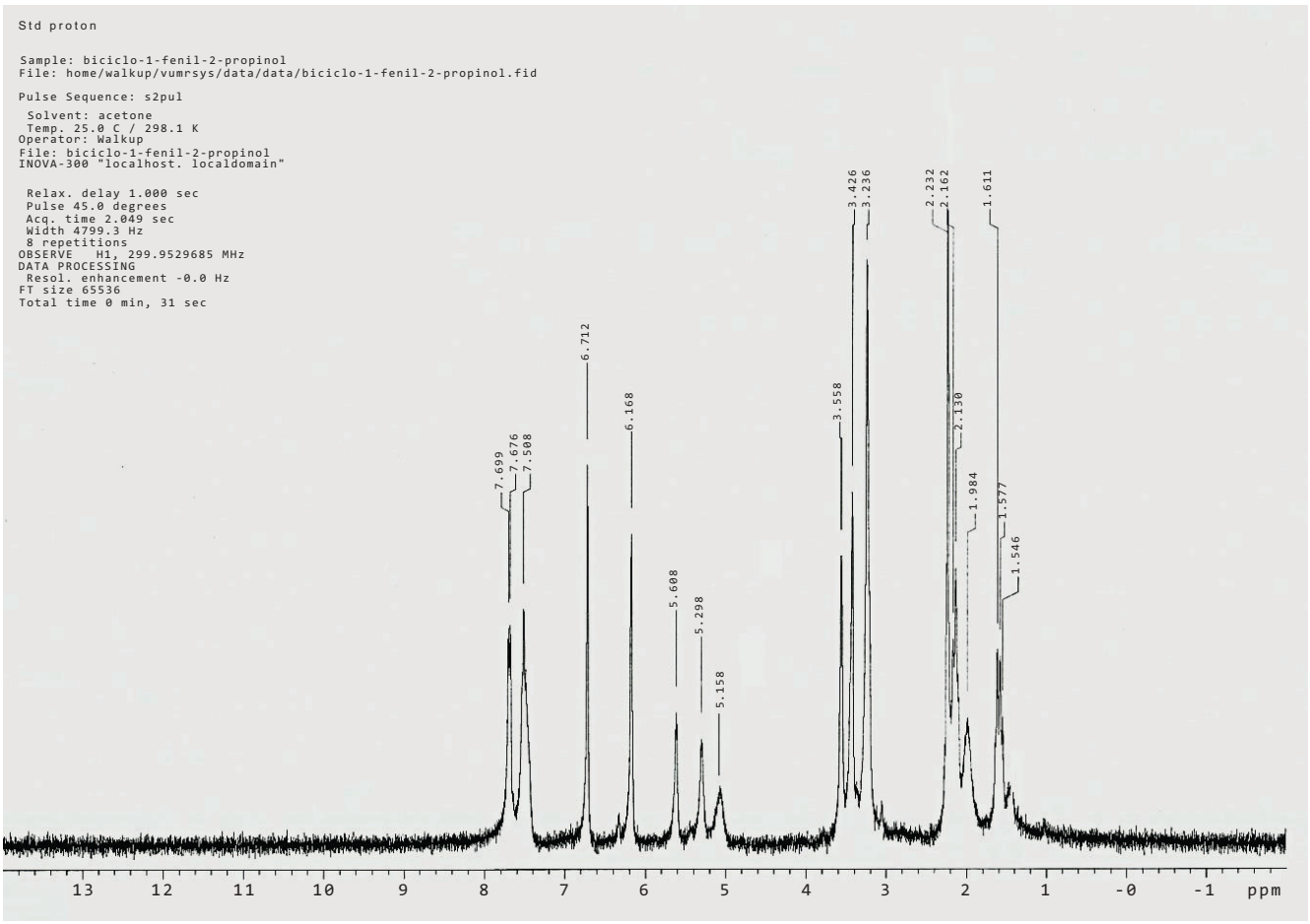

Fig. (2). The scheme shown ^{1}HNMR spectrum from compound **2**. Analyzed with a Varian VXR300/5 FT NMR apparatus at 300 MHz in $CDCl_3$. Axis abscissa (ppm). ppm = parts per million.

Table 2. Values of both ^{1}H NMR and ^{13}C NMR (500 MHz, Chloroform-*d*) spectra of compound 3.

δ_H: 0.80 (s, 6H), 0.97-1.56 (m, 16H), 1.69 (m, 1H), 1.76 (m, 3H), 1.80-2.10 (m, 6H), 2.28 (m, 1H), 2.45-2.96 (m, 7H), 3.37-3.41 (m, 2H), 3.44 (m, 2H), 3.82-3.92 (m, 2H), 5.50-6.00 (m, 2H), 6.28 (broad, 4H), 6.44 (m, 1H), 6.45 (m, 1H), 6.50 (m, 1H), 6.64 (m, 2H), 6.82 (m, 1H), 7.65-7.68 (m, 10H) ppm.
δ_C: 12.78, 23.87, 25.84, 27.77, 29.67, 31.16, 35.66, 38.91, 41.47, 42.83, 42.94, 44.02, 44.15, 47.23, 49.82, 53.38, 55.68, 56.02, 76.49, 79.11, 81.24, 108.41, 108.73, 114.46, 127.88, 128.44, 128.55, 128.58, 128.83, 129.11, 131.34, 134.18, 135.63, 140.96, 143.33, 143.37, 144.01, 145.75, 146.03, 149.78, 153.83, 190.34, 205.64 ppm.

evaluated with SPARTAN'06 software, using Hartree-Fock method at 321-G level [42]. The results (Table **3**, Fig. **4**) showed that LUMO values were lower for compound **3** compared with **2**. In addition, HBD and HBA values were different for two compounds, and the data indicate that **3** could have a different electron donation ability compared to **2**. This phenomenon could result in differences in the activity exerted by compounds **2** and **3** in some biological systems as it happens with other types of drugs [24]; however, it is important to mention that other physicochemical parameters could be involved in their biological activity.

4.2. Physicochemical Parameters

To evaluate the above mentioned hypothesis, in this study, some reports [43] were analyzed which indicate that several physicochemical parameters are used as predictors of lipophilicity degree of some compounds such as logP and π. Therefore, a

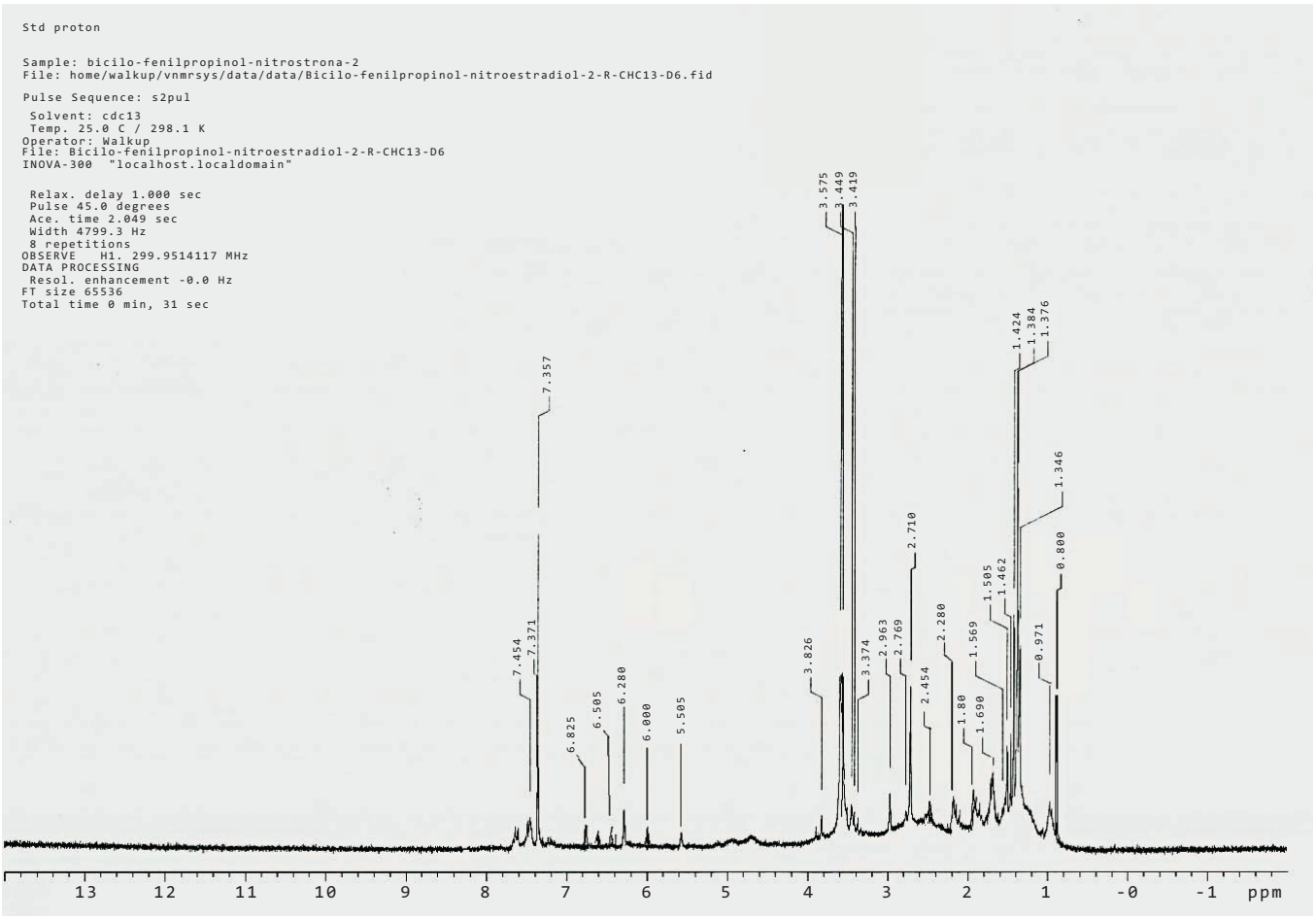

Fig. (3). The scheme shown ^{1}HNMR spectrum from compound **3**. Analyzed with a Varian VXR300/5 FT NMR apparatus at 300 MHz in $CDCl_3$. Axis abscissa (ppm). ppm = parts per million.

Table 3. Physicochemical parameters involved chemical structure of compounds 2 and 3. The values were calculated using both ACDLabs and Spartan software.

Parameters	2	3
Molar Refractivity (cm^3)	122.25	270.91
Molar Volume (cm^3)	318.80	730.50
PSA ($Å^2$)	67.58	104.75
Dipole Moment (debyete)	2.77	0.90
Polarizability	75.28	118.50
E. HOMO (Ev)	-8.62	-7.89
E. LUMO (Ev)	3.35	3.03
HBD	4	8
HBA	2	4

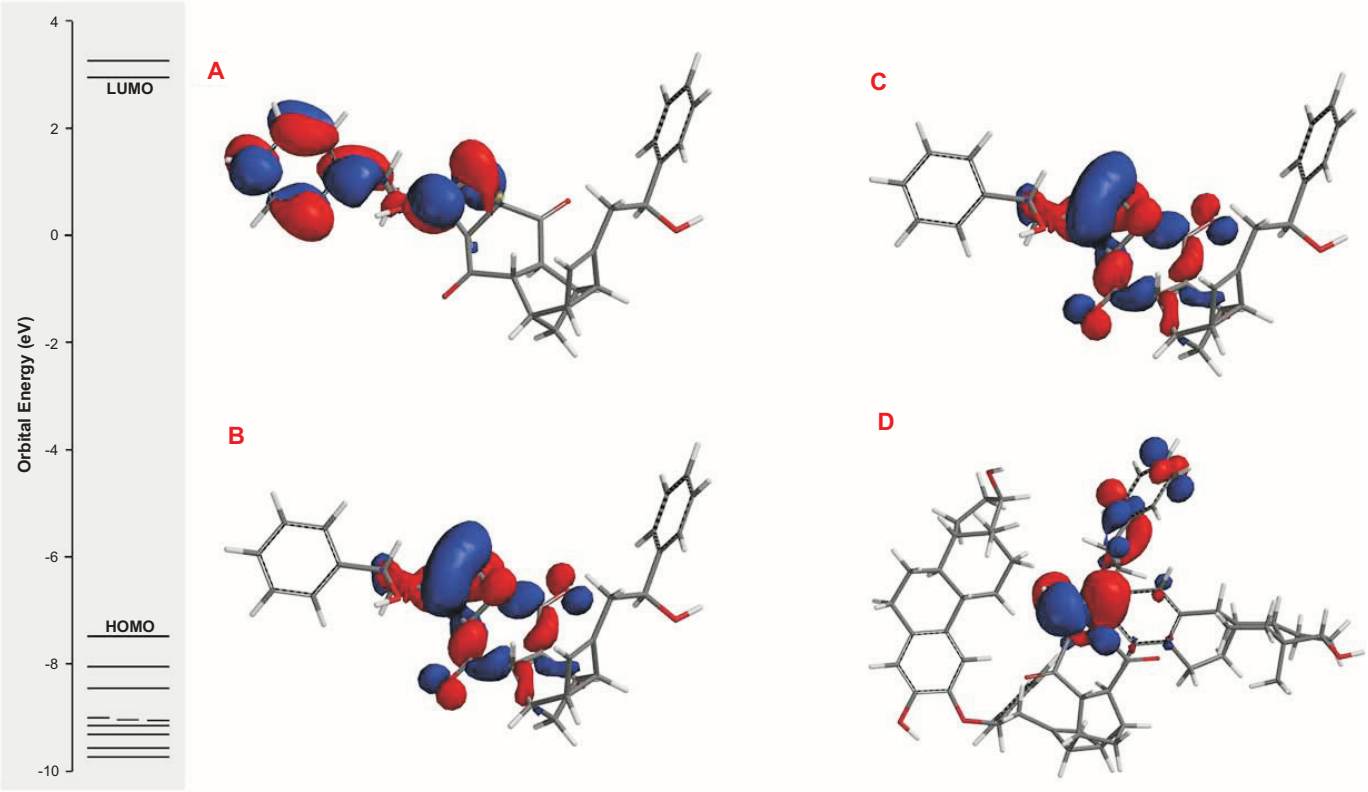

Fig. (4). In the scheme are showed both HOMO and LUMO for compounds **2** (left) and **3** (right). Visualized with SPARTAN´06 program. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

theoretical analysis of lipophilicity degree of both compounds **2** and **3** was evaluated using the parameters logP and π (Table **4**). It is noteworthy that logP (logKow) determines the lipophilicity degree; therefore, logKow represents the lipophilic effects of all the molecules. The result shows that logKow of compound 2 is lower compared with **3**; these data indicate that compound **3** could have a higher lipophilicity degree. However, this phenomenon may be conditioned by other physicochemical parameters involved in the chemical structure of both compounds **2** and **3** such as molar volume (MV) and molar refractory (MR) that are two physicochemical parameters which could produce several changes in some biological models. It is important to mention that these physicochemical factors are tools that have been correlated with several biological properties that depend on the characteristics of substituents attached to a constant reaction center [44]. To evaluate both MV and MR parameters in this study, a previously method reported was used [45]. The theoretical results (Table **3**) showed that MV and MR were higher for compound 3 compared with **2**. These data suggest that steric hindrance, conformational preferences, and internal rotation could be other factors that influence the biological activity of compound **3** on some biological models.

4.3. Pharmacophore Modeling

For several years, the pharmacophore method has become one of the major tools in drug discovery; for example, there are some studies that indicate that the pharmacophore is the three-dimensional orientation adopted by the functional groups of a molecule to be able to interact with some proteins [46]. This pharmacophore model can provide a new insight into the design of novel molecules that can enhance or inhibit the function of the target and will be useful in drug discovery strategies. Therefore, in this study, LigandScout software [25, 26] was used to develop a pharmacophore model for compounds **2** and **3**. The results (Table **3** and Fig. **5**) indicated different types of functional groups involved in compounds **2** and **3** that can interact *via* hydrophobic interactions or as hydrogen bond acceptors or as hydrogen bond donors with some biomolecules. This phenomenon could

Table 4. Physicochemical parameters involved in the chemical structure of both compounds 2 and 3.

Compounds	Fragment	Value
2	$-CH_2-$ [aliphatic carbon]	0.4911
	-CH [aliphatic carbon]	3.6140
	=CH- or =C< [olefinc carbon]	1.5344
	-OH [hydroxy, aliphatic attach]	-2.8172
	Aromatic Carbon	3.5280
	-C(=O)- [carbonyl, aliphatic attach]	-3.1172
	Multi-alcohol correction	0.4064
	Fused aliphatic ring unit correction	-1.3684
	Internal aliphatic fused-ring ketone cor.	0.7044
	Equation Constant	0.2290
	π	3.6945
	Log Kow	3.2045
3	$-CH_3$ [aliphatic carbon]	1.0946
	$-CH_2-$ [aliphatic carbon]	6.3843
	-CH [aliphatic carbon]	5.7824
	=CH- or =C< [olefinc carbon]	1.5344
	-OH [hydroxy, aliphatic attach]	-2.8172
	Aromatic Carbon	7.0560
	[hydroxy, aromatic attach]	0.9604
	[oxygen, one aromatic attach]	-0.9328
	-C(=O)- [carbonyl, aliphatic attach]	-3.1172
	-tert Carbon [3 or more carbon attach]	0.5352
	Multi-alcohol correction	0.4064
	Fused aliphatic ring unit correction	-3.0789
	Ring reaction -> alkyloxy ortho to -OH	-0.5120
	Equation Constant	0.2290
	π	8.3993
	Log Kow	11.6038

be translated as changes in the activity of a biological system.

4.4. Biological Activity

To evaluate the hypothesis above mentioned, in this study, the biological activity of bis-steroid-methanocyclobuta-naphthalene-dione derivative (compound **3**) against ischemia/reperfusion injury (translated as infarct size) was evaluated using 2-nitroestrone and compounds **1** or **2** as controls. The results showed that compound **3** significantly decreases infarct size (expressed as the percentage of the area at risk) compared with vehicle-treated hearts, 2-nitroestrone and compounds **1** or **2**

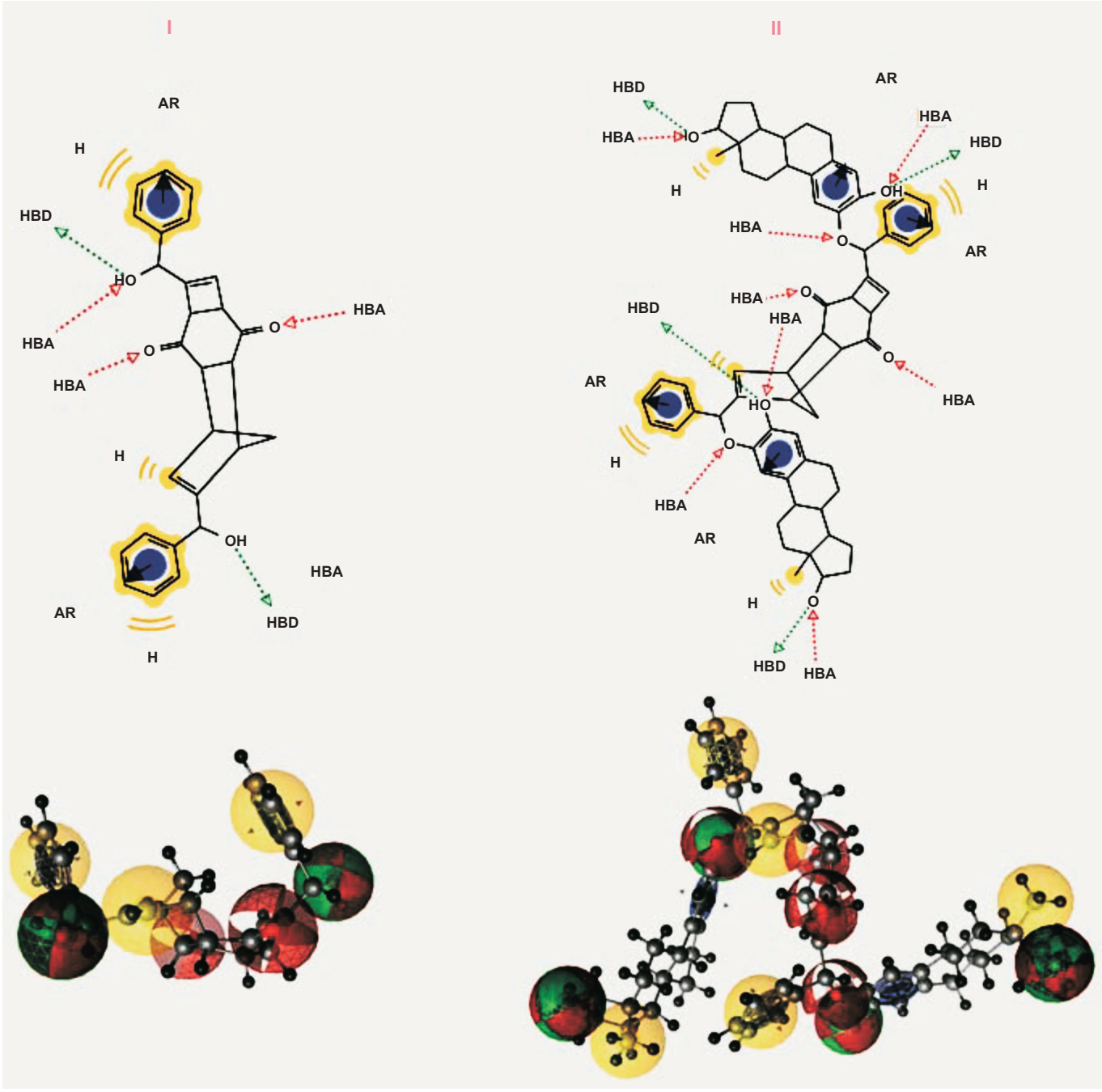

Fig. (5). Scheme represents a pharmacophore model from both compounds **2** (I) and **3** (II) using the LigandScout software. The model involves acceptors (HBA, red) and hydrogen bond donor (HBD, blue). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(Figs. **6** and **7**). This phenomenon indicates that the biological activity exerted by compound **3** against ischemia/reperfusion injury which is translated as the infarct area depends on the functional groups involved in the chemical structure of compound **3**. Analyzing these results other experiments were carried out at different doses in order to evaluate changes in the biological activity exerted by compound **3** on ischemia/reperfusion injury. The results (Fig. **8**) show that compound **3** decreases the myocardial injury in a dose-dependent manner. By analyzing all these data and other reports, it can be said that that some drugs such as noradrenaline, dobutamine, levosimendan, and milrinone may exert effects against ischemia/reperfusion injury [47-49]. In this study, the biological activity of these drugs was evaluated to compare with the effect induced by compound **3**

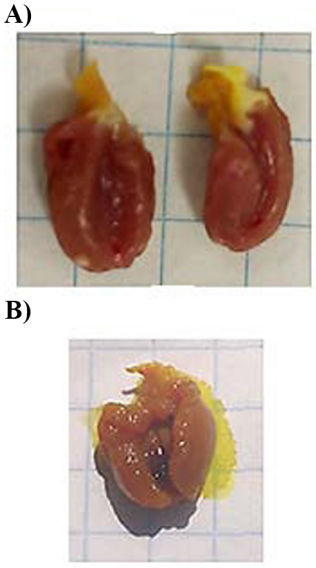

Fig. (6). Comparison of cardioprotective effect of the bis-steroid-methanocyclobuta-naphthalene-dione derivative (compound **3**) (**A**) at a dose of 1 nM with the control (**B**) on the functional recovery of rat hearts subjected to ischemia/reperfusion. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

on ischemia/reperfusion injury. The results (Fig. **9**) showed that compound **3** significantly decreased the infarct area in a similar manner compared to levosimendan [calcium sensitizer] [50]. However, this effect was different from the biological activity exerted by noradrenaline, dobutamine, and milrinone against ischemia/reperfusion injury. This phenomenon suggests that compound **3** could induce changes in calcium levels and left ventricular pressure just as levosimendan [51]. To evaluate this hypothesis, in this study, some experimental alternatives were used, where the activity of the BAY K 8644 drug (calcium channel agonist) [52] on left ventricular pressure was evaluated for comparing it with the biological activity exerted by compound **3**. The results (Fig. **10**) showed that compound **3** increased the left ventricular pressure in a dose-dependent manner and this effect was similar to the effect exerted by Bay K 8644 drug. These data indicated that the effect induced by compound **3** could be *via* the calcium channel activation. Therefore, the biological activity of

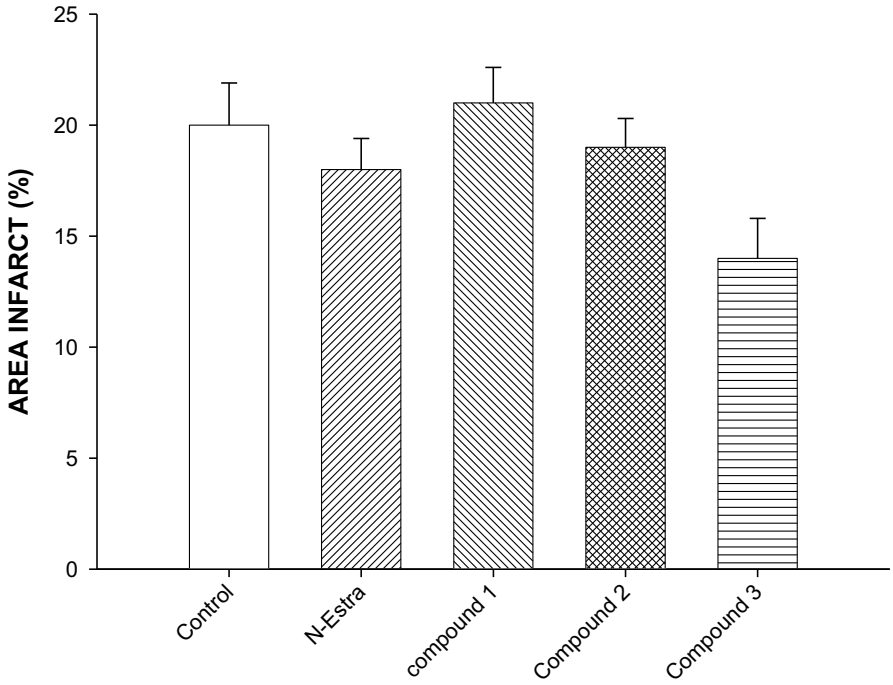

Fig. (7). Effect exerted by the 2-nitroestradiol (N-Estra), the Compounds 1-3 (bis-steroid-methanocyclobuta-naphthalene-dione derivative) on ischemia-reperfusion injury. The hearts perfused with the Krebs-Henseleith solution were subjected to ischemia for 40 minutes by turning off the perfusion system; then, the system was restarted, and the hearts were re-perfused by 40 minutes in absence or presence of either N-Estra or the compounds **1**-**3** at dose of 0.001 nM. The results showed that compound **3** significantly reduced ($p = 0.05$) infarct size expressed as a percentage of the area at risk compared with compounds N-Estra, **1**, **2** and the conditions control. Each bar represents the mean ± S.E. of 9 experiments.

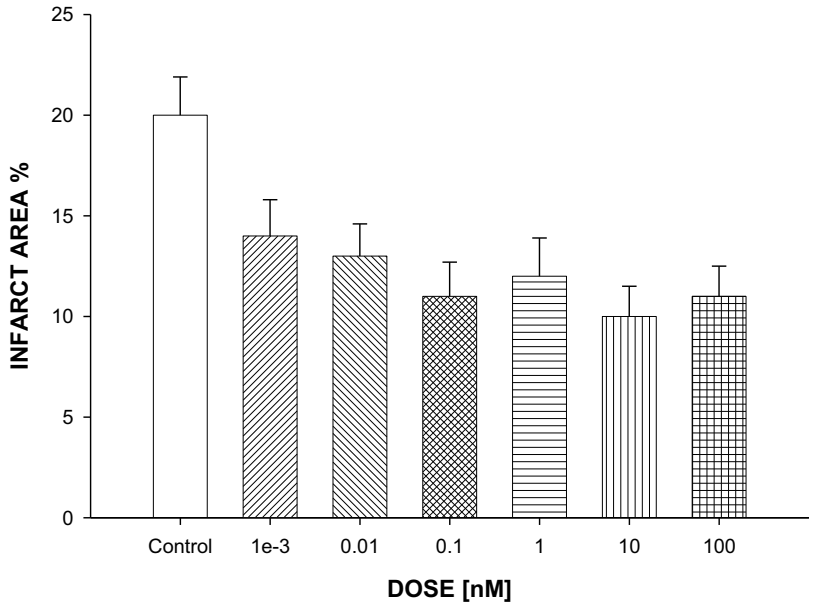

Fig. (8). Effect exerted by the bis-steroid-methanocyclobuta-naphthalene-dione (compound 3) derivative on infarct area. The hearts perfused with the Krebs-Henseleith solution were subjected to ischemia for 40 minutes by turning off the perfusion system; then, the system was restarted, and the hearts were re-perfused by 40 minutes in absence or presence of the compound **3** at dose of 0.001 to 100 nM. The results showed that compound **3** significantly reduced ($p = 0.05$) infarct (size expressed as a percentage of the area at risk) in a dose-dependent manner compared with the conditions control. Each bar represents the mean ± S.E. of 9 experiments.

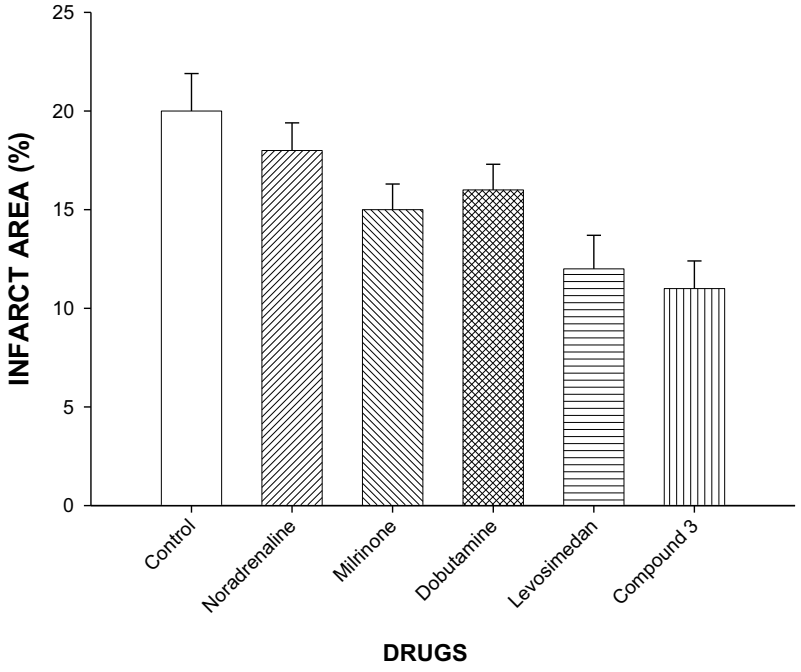

Fig. (9). Biological activity induced by noradrenaline, milrinone, dobutamine, levosimedan and the bis-steroid-methanocyclobuta-naphthalene-dione derivative (Compound 3) against the ischemia-reperfusion injury. The hearts perfused with the Krebs-Henseleith solution were subjected to ischemia for 40 minutes by turning off the perfusion system; then, the system was restarted, and the hearts were re-perfused by 40 minutes in absence or presence of noradrenaline or milrinone or dobutamine or levosimedan or the compound 3 at dose of 0.001 nM. The results showed that compound 3 significantly reduced ($p = 0.05$) infarct size expressed as a percentage of the area at risk in a similar form that levosimedan drug. Each bar represents the mean ± S.E. of 9 experiments.

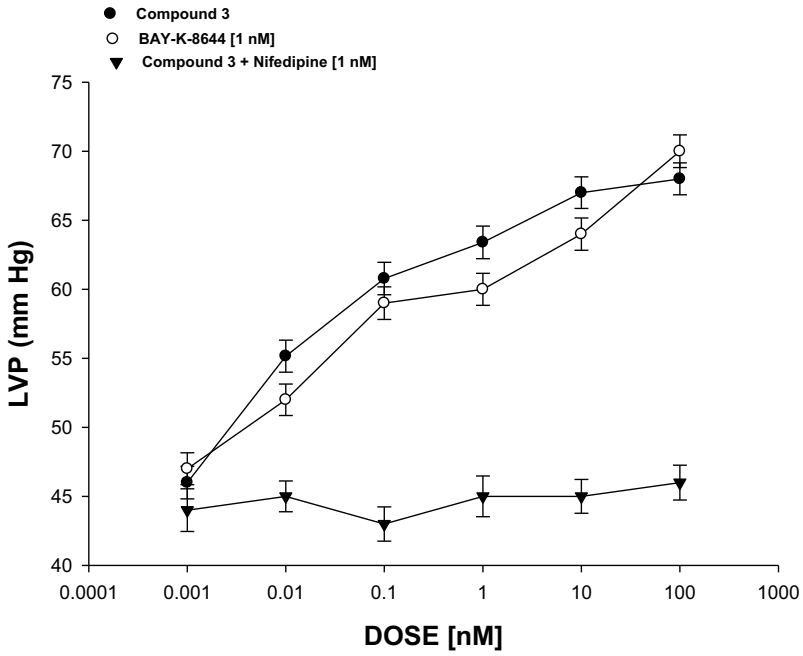

Fig. (10). Effects exerted by the bis-steroid-methanocyclobuta-naphthalene-dione (Compound 3) against left ventricular pressure (LVP) through of calcium cannels. Intracoronary boluses (50 µl) of the compound 3 or Bay-K-8644 [0.001 to 100 nM] were administered and the corresponding effect against the LVP was evaluated. The curve dose-response of exerted by the compound 3 was repeated in absence or presence of nifedipine and the effect was evaluated. The scheme showed that biological activity exerted by the compound 3 was similar at effect induced by Bay-K-8644; however, this effect was inhibited by nifedipine. The effect it is expressed as the area under the curve, and each bar represents the mean ± SE of 9 experiments.

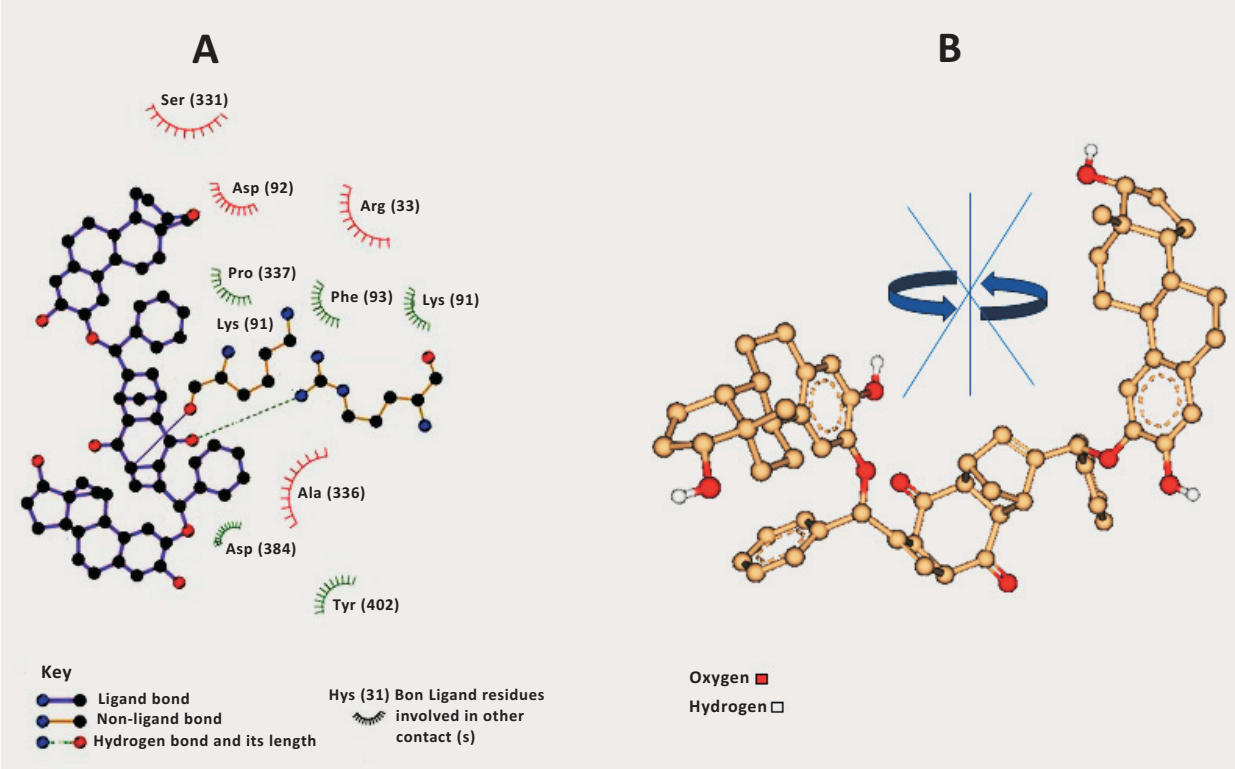

Fig. (11). In the scheme is shown the interaction of 4DEX protein surface with the compound **3 A**; in addition, the scheme B indicate the structure of compound **B**. Theoretical analysis was carried out using the Docking-server. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 5. Aminoacid residues involved between the interaction of the interaction of compound 2, 3 and Bay K8644 with 4DEX protein surface.

Bay K8644	Compound 3
Arg_{66}	Arg_{66}
Lys_{91}	Lys_{91}
Phe_{93}	Asp_{92}
Arg_{228}	Phe_{93}
Ser_{331}	Arg_{228}
Pro_{327}	Ser_{331}
Ala_{336}	Ala_{336}
Pro_{337}	Pro_{337}
Val_{339}	Asp_{384}
Leu_{382}	Tyr_{402}
Asp_{384}	Tyr_{406}

compound **3** on left ventricular pressure was evaluated in the absence or presence of nifedipine (calcium channel antagonist). The results showed that compound **3** increased the left ventricular pressure in a dose-dependent manner; nevertheless, this effect was inhibited by nifedipine. All, these data indicate that the biological activity of compound **3** against left ventricular pressure was *via type-L* calcium channel activation. The mechanism involved in the pharmacological activity of compound 3 exerted on left ventricular pressure is translated as decrease of the area of infarct; however, to validate this hypothesis, in this study, the interaction of compound **3** with calcium channel surface was evaluated.

4.5. Interaction Theoretical (Protein-ligand)

It noteworthy that the transient formation of binary complexes between the ligands and target biomolecules can mediate many biochemical activities of some cellular functions; therefore, cell life is regulated by several biological processes through the receptor-ligand interaction in which the longer the ligand remains with its receptor; the longer the biological effect sustains [52]. In this way, some theoretical models have been used to predict the interaction of some drugs with protein or enzymes [53]. Therefore, in this study, a theoretical analysis of the interaction of compound **3** was carried out on 4DEX protein (calcium channel, databank proteins) [54] using Bay K 8644 drug (calcium channel agonist) as a control in a Docking model [31]. The data showed some similar amino acid residues such as Arg_{66}, Lys_{91}, Phe_{93}, Arg_{228}, Ser_{331}, Ala_{336}, Pro_{337} and Asp_{384} to be involved in the interaction between the compound **3** with 4DEX protein surface compared with Bay K8644 (Fig. **11**, Table **5**). These data suggest that this phenomenon could be conditioned by the different conformations adopted by compound **3** or the duration of binding between the steroid-derivative and the amino acid residues involved in 4DEX protein surface.

4.6. Distances Involved of Protein-ligand Interaction

In order to evaluate the above mentioned hypothesis, in this study, a theoretical analysis was carried out to predict the interaction between compound **3** with calcium channel (4DEX) using the SeeSAR software [21]. The molecular coupling showed differences in the distance of binding between some amino acid residues involved in the surface of the 4DEX protein with the functional groups involved in the chemical structure of compound **3**. This phenomenon indicates that the interactions between functional groups and amino acid residues such as carbonyl-Pro_{337}, hydroxyl-Lys_{91}, and ether-Pro_{337} could be the main responsible for the activation of the calcium channel. Here, it is important to mention that there are some studies [55] which suggest that non-covalent distances could be very repulsive between protein-ligand interactions when are characterized by classical force fields. Therefore, in this study, the distance values involved between the interaction of compound **3** (bis-steroid-methanocyclobuta-naphthalene-dione derivative) with the calcium channel (4DEX) incorporate hydrogen-bonding and some non-covalent distances (Fig. **12**); for example, the interaction of phenyl group involved in the chemical structure of compound **3** (which is binding to ether group) with Phe_{93} aminoacid *via* π-π bound. All these data suggest that this phenomenon could be responsible for the biological activity of compound **3** against ischemia-reperfusion injury translated

Table 6. Distance between the functional groups involved in bis-steroid-methanocyclobuta-naphthalene-dione derivative (compound 3) with the aminoacid residues of 4DEX protein surface.

Functional Groups	Carbonyl (Å)	Hydroxyl (Å)	Ether (Å)
Aminoacid residues	Arg_{66} (6.93)	Lys_{91} (12.90)	Arg_{66} (12.25)
	Lys_{91} (10.16)	Arg_{228} (11.08)	Pro_{337} (3.35)
	Asn_{92} (11.17)	Ser_{331} (12.85)	Phe_{93} (7.67)
	Ala_{336} (12.25)	Asp_{384} (12.19)	Arg_{228} (9.27)
	Pro_{337} (2.84)	-	Ser_{331} (10.37)

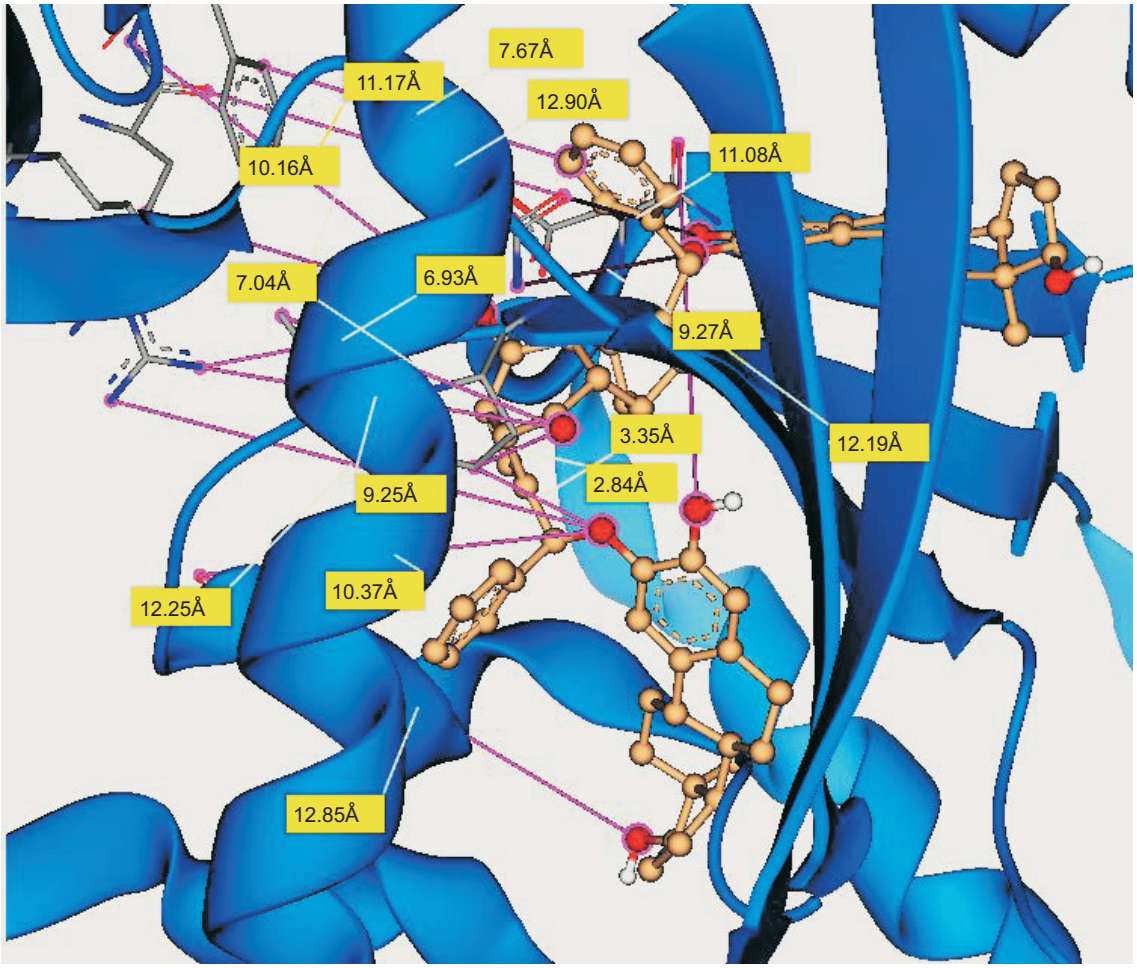

Fig. (12). Distance between functional groups of compound **3** with aminoacid residues of 4DEX protein surface. Visualized with SeeQSAR software. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

as an increase in the left ventricular pressure and a decrease in the area of infarction (Table **6**).

CONCLUSION

The biological activity of the bis-steroid-methanocyclobuta-naphthalene-dione derivative is particularly interesting, due to its cardioprotective effect exerted on the ischemia/reperfusion injury, which is translated by a decrease in the area of infarction and increase in the pressure of the left ventricle through the activation of *type-L* calcium channels. This phenomenon could constitute a new therapy for ischemia/reperfusion injury; however, it is necessary to carry out some toxicity studies to

evaluate if it could be considered for this type of clinical pathology.

AUTHORS' CONTRIBUTIONS

Figueroa-Valverde Lauro, Diaz-Cedillo Francisco, Rosas-Nexticapa Marcela, synthesized and evaluated the biological activity of the compounds involved in this study. Hernandez-Vasquez Patricia, Mateu-Armand Virginia, Pool Gómez Eduardo, Lopez-Ramos Maria, Hau-Heredia Lenin, Alfonso-Jimenez Alondra1, Borges-Ballote Yaritza, and Cabrera-Tuz Jhair, evaluated the theoretical and biological activity of the compounds.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the University Autonomous of Campeche, Mexico, (No. PI-420/12).

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are basis of this research. All the procedures followed on animals were in accordance with the guide for the care and use of laboratory animals.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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