Letters in Applied NanoBioScience
Platinum Open Access Journal (ISSN: 2284-6808)

Article
Volume 9, Issue 3, 2020, 1284 - 1293
https://doi.org/10.33263/LIANBS93.12841293

Biological Activity Exerted by *Crocodylus moreletii* Oil Against Heart Failure using an Ischemia/Reperfusion Model

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Scopus Author ID 55995915500
Received: 10.06.2020; Revised: 1.07.2020; Accepted: 3.07.2020; Published: 7.07.2020

Abstract: Several bioactive substances have been isolated from some crocodiles for therapeutic purposes; however, there is scarce information on the biological activity of *Crocodylus moreletii* against heart failure. The aim of this investigation was to evaluate the biological activity of *Crocodylus moreletii* oil against either heart failure and perfusion pressure. Fatty acids involved in the *Crocodylus moreletii* oil was determinate by gas chromatography analysis. In addition, the effect exerted by *Crocodylus moreletii* oil against heart failure (translated as infarct area) was evaluated using either sodium oleate or methyl linolelaidate as controls in an ischemia-reperfusion injury model. In addition, the biological activity of either *Crocodylus moreletii* (Duméril & Bibron, 1851) oil or sodium oleate or methyl linolelaidate on perfusion pressure was evaluated using an isolated rat heart model. The results showed a high concentration of linolelaidate (23.3%) and oleate (20.3%) fatty acids in the sample from *Crocodylus moreletii*. Also, the results of biological evaluation shown that both *Crocodylus moreletii* oil and sodium oleate decreased the infarct area through increase either perfusion pressure and resistance coronary compared with methyl linolelaidate. In conclusion, the results suggest that both *Crocodylus moreletii* oil and sodium oleate can exert changes on perfusion pressure and coronary resistance translated as a decrease of infarct area and consequently bring a cardioprotective effect.

Keywords: Crocodylus moreletii; heart failure; oleate; infarct area.

1. Introduction

Heart failure is a major cause of cardiovascular mortality and morbidity, among patients older than 65 years old [1-3] there are several risk factors which may contribute to develop this clinical pathology such as hypertension [4], obesity [5], diabetes [6] and others. It is important to mention that some drugs have been used for the treatment of heart failures such as enalapril

[7], nesiritide [8], digoxin [9], spironolactone [10], milrinone [11], dobutamine [12], and levosimendan [13]. However, some of these drugs can produce secondary effects such as arrhythmias [11], hypotension [14], and hyponatremia [15]. In the search for new alternative therapeutics, traditional medicine has been used to heart failure [16]. For example, the use of Moku-boi-to extracts in patients with heart failure [17]. Other data showed that *Panax ginseng*, *Ophiopogon japonicus*, and *Schisandra chinensis* extracts exert benefit against heart failure patients [18]. In addition, the buyang huanwu decoction extract has been asses to protect heart failure in hospitalized patients [19].

On the other hand, there are some studies that indicate that some bioactive chemical substituents isolated from other reptiles (crocodiles) may have pharmacological activity on some inflammatory activity which is a factor involved in heart failure; for example, a study showed some peptides (KF7, KIYFPHF, KF7, KIYFPHF) isolated of *Crocodylus siamensis* exert anti-inflammatory and antioxidant activity *in vitro* [20]. In addition, a study shown that some hemoglobin hydrolysates isolated from *Crocodylus siamensis* can be used against inflammation and oxidative stress-related disorders [21, 22]. These results indicate that some bioactive compounds isolated from Crocodiles may exert pharmacological activity against some factor risk involved in heart failure; however, However, there is little information on the effect exerted by some bioactive substances involved in *Crocodylus moreletii* oil against heart failure. Analyzing these data, the objective of this study was to evaluate the biological activity exerted by *Cocodrylus moreletii* oil against heart failure using a model of ischemia-reperfusion injury.

2. Materials and Methods

2.1. General methodology.

The reagents were used in this investigation were acquired from Sigma-Aldrich Co., Ltd. In addition, the sample of Crocodylus moreletii [23] was obtained from the Wotoch Aayin farm located at 24919 Isla Arena, Campeche, Mexico.

2.2. Preparation of Crocodile Oil.

Crocodylus moreletii oil was extracted using a previously reported method as follows; the fats were extracted with petroleum ether (boiling process 30-60°C) in an ultrasonic apparatus at room temperature (three times, 2 hours each). The extractions were collected together and concentrated in a vacuum at 50°C by a rotary evaporator. The oil was then evaporated by a vacuum dryer until it reached a constant weight.

2.3. Gas Chromatography analysis of Crocodile Oil.

The chromatography analyses were determinate using a Finnigan Trace GC Ultra gas chromatography coupled to a Finnigan Polaris Q Quadrupole Ion Trap mass spectrometer (70 eV) and RTX-5MX capillary columns (diphenyl-dimethypolysiloxane (5:95), 30 m × 0.25 mm, i.d. × 0.25 μm) following a temperature program of 70°C for 1 min, rising at 20°C/min to 250°C. It is noteworthy that helium carrier gas, at a flow of 1 ml/min, was used. Then, 1 μL of the sample* was inserted into the apparatus to determine its composition. It is important to mention that peak areas obtained were used for their quantitation. In addition,

identification of fatty acids was achieved by comparison of retention times with the retention times of authentic standard fatty acid methyl esters.

2.4. Biological methods.

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and use Committee of University Autonomous of Campeche (no. PI-420/12) and were in accordance with the Guide for the Care and Use of Laboratory Animals [24]. Male Wistar rats, weighing 200-250 g, were obtained from University Autonomous of Campeche. It is important to mention that all drugs were dissolved in methanol, and different dilutions were obtained using Krebs-Henseleit solution (≤0.01%, v/v).

2.5. Experimental design.

Briefly, the male rat (200-250 g) was 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 non-cardiac tissue and retrograde perfused via a non-circulating perfusion system at a constant flow rate. The perfusion medium was the Krebs-Henseleit solution (pH = 7.4, 37 ^{o}C) composed of (mmol) 117.8, NaCl; 6, KCl; 1.75, CaCl2; 1.2, $NaHPO_4$; 1.2, $MgSO_4$; 24.2, $NaHCO_3$; 5, glucose; 7 and 5, sodium pyruvate. The solution was actively bubbled with a mixture of O_2/CO_2 (95: 5/5%). 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 15 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

2.5.1. Perfusion Pressure Evaluation of measurements of perfusion pressure changes induced by drug administration.

In this study, the perfusion pressure was assessed using a pressure transducer connected to the chamber where the hearts were mounted, and the results entered a computerized data capture system (Biopac).

2.5.2. First Stage. Biological activity exerted by the oil of either Crocodylus moreletii or methyl linolelaidate or sodium oleate against heart failure.

Then, 15 minutes of equilibration time, the hearts were subjected to ischemia for 40 minutes by turning off the perfusion system. Following this period, the system was restarted, and the hearts were re-perfused by 40 minutes with the Krebs-Henseleit solution. The hearts were randomly divided into 4 major treatment groups (Table 1) as follows:
Group I. Hearts were subjected to ischemia/reperfusion but received vehicle only (Krebs-Henseleit solution).
Group III. Hearts were subjected to ischemia/reperfusion and treated with *sodium oleate* at a dose of 0.2 mM/min during the entire period of reperfusion ischemia period (for 18 minutes).
Group III. Hearts were subjected to ischemia/reperfusion and treated with *methyl linolelaidate* at a dose of 0.2 mM/min during the entire period of reperfusion ischemia period (for 18 minutes).

Group IV. Hearts were subjected to ischemia/reperfusion and treated with oil of *Crocodylus moreletii* at a dose of 10 µl/min during the entire period of reperfusion ischemia period (for 18 minutes).

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. It is important to mention that fluorescein was used to demarcate the tissue that was subjected to regional ischemia. Then, the heart was removed from the perfusion apparatus and cut into two transverse sections at right angles to the vertical axis to characterize the area of infarction. It is important to mention that the areas of the normal left ventricle non-risk region, area at risk, and infarct region (expressed as the percentage of the left ventricle, n = 6) were determined using a method previously reported [25].

2.5.3. Second Stage. Biological activity induced by either oil Crocodylus moreletii (CM) or methyl linolelaidate (ML) or sodium oleate (SO) on perfusion pressure.

Intracoronary boluses (50 µL) of either CM or ML [2 mM] or SO [2 mM] were administered, and the corresponding effect on the left ventricular pressure was evaluated.

2.5.4. Effects exerted by either oil Crocodylus moreletii (CM) or methyl linolelaidate (ML) or sodium oleate (SO) on coronary resistance.

Coronary resistance in the absence (control) and presence of either CM or ML [2 mM] or SO [2 mM] was evaluated. The effects were obtained in isolated hearts perfused at a constant flow rate of 10 ml/min. Since a constant flow was used, changes in coronary pressure reflected the changes in coronary resistance.

2.5.5. Statistical analysis

The obtained values are determinated as average ± SE. The data obtained were put under an analysis of variance (ANOVA) with the Bonferroni correction factor using the SPSS 12.0 program [26]. The differences were considered significant when it was equal to or smaller than 0.05.

3. Results and Discussion

3.1. Chromatographic analyses.

In this study, both saturated and unsaturated fatty acids were determined though a chromatographic analysis; the results showed a high concentration of linolelaidate (23.3%) and oleate (20.3%) fatty acids in the sample from *Crocodylus moreletii* (Figure 1 and Table 1). It is noteworthy that peaks of chromatogram from *Crocodylus moreletii* were identified by comparison of their retention times with those of individual, purified standards based on lipids that have been characterized in literature [27].

3.2. Biological activity.

In this study, the biological activity exerted by *Crocodylus moreletii* oil against heart failure was evaluated using an ischemia/reperfusion model. In this way, the hearts were subjected to ischemia/reperfusion and treated in the absence (received vehicle only; Krebs-Henseleit solution) or presence of *Crocodylus moreletii* oil a dose of 5 to 30 mg/min before

ischemia period (for 40 minutes) and during the entire period of reperfusion (40 min). The results showed that *Crocodylus moreletii* oil decrease infarct size (determinated as a percentage of the area at risk) in a dose-dependent manner compared (Figures 2 and 3) with the conditions control. This phenomenon could be to some substance such as fatty acids involved in the *Crocodylus moreletii* oil; here, it is important to mention that there are studies that suggest that either cis or trans-unsaturated fatty acids may exert some biological activity on heart [28-30].

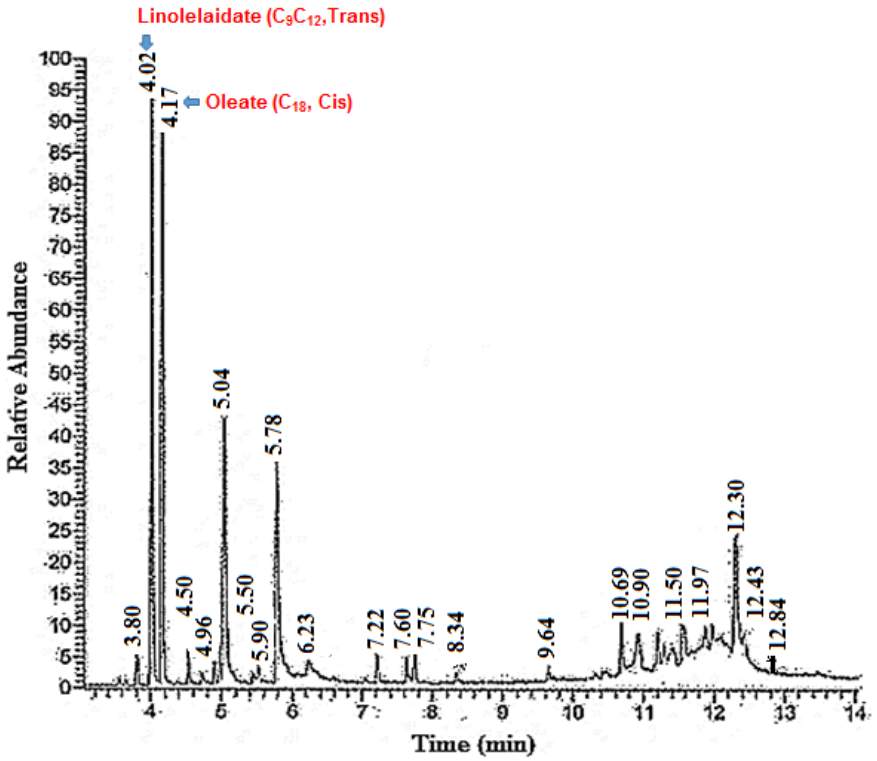

Figure 1. Chromatogram of oil from *Crocodylus moreletii* showing fatty acid peaks.

Table 1. Percentage (%) of saturated fatty acids of oil from *Cocodrylus moreletii* analyzed by GC-MS and their retention times (Rt).

Oil	%	Retention Time
Araquidate	3.2	4.9
Dodecyl acetate[1]	3.2	7.6
Eicosenoate	0.4	11.9
Elaidate	1.3	11.5
Erucate	1.5	3.8
Estereate	4.1	5.9
Heptadecanoate	0.5	6.2
Hexadecyl acetate[1]	5.1	10.9
Linoleate	6.2	12.
Linolelaidate	23.3	4.0
Miristate	1.6	7.7
Oleate	20.3	4.1
Palmitate	13.4	5.0
Palmitoleate	1.4	7.2
Pentadecanoate[1]	0.2	5.5
Tetradecyl acetate[1]	12.3	10.6
Tridecanoate	0.7	8.5
Undecanoate	1.3	11.2
$C_{15}H_{27}$[2]	1.2	4.5

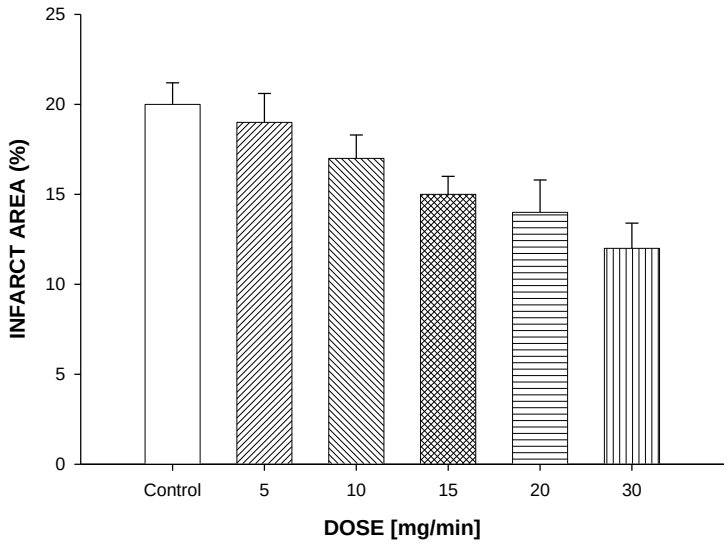

Figure 2. Effect exerted by the oil from *Crocodylus moreletii* against the infarct area. The experimental data found shown that *Crocodylus moreletii* oil significantly decreased (p = 0.05) infarct size (expressed as a percentage of the area at risk) in a dose-dependent manner compared with the vehicle-treated hearts. The values indicate the mean ± S.E. of 6 experiments.

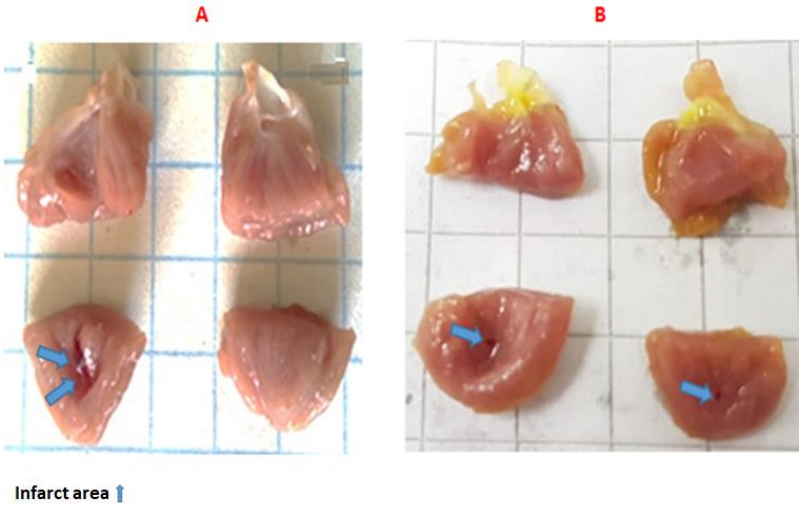

Figure 3. The scheme has shown that myocardial tissue infarction is higher in a vehicle (A) treated rats subjected to occlusion (for 40 min and reperfusion for 40 min); however, in the presence of the *Crocodylus moreletii* oil (B; 30 mg/ml) this phenomenon was lower.

For example, some studies have shown that trans-unsaturated fatty acids can induce adverse effects on the heart through increased blood lipid levels, which translates to an increase in LDL-cholesterol concentration and a decrease in HDL-cholesterol [31, 32]. In addition, other studies have shown that cis-unsaturated fatty acids have a favorable effect on the serum lipoprotein profile. In this way, a study showed that cis-unsaturated fatty can significantly decrease the serum concentrations of total cholesterol, LDL-cholesterol, and apo-B100 and the total to HDL-cholesterol [33]. Analyzing these data, in this study, the effect of either methyl linolelaidate (trans-unsaturated fatty acid) or sodium oleate (cis-unsaturated fatty acid) against heart failure was evaluated to compare with the biological activity exerted by *Crocodylus moreletii* oil. The results showed that sodium oleate decreases the infarct area in a similar manner to *Crocodylus moreletii* oil (Figure 4); these data suggest that *Crocodylus moreletii* oil

exerts a protective effect through of interaction of sodium oleate with some endogenous substance.

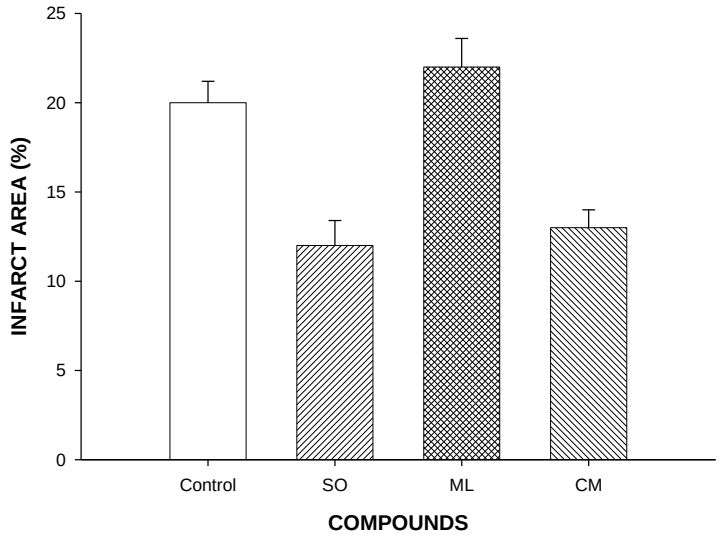

Figure 4. Biological activity induced by either *Cocodrylus moreletii* oil (CM; 5 mg/min) or methyl linolelaidate (ML; 0.2 mM/min) or sodium oleate (SO; 0.2 mM/min) against infarct area. The experimental data found shown that CM significantly ($p = 0.05$) reduced infarct size expressed as a percentage of the area at risk in similar form to effect exerted by SO. The values indicate the mean ± S.E. of 6 experiments.

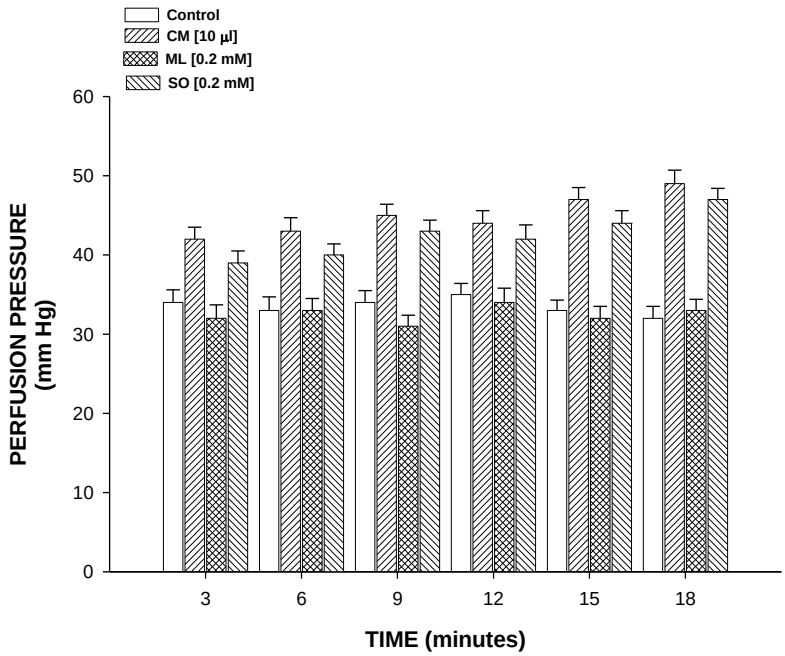

Figure 5. Biological activity induced by either *Cocodrylus moreletii* oil (CM; 5 mg/min) or methyl linolelaidate (ML; 0.2 mM/min) or sodium oleate (SO; 0.2 mM/min) on perfusion pressure. The experimental data found shown that CM and SO increase the perfusion pressure through time (3-18 min) compared with ML and the conditions control. The values indicate the mean ± S.E. of 6 experiments.

Analyzing these data and other reports which indicate that sodium oleate can induce changes in blood pressure levels [34]; in this study, the effect exerted by either *Crocodylus moreletii* oil or methyl linolelaidate (trans-unsaturated fatty acid) or sodium oleate (cis-unsaturated fatty acid) against perfusion pressure was evaluated. The results showed that both *Crocodylus moreletii* oil and sodium oleate increase the perfusion pressure in a time-dependent

manner. Here, it is important to mention that some vasoactive substances can modify the perfusion pressure through of inducing changes in resistance coronary [25]; analyzing these data, in this investigation the biological activity induced by either *Crocodylus moreletii* oil or methyl linolelaidate (trans-unsaturated fatty acid) or sodium oleate (cis-unsaturated fatty acid) against perfusion pressure was evaluated (Figure 5).

The results have shown that both *Crocodylus moreletii* oil and sodium oleate increase the coronary resistance at a flow of 10ml/min (Figure 6). All these data suggest that both Crocodylus moreletii oil and sodium oleate exert changes in the perfusion pressure, and coronary resistance translated as a decrease of infarct area and consequently brings a cardioprotective effect.

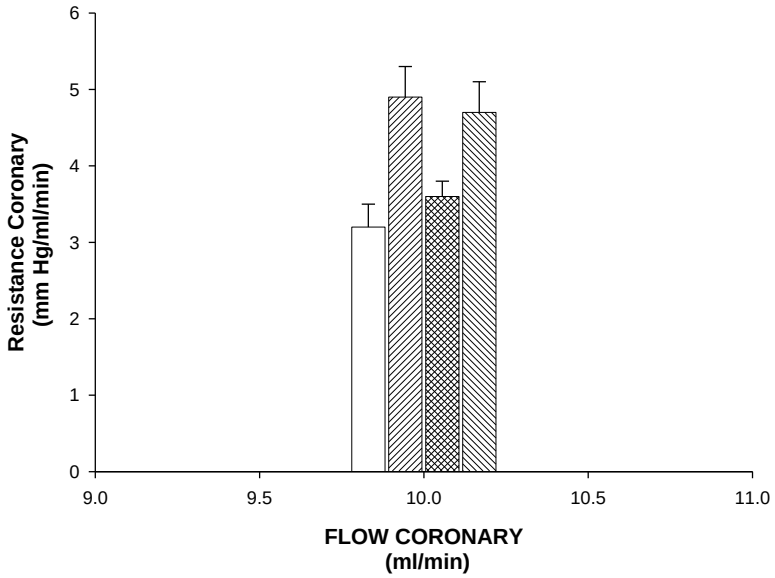

Figure 6. Activity exerted by either *Cocodrylus moreletii* oil (CM; 30 mg/min) or methyl linolelaidate (ML; 0.2 mM/min) or sodium oleate (SO; 0.2 mM/min) on perfusion pressure. on coronary resistance. The results show that coronary resistance was higher ($p = 0.05$) in the presence of either CM or SO compared with ML and the control conditions. Each bar represents the mean ± S.E. of 6 experiments.

4. Conclusions

In this study, the biological activity exerted by *Crocodylus moreletii* oil against heart failure is reported. The *Crocodylus* moreletii oil may constitute a novel therapy for heart failure translated as a decrease in heart failure; however, it is important to carry out toxicity studies of this compound to rule out any adverse effect.

Funding

This research received no external funding.

Acknowledgments

To Benjamin Valverde and Raquel Anzurez, for their unconditional support on this manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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