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SHORT COMMUNICATION

The Pulmonate Limpet *Siphonaria lessoni*: Pharmacological Study of Norsiphonarienone, a Polipropionate

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ABSTRACT

We presented a marine natural product, norsiphonarienone, isolated from *Siphonaria lessoni*, a pulmonate limpet of Central Chile. The norsiphonarienone was pharmacologically studied in rat thoracic aorta with endothelium previously contracted with phenylephrine. This technique has been widely used to describe pharmacological characterization and seek for mechanisms of action of natural products and other physiological phenomena. The main characteristics described are pD_2 (-log EC_{50}) and maximum effect or maximal contraction (E_{max}). The results show that norsiphonarienone induced a significant difference in the maximal contraction and also in the pD_2 (-log EC_{50}).

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INTRODUCTION

Siphonaria lessoni (Blainville, 1824) is the only a small sized pulmonate limpet inhabiting the rocky intertidal zone from Peru to Cape Horn (Chile) in the Pacific Ocean. Also, along the shores of Argentina, Uruguay and the Falkland Islands (Malvinas) in the Atlantic Ocean (Aguilera and Navarrete, 2012).

Pulmonate mollusks of the genus *Siphonaria* are commonly called false limpets. The *Siphonaria* genus lives in the intertidal zone, with highest densities in the high and mid zones, while *Nacella magellanica* dominates the lower zone (Bazterrica *et al*., 2007). *Siphonaria lessoni* is one of the most abundant species at intertidal and coastal fringe levels, although found subtidally (Rovirosa *et al*., 1991). From the Chilean coast, polypropionate type structures of *S. lessoni* mollusk have been described (Rovirosa *et al*., 1991).

Biological and biomedical studies are scarce (Bano and Ayub, 2012; Krishnan *et al*., 2015). Also, in drug seeking of marine origin, there are some studies about the cardiovascular effect of diacetyl epitaondiol, a diterpenoid from Easter Island algae (Chile) studied with good results (Martinez *et al*., 1997).

Described here, is the study of the pharmacological activity of norsiphonarienone (Fig. 1), a polipropionate isolated from *S. lessoni*, using rat isolated aortic rings, a technique extensively utilized (Vinet *et al*., 2014; Fuentes *et al*., 2015). On the other hands, there is evidence indicating that many phytochemical from different natural sources may protect the endothelium. On this basis, many extracts and isolated phytochemicals have been tested in this model.

MATERIALS AND METHODS

Adults Wistar rats of both sexes weighing 300-450 g (intact, n=6), were used in this study in accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda, USA 2011, and the University of Santiago of Chile Ethical Committee. We described a modified method of Illanes *et al*. (1993) and Zamorano *et al*. (1995) in the determination of aorta contractility. The thoracic aorta was carefully excised and placed in a Petri dish containing Krebs-Henseleit modified buffer (in mM: NaCl 122; KCl 4.7; $NaHCO_3$ 15.5; KH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 2.0; glucose 11.5; EDTA 0.026; pH 7.4) at room temperature. Aorta was dissected, cleaned of connective tissue and divided into 5mm rings segments. The rings were suspended between two L shaped stainless steel hooks and placed in a 20-30 ml organ chambers containing modified Krebs-Henseleit buffer, maintained at 37°C and supplied continuously with a 95% O_2 and 5% CO_2 gas. Isometric tensions were measured using a force displacement transducer connected to a polygraph. The rings were allowed to equilibrate in the tissue bath for 60

minutes under an optimal resting tension of 1.5g. Rings were progressively stretched at least three times with a depolarizing 70 mM KCl solution (in mM: NaCl 52; KCl 70.0; $NaHCO_3$ 15.5; KH_2PO_4 1.2; $MgCl_2$ 1.2; $CaCl_2$ 2.0; glucose 11.5; EDTA 0.026; pH 7.4) until the contractile response was maximal (optimal and reference tension). Aortic rings were repeatedly washed and allowed to re-equilibrate for an additional 30 minutes. The preparation was ready to evaluate relaxation or contraction activities. The analysis of the effect of drug or extract on aortic reactivity included maximal relaxation or maximal contraction (Cmax) and the concentration causing 50% of the maximal response (EC_{50}), expressed as pD_2 (-log EC_{50}). Integrity of endothelium may be assessed by testing the relaxation produced by the addition of acetylcholine (1µM) in phenylephrine (PE), (0.1-1 µM) precontracted rings. When relaxation was evaluated, rings were usually precontracted with an alpha1 adrenergic agonist (i.e. phenylephrine 0.1-1 µM) and once a stable contraction was achieved, cumulative concentration-response curve was obtained by a stepwise increase in the drug or extract concentration (usually in the range from 10^{-9} to 10^{-4} M). Response was measured as a percentage of relaxation from the precontracted level, considering the baseline as 100% relaxation.

When contraction was evaluated, drug or extract was added directly on aortic rings under basal tension by cumulative addition to obtain a concentration-response curve. The analysis of the effect of drug or extract also included maximal contraction (Cmax) and EC_{50}, as previously described. Rings were repeatedly washed and allowed to equilibrate for an additional 30 min before testing the extracts. Subsequently, rings were precontracted with PE (0.1µM) and once a stable contraction was achieved, cumulative concentration-response curves were obtained by a stepwise increase in the extract concentration.

We worked with 300 specimens of *S. lessoni* which, once collected, were immersed in acetone. After extraction on exhaustive and subsequent evaporation of the solvent, an extract was obtained. Then, it was subjected to chromatographic separation. Finally 10mg of norsiphonarienone was obtained and identified by the usual spectroscopic methods (Rovirosa *et al*., 1991).

Statistical analysis: Data obtained from vascular reactivity studies were expressed as means±SEM. Analysis of variance (ANOVA) followed by a Tukey test to evaluate the statistical significance of vascular reactivity results. All these analysis were carried out by using the GB-STAT 3.0 program. Differences were considered significant with probability values less than 0.05.

RESULTS AND DISCUSSION

The importance of this type of study is mainly based on the compounds of marine origin are isolated in very low concentrations which hinders their biological studies and when a good property is found, a limitation occurs in their study. Norsiphonarienone is an example of such structures. On the other hand, the popularity of the isolated aorta as *in vitro* model is closely associated to endothelial function. As we know, endothelium plays a key role on vascular relaxation and its dysfunction is characterized by a shift of the actions of the endothelium toward reduced vasodilation.

The results of experiments done with norsiphonarienone are shown in Fig. 2. There was an inhibitory effect on the contraction produced by phenylephrine in isolated aortic rings with intact endothelium. The concentration-response curve obtained by adding cumulative amounts of an aqueous solution of norsiphonarienone, showed a decrease in the maximum contractile response in a dose-dependent fashion. It was observed that the dose response of norsiphonarienone + phenylephrine (10^{-10} to 10^{-5} M) curve was shifted to the right compared to the control phenylephrine curve. The vehicle only produced a non-significant inhibition compared with phenylephrine + norsiphonarienone curve and the control curve (n=6 in each case). Dysfunction of the endothelium has been implicated in the pathophysiology of different forms of cardiovascular disease, including hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure (Vinet *et al*., 2014). Fig. 2 clearly shows that norsiphonarienone induced a concentration-dependent vasodilation of aortic rings.

Table 1 shows the pharmacological parameters, maximal effect (Cmax) and the concentration representing the 50% of the maximal effect (pD_2). It is observed that both Cmax and pD_2 presented significant difference ($P<0.02$ and $P<0.001$, respectively). PD_2 values obtained for phenylephrine in our results differ from those obtained by other authors (Martinez *et al*., 1997). This can be explained by physiological phenomena that produce changes in vascular reactivity in isolated rings (*in vitro*) such as:

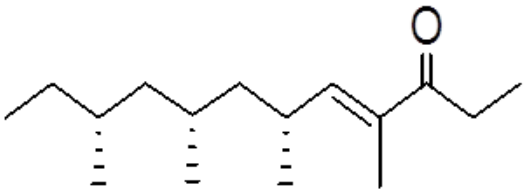

Fig. 1: Norsiphonarianone structure

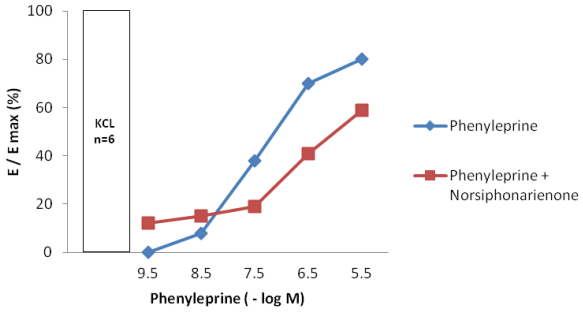

Fig. 2: Effect of phenylephrine alone and phenylephrine plus norsiphonarieonone on the contractile response of isolated aortic rings with intact endothelium.

Table 1: Pharmacological parameters (Cmax and pD_2) in the effect of phenylephrine (control) and phenylephrine plus norsiphonarienone in rat isolated aortic rings. Cmax = Agonist maximal contraction x 100/ maximal contraction with 70 mM KCl; pD_2 = -log EC_{50}; *P<0.02; **P<0.001.

Drug (n=6)	C_{max}	pD_2
phenylephrine (control)	80.5±6.5*	5.91±0.10**
phenylephrine + norsiphonarienone	59.2±4.6	5.42±0.02

Regarding the weight and age of the rats it is known that the endothelium-dependent effects in rats decrease with age of the animals (Ibarra *et al*., 1995).

Regarding the sex, vascular reactivity in rat aortas differs with sex and in normal female rats varies with the estrous cycle. There are also influenced by other hormonal phenomena (Zamorano *et al*., 1995).

The blocking effect of norsiphonarienone on the response to phenylephrine may depend on the presence of endothelium. The idea is to find a possible mechanism of action through an endothelial-derived relaxing factor with a possible modulatory role in the contraction of isolated rat aorta.

Marine natural products present an unexplored new alternative for searching new drugs, despite the fact that they are usually poorly soluble in water, making them difficult to study in living beings and also the low performance in obtaining organic skeletons.

Our results in isolated aorta from rat, with the polipropionate isolated from a Chilean central zone mollusk is a pioneer result in this type of study because most studies on isolated rat aorta are made from plant extracts being the first of them published by Martinez *et al*. (1997) with a compound isolated from a lake of Easter Island. We can conclude that these compounds could present cardiovascular protective effects.

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Authors contribution: JR and ASM contributed to polipropionate isolated from a Chilean central zone mollusk. Luisauris Jaimes for drawing the diagrams and graphics. Raúl Vinet made the statistical calculations and wrote part of the manuscript. JLM was responsible for the design and analysis of all the experimental data: CL wrote the entire manuscript and discussion.

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