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In-silico and *in-vivo* evaluation of the Cardiovascular effects of five *Leonotis leonurus* diterpenes

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ABSTRACT

Background: *Leonotis leonurus* extracts and compounds have been extensively studied for pharmacological effects. However, most of the diterpenes isolated from the plant have not been evaluated as possible contributors to the cardiovascular effects of the plant extracts. In this study, computational modelling was used to predict the drug-likeness and cardiovascular effects of five diterpenoids *of* L. *Leonurus*. The predicted results were then subsequently compared with results obtained from anaesthetized normotensive Wistar rats to determine the most likely lead compounds for drug development.

Methods: Molecular operating environment (MOE) software was used to assess the drug-likeness and molecular docking interactions between the diterpenoids and the angiotensin-converting enzyme (ACE) (PDB; 2 × 8Z), the angiotensin receptor (AT_1) (PDB; 3R8A) and the β_1 receptor (PDB; 2Y04). The predicted cardiovascular effects were assessed in the anaesthetized normotensive Wistar rat model.

Results: Dubiin and saponified dubiin were the most drug-like, while DC9 was the least drug-like diterpene. The interactions between the ACE and marrubiin and saponified dubiin were similar to ACE and captopril but lacked interactions with the zinc ion. None of the compounds interacted with the angiotensin receptor similar to HIG (native ligand), suggesting there was no AT_1 blockade. Binding with the β_1 receptor was similar to that of salbutamol, suggesting a β_1 agonist activity. In the *in vivo* study, statistically significant ($p < 0.05$) increases in SP, and MAP were observed with hispanol and DC9, while a significant ($p < 0.05$) increase in HR occurred with the administration of hispanol only.

Conclusion: As predicted from the *in silico* studies, none of the five diterpenes acted as inhibitors of ACE or AT_1. Hispanol produced cardiovascular effects suggestive of β_1 agonism

Abbreviations: 3D, Three-dimensional; ACE, Angiotensin converting enzyme; ANOVA, Analyses of variance; AT1, Angiotensin II receptor type I; BBB, Blood brain barrier; BP, Blood pressure; Ca^{2+}, Calcium channels; CMC, Comprehensive medicinal chemistry; DC, Diterpenoid compound; DP, Diastolic pressure; HBA, Hydrogen bond acceptors; HBD, Hydrogen bond donors; HIV, Human immunodeficiency virus; HR, Heart rate; K^+, Potassium channels; *L. Leonurus*, *Leonotis Leonurus*; LOGP, Octanol-water partition coefficient; MAP, Mean arterial pressure; MDDR, Mdl drug data report; MOE, Molecular operating environment; MORF, Molar refractivity; MW, Molecular weight; NAC, No acids; NAT, Total number of atoms; PDB, Worldwide protein data bank; PSA, Polar surface area; QSAR, Quantitative structure-activity relationship; RIGB, Rigid bonds; ROTB, Rotatable bonds; SP, Systolic pressure; TOHB, Total number of hydrogen bonds; Zn^{2+}, Zinc ion; ΔG_b, Binding free energy.

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in vivo. The *in silico* predictions correlated well with *in vivo* observations and allowed for improved determination of the ideal lead compound for drug development.

Introduction

The incidence of cardiovascular diseases (CVDs) is on the increase worldwide, and in Africa, about 1.3 million new cases are reported yearly [1]. The management of CVDs mostly requires chronic therapy, which although largely effective, can be expensive and presents with serious adverse effects [2]. This necessitates the continuing need for cheaper and more effective options including those derived from lead compounds from medicinal plants [3–5]. The use of computational modelling in predicting the drug-likeness and biological activity of these lead compounds has been identified as a way to mitigate the huge costs and high failure rates associated with the drug discovery process [6–8].

Leonotis leonurus (Lamiaceae) R.Br. is a traditional medicinal plant, indigenous to southern Africa. It is used for the treatment of a wide variety of ailments [9]. Extracts of the plant have been reported to affect blood pressure (BP) and heart rate (HR) in *in vivo* and *in vitro* studies [10–12]. Diterpenes, the major compounds isolated from the plant [13–15], have been postulated to be responsible for the reported cardiovascular effects of its extracts [16,17]. Although Obikeze *et al*., [15] reported an increase in BP and HR from a diterpene they isolated from the plant, the majority of diterpenes reported as isolated from L. *Leonurus* since the early 60′s, are yet to be evaluated for their effects on the cardiovascular system [13,18,19]. The anaesthetised normotensive Wistar rat model was used in the present study as it is particularly useful in evaluating the hypotensive, hypertensive, and chronotropic effects of compounds whose cardiovascular effects are unknown [20,21]. There is no previous study in literature which used *in silico* investigation to assess the potential bioactivity of L. *leonurus* specifically. Thus, the present study investigated the possible contribution of five diterpenes of L. *leonurus* to the previously reported cardiovascular effects of the plant extracts using *in silico* experiments, and furthermore compared the predicted results obtained from the *in silico* experiments with the subsequent results obtained from the *in vivo* study in anaesthetized normotensive Wistar rats.

Material and methods

Compounds

Dubiin, hispanol, saponified dubiin, DC9 and marrubiin were obtained from Prof Davies-Coleman at the University of the Western Cape (UWC), Cape Town, South Africa, and NMR data compared to literature to authenticate the identities of the compounds (NMR data not included). All compounds were sparingly soluble in water and were dissolved in normal saline with the aid of two (2) drops of tween 80.

Preparation of compounds and receptors

The chemical structures of the compounds were drawn on ChemBioDraw® Ultra 13, converted to 3D structures using ChemBio3D® Ultra 13 (Cambridgesoft, USA) and the simplified molecular-input line-entry system (SMILES) obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/). SMILES were then uploaded to the molecular operating environment (MOE) software (Chemical computing group Inc, USA), and the energy minimised using MM2 Force field preparative to molecular descriptor calculations and the molecular docking. X-ray crystal structures (3D) of the target receptors - angiotensin converting enzyme (ACE) (PDB:2 $\times$ 8Z), angiotensin receptor (AT_1) (PDB:3R8A) and β_1 receptor (PDB:2Y04) were downloaded from the protein data bank (http://www.rcsb.org), loaded unto MOE and structural issues corrected using the software's structure preparation application before docking with the compounds from *L. leonurus*. Discovery Studio® 0.4 software (Client, USA) was used to visualize molecular docking between the compounds and the protein targets.

Animals

Male normotensive Wistar rats between 250–350 g and 3–5 months old from the animal unit, School of Pharmacy, UWC, were housed in standard rat cages at 24 °C, with a 12:12-h light-dark cycle, with free access to both food and water [21,22]. Ethics approval for the study was obtained from the UWC Animal Research Ethics Committee (approval reference number 13/1/17), and the study was conducted according to the European Union directive on the use of animals for scientific purposes (EU Directive 2010; 2010/63/EU), and the Principles of Laboratory Animal Care [22].

Experimental detail

Drug-likeness

Molecular weight (MW), number of atoms (NAT), number of hydrogen bond donors (HBD), number of hydrogen bond acceptors (HBA), total number of hydrogen bonds (TOHB), molecular polar surface area (PSA), molar refractivity (MORF), number of rotatable bonds (ROTB), number of rings (NRING), partition coefficient (LOGP), number of rigid bonds (RIGB), and number of acids (NAC) for each of the compounds was calculated in MOE, and used to predict bioavailability based on five rules of drug-likeness (Lipinski's rule of five, Ghose filter, Veber filter, Blood-Brain Barrier likeness (BBB) and MDDR-like rule) [23–28].

Molecular docking

Molecular docking between the proteins and the corresponding native ligands (captopril, HIG and salbutamol) were carried out to validate the docking protocol and identify the binding sites for each protein. Various 'poses' were generated for each compound, and free energy of binding (ΔGb) scores calculated. The ten best 'poses' were then visualised, compared to the native ligand, and the best conformation (with the best combination of binding interaction similar to the native ligand and low binding energy) identified.

Cardiovascular effects in anaesthetised normotensive rats

Evaluation of the cardiovascular effects of the compounds was carried out according to the method previously described [15,29]. The test animals received dubiin, saponified dubiin, DC9, hispanol, and marrubiin (0.5 - 60 mg/kg), while the negative control received normal saline and two (2) drops of tween 80. Each group consisted of six (6) rats. All chemicals used were of analytical grade. Animals were anaesthetized using sodium pentobarbital (40 mg/kg; I.P) and the external jugular vein and femoral artery exposed and cannulated using heparinized polyethylene catheters to respectively infuse the test compounds and measure the cardiovascular parameters [systolic pressure (SP); diastolic pressure (DP); mean arterial pressure (MAP) and heart rate (HR)]. The Chart for Windows v5 software was used to record cardiovascular parameters from the femoral catheter via a BP transducer and amplifier connected to the PowerLab® 4/20T (All AD Instruments, Australia).

Data analysis

Mean change (Δ mean $\pm$ S.E.M) in SP, DP, MAP and HR after the administration of dubiin, saponified dubiin, DC9, hispanol, and marrubiin (0.5 - 60 mg/kg) from baseline values, was calculated and the ANOVA test for statistical significance ($p < 0.05$) carried out using GraphPad Prism® software.

Results

Drug-likeness

Dubiin and saponified dubiin passed all the rules of drug-likeness, while hispanol, DC9 and marrubiin failed the MDDR-like rule for drug-likeness. In addition to this DC9 also failed the Ghose filter rule for drug-likeness (Table 1). From this, dubiin and saponified dubiin were predicted to be the most drug-like of the diterpenes, while DC9 was predicted to be the least drug-like of the diterpenes.

Molecular docking

When docked to PDB; 2 $\times$ 8Z (ACE active site) on MOE, all compounds reported binding energies (ΔGb) similar to that obtained with the native ligand, captopril (−7.958 kcal/mol). Dubiin (ΔGb of −6.612 kcal/mol) reported the highest binding affinity, with DC9, marrubiin and hispanol (ΔGb of −6.498, −6.340 and −6.148 kcal/mol respectively) reporting similar values to dubiin, while saponified dubiin (ΔGb of −5.776 kcal/mol) reported the lowest binding affinity for the ACE (see supplementary data).

With respect to interactions between the diterpenes and the amino acid residues at the active site of PDB; 2 $\times$ 8Z (ACE active site), captopril interacted with four amino acid residues (Lys 495, Tyr 504, His 337, and Tyr 507) via four hydrogen

Table 1
Drug-likeness predictions for the five Leonotis leonurus diterpenoids.

Filters	Dubiin	Saponified- Dubiin	Hispanol	DC9	Marrubiin
Lipinski's rule	Pass	Pass	Pass	Pass	Pass
Ghose filter	Pass	Pass	Pass	Fail	Pass
MDDR-like rule	Pass	Pass	Fail	Fail	Fail
Veber filter	Pass	Pass	Pass	Pass	Pass
BBB likeness	Pass	Pass	Pass	Pass	Pass

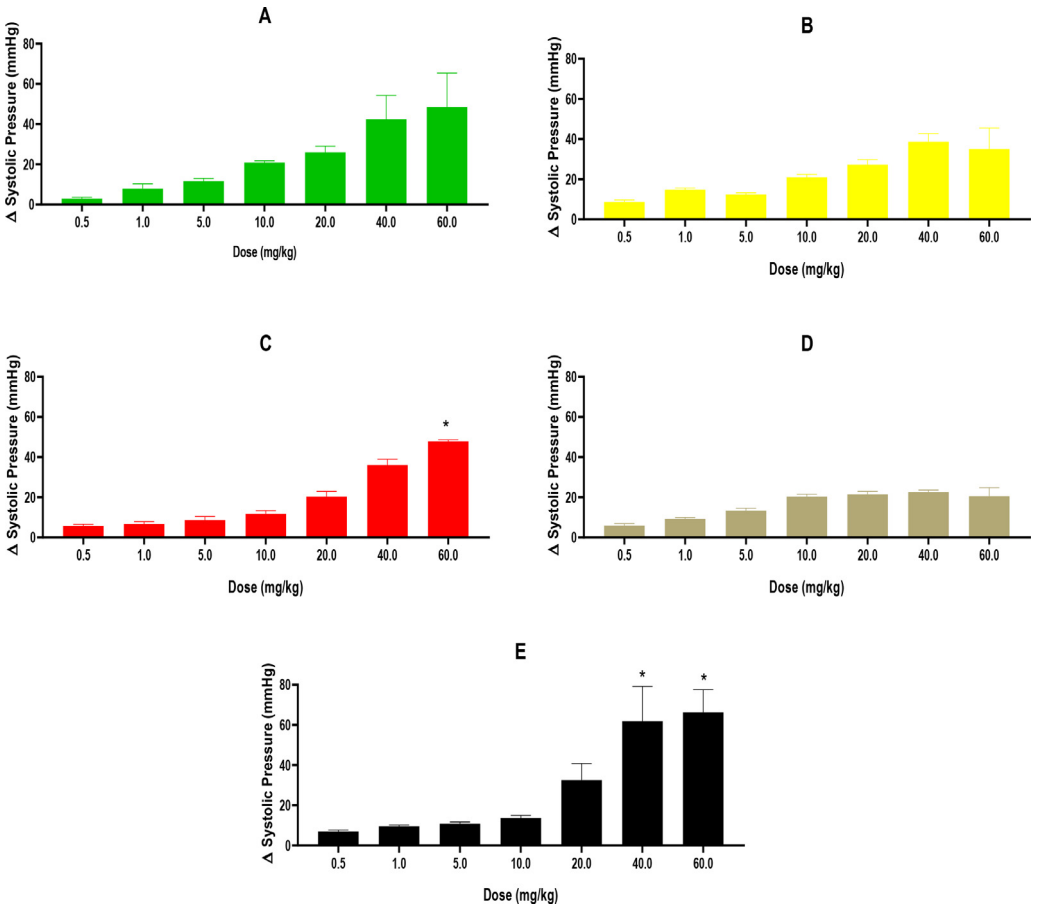

Fig. 1. Effects of various doses (0.5 mg/kg - 60 mg/kg) of dubiin (A), saponified dubiin (B), hispanol (C), marrubiin (D) and DC9 (E) on the change in systolic pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change. $n = 6$ animals.

bonds and with the Zn^{2+} ion of the ACE active site. Dubiin interacted with two amino acid residues (Thr 364 and Asn 261) via two hydrogen bonds. Saponified dubiin interacted with three amino acid residues (Tyr 504, Lys 495 and Gln 265) via three hydrogen bonds. DC9, hispanol and marrubiin interacted with two amino acid residues each (Glu 368 & Thr 364, His 337 & Lys 495, and Asp 360 & Ala 338 respectively) via two hydrogen bonds each (see supplementary data).

When docked to PDB; 3R8A (AT_1 active site), all diterpenes reported ΔGb (−5.306 kcal/mol, −3.509 kcal/mol, −5.214 kcal/mol, −5.671 kcal/mol, and −3.029 kcal/mol for dubiin, saponified dubiin, hispanol, DC9 and marrubiin respectively) that were higher than that obtained with the native ligand, HIG (−9.129 kcal/mol) (see supplementary data).

With respect to interactions between the diterpenes and the amino acid residues at the AT_1 active site, HIG interacted with one amino acid residue (Arg 288) via two hydrogen bonds, while saponified dubiin interacted with two amino acid residues (Ser 342 & Gly 284) via two hydrogen bonds. Dubiin and hispanol each interacted with one amino acid residue (Gly 284) via one hydrogen bond, while DC9 and marrubiin each interacted with one amino acid residue (Ser 342 and Cys 285 respectively) via one hydrogen bond (see supplementary data).

When docked to PDB; 2Y04 (β_1 adrenoceptor active site), all compounds reported binding energies similar to that of the native ligand, salbutamol (ΔGb of −5.654 kcal/mol). Dubiin reported the highest binding affinity (ΔGb of −5.450 kcal/mol), while the binding affinity for saponified dubiin, hispanol and DC9 (ΔGb of −5.315 kcal/mol, −5.264 kcal/mol and −5.270 kcal/mol respectively) were similar to that of dubiin. Marrubiin reported the lowest binding affinity (ΔGb of −5.144 kcal/mol) (see supplementary data).

With respect to the interactions with the amino acid residues at the active site of PDB; 2Y04, salbutamol interacted with four amino acid residues (Asn 329, Asn 310 and Asp 121 via four hydrogen bonds and Val 122 via one π bond). Dubiin, DC9 and marrubiin each interacted with two amino acid residues (Asp 200 and Asn 329) via two hydrogen bonds, while saponified dubiin interacted with two amino acid residues (Asp 200 via two hydrogen bonds and Asn 329 via one hydrogen bond). Hispanol interacted with one amino acid residue Asn 329 via one hydrogen bond (see supplementary data).

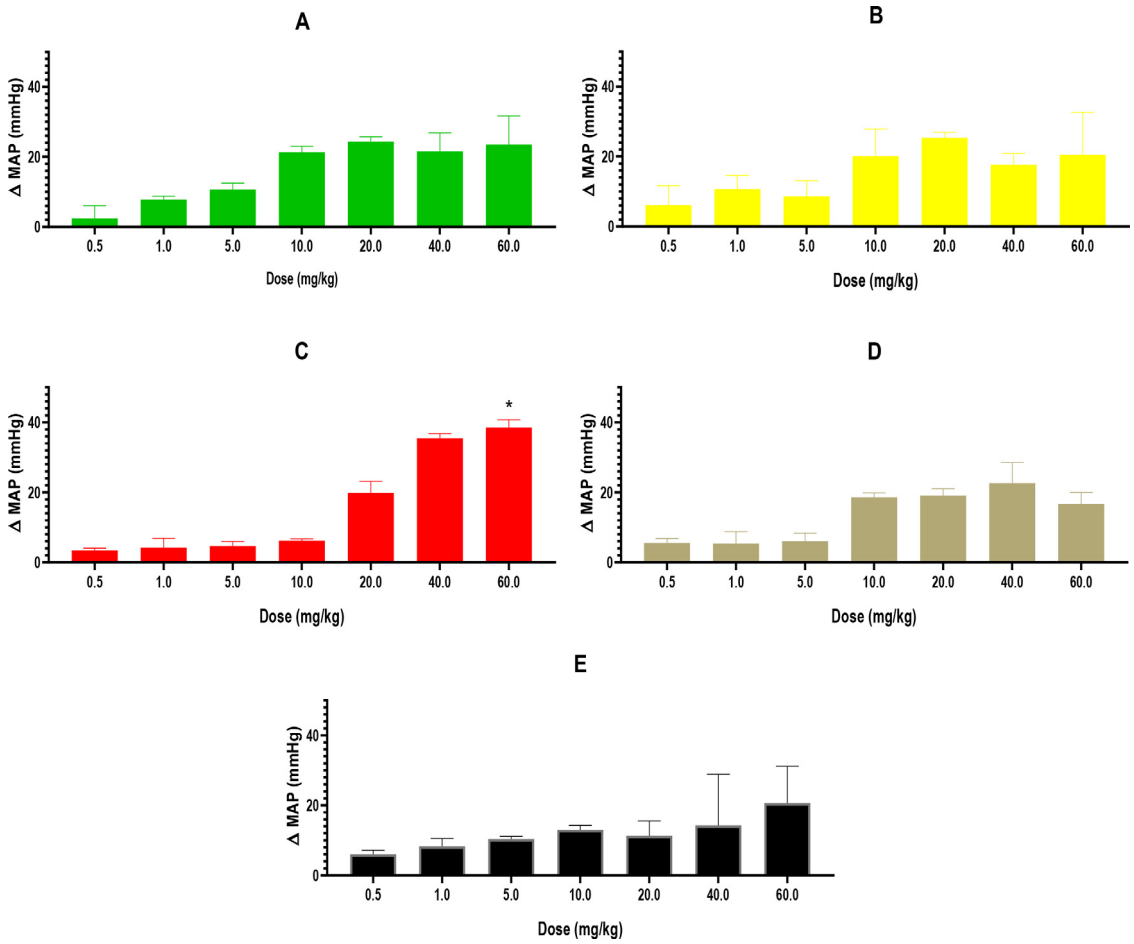

Fig. 2. Effects of various doses (0.5 mg/kg - 60 mg/kg) of dubiin (A), saponified dubiin (B), hispanol (C), marrubiin (D) and DC9 (E) on the change in mean arterial pressure in anaesthetised normotensive Wistar rats. * indicates statistical significant change. $n = 6$ animals.

Cardiovascular effects in anaesthetised normotensive rats

Normal saline and two (2) drops of tween 80 was used as the negative control. The infusion of the negative control had no significant effect on baseline blood pressure or heart rate. When administered to anaesthetized normotensive male Wistar rats, all five compounds produced positive changes to SP. The increases observed were however only dose-dependent for dubiin, hispanol and DC9, while increases observed with the administration of saponified dubiin and marrubiin were neither statistically significant nor dose-dependent. Statistically significant ($p < 0.05$) increases in SP only occurred with the administration of the highest doses of hispanol and DC9 (Fig. 1).

When administered to anaesthetized normotensive male Wistar rats, all five compounds produced positive changes to MAP. The administration of dubiin, hispanol and DC9 led to dose-dependent increases in MAP. Statistically significant ($p < 0.05$) increases in MAP only occurred with the highest dose of hispanol (Fig. 2).

When administered to anaesthetized normotensive male Wistar rats, all five compounds produced positive changes to HR. The increases observed were however only dose-dependent for saponified dubiin, hispanol, marrubiin and DC9. The increases observed with the administration of dubiin were neither statistically significant nor dose-dependent. Statistically significant ($p < 0.05$) increases in HR only occurred with the administration of the highest dose of hispanol (Fig. 3).

Discussion

All five diterpenes passed the rules of Lipinski and BBB-likeness. Thus, are predicted to have good oral bioavailability and the ability to cross the blood-brain barrier. Compared to dubiin and saponified-dubiin, hispanol and marrubiin were less likely to be drug-like. DC9 was expected to be the least likely to produce any effects *in vivo*, since it was the only compound to fail more than one filter. Therefore, in identifying potential new drug compounds, DC9 would be the least favourable of the compounds to require further evaluation for pharmacological effect.

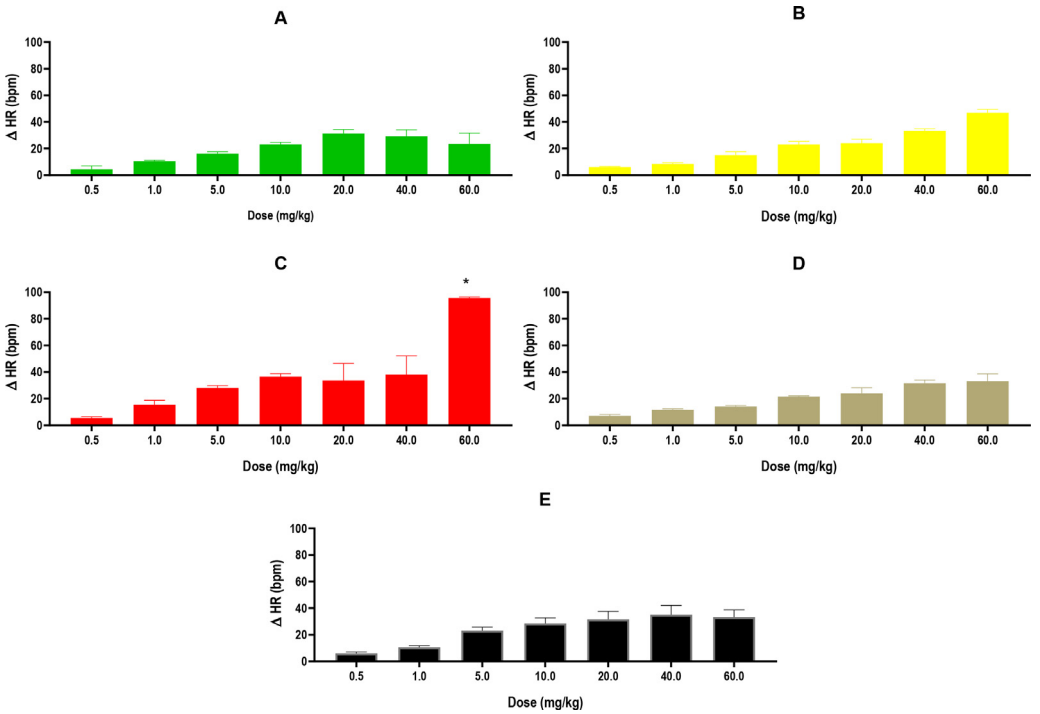

Fig. 3. Effects of various doses (0.5 mg/kg - 60 mg/kg) of dubiin (A), saponified dubiin (B), hispanol (C), marrubiin (D) and DC9 (E) on the change in heart rate in anaesthetised normotensive Wistar rats. * indicates statistical significant change. $n = 6$ animals.

Marrubiin and saponified dubiin interacted with some of the amino acid residues that captopril interacted with, and had similar binding energies to captopril, suggesting that these compounds could have a similar affinity for the ACE receptor as captopril. However, there were some differences in the amino acid residues involved in these interactions, and the lack of any direct interaction between the two compounds and the Zn^{2+} ion, which has been identified as essential for the pharmacological activity of ACE inhibitors, meant it was unlikely that any of the diterpenes would act as inhibitors of ACE *in vivo* [30,31]. The increases in BP observed in the *in vivo* study correlated with this prediction.

All the compounds tested exhibited binding energies greater than that observed with HIG (AT_1 blocker), suggesting a low affinity for the $^{AT}{}_1$ receptor [30,31]. This suggests that these compounds would not produce pharmacological effects indicative of angiotensin receptor stimulation (an increase in blood pressure, with no effect on HR) in *in-vivo* studies [32].

All five compounds had binding interactions and binding energy similar to salbutamol, suggesting that these compounds could produce $\beta 1$ agonist activity (increase in HR and BP), similar to that observed with salbutamol, when tested *in-vivo*. The increases in BP observed *in vivo* with saponified dubiin, hispanol and DC9 were similar to those previously reported with the aqueous and methanol extracts, and a diterpene from the same plant. It was also consistent with the previously reported cardiovascular effects of other diterpenes [11,12,15]. Marrubiin has been previously reported to produce vasorelaxant effects in the isolated rat aorta due to Ca^{2+}channel blockade, a mechanism that has also been associated with the cardiovascular effects of some other diterpenes [33,34]. In this study, none of the diterpenes produced a reduction in BP indicative of a vasorelaxant effect. The lack of a vasorelaxant effect with marrubiin in this study as opposed to that reported in other studies could possibly be an indication of the non-drug like nature of the compound, as predicted by the MDDR-like rule. Interestingly though, both hispanol and DC9 produced cardiovascular effects *in vivo* despite having also failed the MDDR-like rule.

As predicted by the *in silico* data, none of the compounds produced ACE inhibition-mediated decreases in blood pressure. DC9 produced increases in BP without increasing HR. This could be due to α_1 agonism and not angiotensin receptor agonism as the *in silico* data predicted no close affinity to the AT_1 receptor. Hispanol produced statistically significant ($p < 0.05$) increase in HR at the highest dose suggestive of a β_1 agonist effect, which correlated with the predictions from the *in silico* computational modelling data [35]. Although other studies have examined extracts or compounds of L. *leonurus* for cardiovascular activity this study is the first to specifically report on the cardiovascular effects of dubiin, saponified dubiin, hispanol and DC9. None of the previous studies on compounds isolated from the plant had utilised *in silico* techniques to evaluate both the drug-likeness and potential bioactivity of these compounds, and thus provide important information on their suitability as lead compounds for drug discovery. The process of drug discovery has continually gotten more expensive, and *in vivo* models are becoming more difficult to justify and use in the early stages of this process. The use of *in silico* drug discovery methods such as computational modelling offers a way for African scientists to significantly contribute to the

drug development process to address the current healthcare challenges within the continent such as CVDs. The results of this study as such contributes to aspiration 1 of the African Union's Agenda 2063 by both increasing the body of knowledge on the cardiovascular effects of the constituents of L. *leonurus* which is used in traditional medicines in Southern Africa. The study also provides foundational knowledge for further studies on the development of novel drugs for the treatment of cardiovascular diseases, which is a growing proportion of the disease burden on the continent.

Conclusion

Of the five diterpenes tested, only hispanol and DC9 produced some cardiovascular effects *in vivo*. The cardiovascular effects of hispanol correlated with the *in silico* β_1 agonist effect predicted using computational modelling tools. The results obtained from this study suggest that computer modelling is a valuable alternative tool in the drug discovery process.

Author contributions

KO, and AS conceptualised the study, carried out the investigation and acquired the data. KO, AS and IR analysed the data. AS and IR prepared the original draft manuscript. IR Data curation and KO reviewed and edited the manuscript. All authors have approved the final article.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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