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Article

Extract from leaf of *Psidium guajava* L depresses the guinea pig atrial contractility by interfering with potassium and calcium channels

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The negative inotropic effect of aqueous fraction (AqF) obtained from the acetic extract of *Psidium guajava* L leaf was investigated on the guinea pig left atrium. Myocardial force was measured isometrically (27 ± 0.1 °C, 2 Hz). AqF (100 µg/ml) reduced contractility of about 85 ± 9.4 % (n = 4, $p < 0.001$, F_{calc} = 51.70, $F_{(0.01; 4; 21)} = 5.09$, EC_{50} = 14.28 ± 3 µg/mL) in a concentration-dependent fashion. This effect was reduced by 20 mM of tetraethylammonium (TEA), increasing EC_{50} to 50 ± 7 µg/ml (n = 4, $p < 0.001$, F_{calc} = 282.13; $F_{(0.01; 21; 66)} = 2.36$). AqF (100 µg/ml) shifted to the right the $CaCl_2$ concentration-effect curve, increasing the EC_{50} from 2170 ± 112 to 2690 ± 132 µM (n = 3, $p < 0.001$, F_{calc} = 220.80 ; $F_{(0.01; 29; 60)} = 2.19$). L-NAME (100 µM) did not modify the AqF inotropic effect (n = 3, $p > 0.05$) sugesting that the oxide nitric pathway did not participate of the action mechanism of AqF. We can conclude that AqF depresses the atrial contractile by reducing the calcium entry in myocardial cells and also by openenig potassium channels of cardiac tissue.

Uniterms: *Psidium guajava*/acetic extract/ inotropic effect. Inotropic effect/experimental study. Myocardium/contraction/experimental studies. Myocardial cells/experimental studies.

O efeito inotrópico da fração aquosa (AqF) do extrato acético das folhas de *Psidium guajava* L. foi investigado em átrio esquerdo de cobaia. A força miocárdica foi medida isometricamente (27 ± 0,1 °C; 2 Hz). A AqF (100 µg/mL) reduziu a contratilidade em até 85 ± 9,4 % (n = 4; $p < 0,001$; F_{calc} = 51,70; $F_{(0,01; 4; 21)} = 5,09$; CE_{50} = 14,28 ± 3 µg/mL) de forma dependente da concentração. Este efeito foi reduzido pelo tetraetilamônio (TEA, 20 mM) que também aumentou a CE_{50} de 14,28 ± 3 µg/mL para 50 ± 7 µg/mL (n = 4; $p < 0,001$; F_{calc} = 282,13; $F_{(0,01; 21; 66)} = 2,36$). A AqF (100 µg/mL) deslocou para a direita a curva concentração-efeito do $CaCl_2$, aumentando a CE_{50} de 2170 ± 112 para 2690 ± 132 µM (n = 3; $p < 0,001$; F_{calc} = 220,80 ; $F_{(0,01; 29; 60)} = 2,19$). Por outro lado, o L-NAME (100 µM) não alterou o efeito inotrópico da AqF (n = 3; $p > 0,05$), sugerindo que a via do óxido nítrico não participa do mecanismo de ação da AqF. Conclui-se que a AqF deprime a contratilidade atrial por reduzir a entrada de cálcio nas células miocárdicas e por abrir canais de potássio deste tecido.

Unitermos: *Psidium guajava*/extrato acético/efeito inotrópico. Efeito inotrópico/estudo experimental. Miocárdio/contração/estudo experimental. Células miocárdicas/estudo experimental.

INTRODUCTION

Psidium guajava L (Myrtaceae), commonly called guava (*"goiaba"* in Brazil), is widely found in the whole world. Teas prepared from its leaves are commonly used to treat colic, diarrhea, cough, gingivitis, scurvy, uterine bleedings, bronchitis, arterial hypertension, and some intestinal parasitosis (Coe, Anderson, 1996; Ramirez *et al*., 1988). The following effects of *P. guajava* leaf extracts were reported: slowed locomotion (Lutterodt, Maleque, 1988), depression of the central nervous system (Olajide *et al*., 1999), inhibition of the retroviral reversal transcriptase (Suthienkul *et al*., 1993), antimutagenic action (Grover, Bala, 1993;

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Matsuo *et al*., 1994), antimalarial agent (Gessler *et al.*, 1994), cytotoxic (Arisawa, 1994), antidiabetic (Cheng, Yang, 1983; Hsu, Cheng, 1992; Roman-Ramos *et al*., 1995), antipyretic and antiinflammatory (Hussan *et al*., 1995), antibiotic (Cuellar-Cuellar *et al*., 1984; Le Grande, 1989), amoebicide (Tona *et al*., 1998), giardicide (Ponce-Macotela *et al*., 1994), antiallergic (Kossuge *et al*., 2000), antioxidant (Qian, Nihorimbere, 2004), and myocardial protection against injury promoted by ischemia-reperfusion maneuvers (Yamashiro *et al*., 2003). However, very little information could be found dealing with *P.guajava* extracts on the mammalian myocardium. Conde-Garcia *et al*. (2003) showed that its crude hydroalcohoolic extract depresses the guinea pig atrial contractility. This effect was completely abolished by atropine sulfate. Gondim *et al*. (2006) reported that the aqueous fraction of acetic extract induced a complete atrioventricular block on isolated guinea pig heart. Therefore, this paper aimed to shed light on the action mechanism of *P.guajava* extract on the myocardial tissue.

MATERIAL AND METHODS

Ethical procedures

Experiments and animal handling followed the rules of the Brazilian College for Animal Experimentation (COBEA).

Botanical material

Psidium guajava leaves were collected in January 2004 from agrotoxic-free tree (11° 00' 50'' S, 037° 04' 35'' W). A plant voucher was identified and deposited in the Herbarium of the Federal University of Sergipe (ASE 03304, sample number 008076).

Drugs

Hexane was purchased from SYNTH (Brazil), chloroform from GRUPO QUÍMICA (Brazil), potassium chloride, glucose, and sodium bicarbonate from MERCK S.A. Indústrias Químicas (Brazil), acetone P.A., ethanol P.A., methanol P.A., acetic acid P.A., magnesium chloride, and sodium dihydrogen phosphate were from VETEC Química Fina (Brazil), sodium chloride from QUIMIS (Brazil), atropine sulfate, tetraethylammonium chloride (TEA) and N^{ω}-Nitro-L-arginine-methyl ester (L-NAME) from SIGMA-ALDRICH (USA).

Extract preparation

P. guajava leaves were carefully cleaned and dried (50 ± 2 ºC, 10 days) before extraction in a Soxhlet apparatus. In order to remove substances of low polarity, 250 g of dry leaves were extracted with hexane followed by chloroform. Secondary metabolites of intermediate polarity were removed by acetone, ethanol, and methanol before using glacial acetic acid to extract polar compounds. The acetic extract was concentrated under low pressure in a rotative evaporator (TE-210, TECNAL, Brazil) and in a thermostable warmed environment (50 ± 2 °C) and then stored at -20 °C (FRICON – VCV -1C PVR, Brazil). The aqueous fraction (AqF) was obtained from the acetic extract by carefully dissolving it in Tyrode solution (Dorigo *et al*., 1990) to eliminate insoluble residues.

Phytochemical screening

The main AqF constituents were qualitatively determined according to the procedures proposed by Dominguez (1973).

Experimental assembly

Adults guinea pig (*Cavia porcellus*) of both gender (300 to 500 g) were used. Animals were sacrificed by a blow applied to the skull base and their hearts were rapidly removed. The left atrium was separated from the heart and mounted inside an organ bath (5 mL). Contraction force was determined isometrically (FTA10 HP/SUNBORN, Chicago, IL, USA). The atrium was bathed by a Tyrode solution (Dorigo *et al*., 1990) placed in an organ chamber, where it was oxigenized and balanced with a carbogen mixture (95% O_2 + 5% CO_2). The atrial temperature was kept constant (27 ± 0.1 °C) and it was continuously monitored by a small thermistor placed inside the bath (RADIOSHACK, model 63-1009A, China). The atrium was stretched to reach a resting tension of 9.81 mN (1 gram force) and remained under electrical field stimulation (DIGITIMER D4030, 3072, England). Contraction forces were recorded by a thermo-paper polygraph (HP 8805B, 7754A, 7754B, EUA) and simultaneously stored in a computer (A/D converter: DI 400; WINDAQ PRO AQUISITION, DATAQ Instruments, USA).

Data automatic processing

Contraction forces were automatically processed by the softwares PRAEPARATOR and CONEXON, both developed by Eduardo Antonio Conde-Garcia, MD, PhD,

from the Department of Physiology, Federal University of Sergipe (Patent number 00051104, *Instituto Nacional de Propriedade Industrial*, INPI, *Ministério do Desenvolvimento, Indústria e Comércio Exterior*/Brasília, DF, Brazil).

Experimental protocols

The inotropic effect was studied by cumulatively adding AqF to the organ bath (0.5 to 120 µg/mL). Concentration-effect curves were obtained before and after incubating the atrium with 20 mM of TEA in order to evaluate the involvement of potassium channels.

Contribution of the cellular calcium inward current to the AqF inotropic effect was also investigated. This was done by determining concentration-effect curves obtained by cumulatively adding $CaCl_2$ to the organ bath before and after adding AqF (100 µg/mL). The involvement of the intracellular nitric oxide pathway was evaluated by concentration-effect curves obtained before and after incubating the atria (20 min) with 100 µM of L-NAME (Mori *et al*., 2004).

Statistical analysis

ANOVA followed by the post test of TUKEY (General Linear Model, MINITAB Release 14.13, Minitab Inc.) was used for the statistical analysis to evaluate data of concentration-effect curves. Significance level to reject the null hypothesis was $p < 0.05$. Results are expressed as mean ± standard error of mean.

RESULTS AND DISCUSSION

Phytochemical analysis of AqF showed: 1) flavonoids [magnesium strip (+), fluorescence (++)]; 2) steroids/terpenoids [Lieberman-Buchard (++)]; and 3) tanins [$FeCl_3$ (++), gelatin 0.5 % (++)], but alkaloids were not found [Bouchardat, Mayer, Dragendorff, tungstosilicic acid].

Fig. 1 shows a typical result of the negative inotropic effect produced on the guinea pig atrium by different concentrations of AqF. The effect was concentration-dependent, reducing the atrial contractile force from 13.4 mN (control) to 1.7 mN (100-120 µg/mL). The maximal reduction of force was about 87 % of the control force. Despite its large magnitude, the effect was promptly abolished after removing the AqF from the bath (wsh: washout). Figure 2 depicts a concentration-effect curve of the AqF inotropic effect in guinea pig atria. AqF showed a relative efficacy of 85 ± 9.4 % (EC_{50} = 14.28 ± 3 µg/mL, Hill constant = 1.5, n = 4).

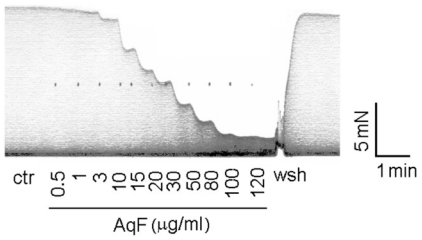

FIGURE 1 - Typical experimental record showing the contractile depressant effect of AqF on the guinea pig left atrium (ctr: control; wsh: washout; 27 ± 0.1 ºC; Stimulation: 2 Hz, 400 V, 0.5 msec).

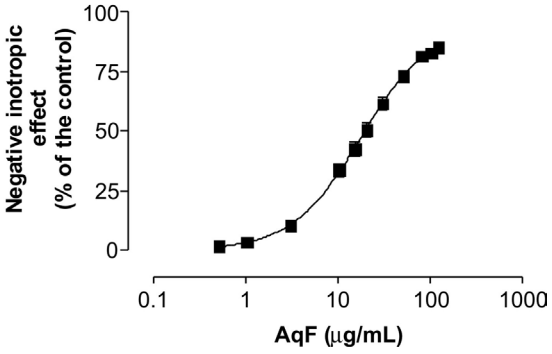

FIGURE 2 - Hill-Lagmuir curve of the depressant effect produced by AqF on the contraction force of guinea pig left atria (n = 4; EC50 = 14.28 ± 3 µg/mL; Relative efficacy: 85 ± 9.4 %; Hill constant: 1.5; 27 ± 0.1 ºC; Stimulation: 2 Hz, 400 V, 0.5 msec).

Conde-Garcia *et al*. (2003) showed that atropine sulfate (1.5 µM) – a non-selective muscarinic receptor antagonist - completely abolished the negative inotropic effect of AqF. This was also confirmed in the present work (data not shown).

It is well known that the activation of muscarinic M_2 receptors induces an increase of the potassium sarcolemmal current leading to a reduction of the action potential duration. This effect contributes to a reduction of the calcium inward current in the cardiac tissue (Dhein *et al*., 2001). The mechanism of action of AqF on the myocardium involves the activation of muscarinic receptors leading to an increase in the repolarizing currents. Thus, the reduction of the atrial contraction force promoted by AqF can be attributed to this effect. To test this hypothesis TEA (20 mM, 10 minutes) was used to block potassium currents (Freeman *et al*., 1992). AqF (100 µg/mL) reduced the atrial force from 13 to 3.5 mN (73 %) but after incubating the preparation with TEA, this effect was reduced (14.1 to 7.8 mN, 45 %) (Fig. 3). Besides that, the atrial response to AqF becames slower. Fig. 4 shows TEA shifting toward right the Hill-Langmuir of AqF concentration-effect curve,

increasing the EC_{50} from 14.28 ± 3 to 50 ± 7 µg/mL (n = 4, $p < 0.001$, $F_{calc} = 282.13$, $F_{(0.01; 21; 66)} = 2.36$). The partial reduction of the AqF negative inotropic effect promoted by TEA suggests that potassium channels could be involved in the AqF mechanism of action on the guinea pig atrium.

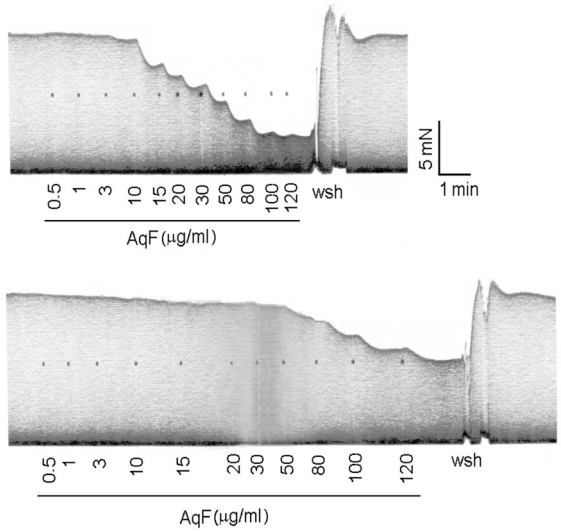

FIGURE 3 - Representative recordings showing the AqF negative inotropic effect obtained before (upper panel) and after (lower panel) adding 20 mM of TEA to the organ bath (wsh: washout, 27 ± 0.1 ºC, stimulation: 2 Hz, 400 V, 0.5 msec).

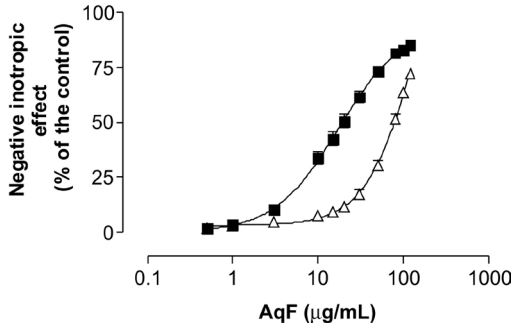

FIGURE 4 - Effect of TEA on the negative inotropic response of AqF in guinea pig left atria. Concentration-response curves were obtained on control (■, EC50 = 14.28 ± 3 µg/mL) and after adding 20 mM of TEA (Δ, EC50 = 50 ± 7 µg/mL) to the organ bath (27 ± 0.1 ºC, stimulation: 2 Hz, 400 V, 0.5 msec, n = 4, , Fcalc = 282.13; F(0.01; 21; 66) = 2.36; * $p < 0.001$).

Another effect directly associated with the activation of muscarinic receptors in cardiomyocytes is the inhibition of the intracellular AMPc-dependent responses. According to Méry *et al*. (1997), in basal conditions, the atrial cells show high adenylyl cyclase activity. Furthermore, these authors mentioned that such enzyme could be inhibited by the M_2 muscarinic receptors activation. As the reduction of intracellular AMPc levels decreases the calcium inward current in cardiomyocytes, it sounds reasonable to hypothesize that AqF could be acting on the guinea pig atrium contractility by reducing the sarcolemmal calcium influx. Experiments were performed to determine the influence of AqF (100 µg/mL) in the calcium concentration-effect curves. Fig. 5 shows that AqF partially inhibited the inotropic effect of $CaCl_2$, shifting the concentration-effect curve to the right and increasing EC_{50} from 2170 ± 112 to 2690 ± 132 µM ($p < 0.05$). Furthermore, the relative efficacy was sligthly reduced in 16 % (n = 3, p = 0.05). The positive inotropic effect produced when the extracellular calcium concentration was increased mirrors an increase of the calcium inward current through the sarcolemma. This information allowed us to suggest that the AqF also reduces the calcium inward current because it displaced to the right the calcium concentration-effect curve.

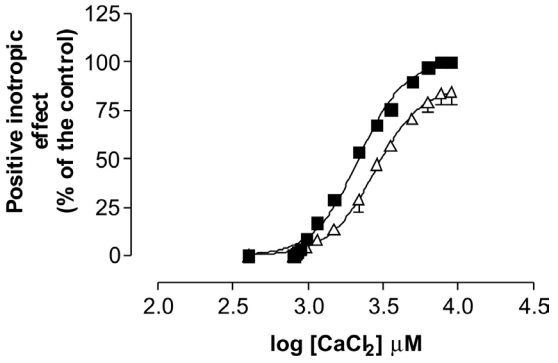

FIGURE 5 - Effect produced by AqF on the positive inotropic response obtained by increasing the extracellular calcium concentration in guinea pig isolated left atria. Curves show data before (■) and after (Δ) adding 100 µg/mL of AqF to the organ bath (Resting tension: 4.9 mN; temperature: 29 ± 0.1 ºC; stimulli: 1.5 Hz; 400 V; 0.5 msec; n = 3 atria; Fcalc = 220.8; F(0.01; 29; 60) = 2.19; * $p < 0.001$).

By using fluorescence microscopy, Dedkova *et al*. (2003) showed that after exposing cat atrial cells to acetylcholine, it was recorded an increase in the intracellular levels of nitric oxide. George *et al*. (1970) and Nascimento *et al*. (2001) reported that myocardial atrial cells submitted to acetylcholine also increase the intracellular GMPc level. In some cardiomyocytes preparations, the high intracellular GMPc concentration antagonizes the activating effect of AMPc on ionic channels. This was attributed to the type-2 phosphodiesterase (PDE) activation by GMPc. Such effect is due to an increase in AMPc degradation and also to an activation of a protein kinase which is dependent on GMPc (PKG) (Vandecasteele *et al*.

2001; Harvey, Belevych, 2003). The increase in GMPc and the reduction in AMPc concentration would lead to a decrease of the calcium L-type current and this should contribute to the negative inotropic effect of AqF. Some experiments were performed before and after incubating the atrium with the L-arginine analogue (L-NAME) to test if the intracellular nitric oxide (NO) pathway could be involved with the AqF negative inotropic effect on the guinea pig atrium. Under L-NAME effect, the production of nitric oxide is disturbed and intracellular level of NO decreases (Jindia *et al*., 1994). The results showed that L-NAME did not change significantly ($p > 0.05$, $n = 3$) the EC_{50} of AqF (12.6 ± 1.08 µg/mL), when compared to the control value (11.7 ± 1.1 µg/mL). Although some reports point towards a participation of the nitric oxide synthase (NOS) in the muscarinic pathway in the myocardium (Dedkova *et al*., 2003; Han *et al*., 1998), our data suggest that such enzyme does not participate on the AqF inotropic effect on the guinea pig atrium.

Our data allow us to conclude that AqF from the acetic extract of *P. guajava* leaf reduced the atrial contractile force. The effect disappeared during the washout and was concentration-dependent. Furthermore, it seems to be associated to an increase of sarcolemal potassium current and to a reduction of the calcium infux through the cellular membrane.

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