Hydroalcoholic extract of leaves of *Arrabidaea brachypoda* (DC.) Bureau present antispasmodic activity mediated through calcium influx blockage

Fabio de Souza Monteiro1* , Jhone Robson da Silva Costa1 , Lenivaldo Jorge Alves Martins2 , Cláudia Quintino da Rocha2 , Antonio Carlos Romao Borges1 , Marilene Oliveira da Rocha Borges1   
1Laboratory of Research and Graduate in Pharmacology, Federal University of Maranhão (UFMA), São Luís, MA, Brasil  
2Laboratory of Advanced Phytomedicine Studies, Federal University of Maranhão (UFMA), São Luís, MA, Brasil  
*Corresponding author: fabio.souza@ufma.br

Abstract

Aim: Since other species of the Bignoniacea Family presented of antispasmodic activity, it was decided, by chemotaxonomic criterion, to determine the antispasmodic activity of the leaves of *Arrabidaea brachypoda*. Methodology: the segments of the rat jejunum were suspended in glass vats containing specific saline solution, at an appropriate temperature, and after stabilization period, were stimulated by a contractile agent to observe the inhibitory or relaxing effect of EH-FAB. Results: EH-FAB showed the presence of 10 compounds, mainly rutin and it has an antispasmodic activity as it inhibits the phasic component and relaxes the tonic component of the contraction in isolated rat jejunum. To assess the mechanism of antispasmodic action, cumulative curves to the CCh were performed in which a non-competitive antagonism was observed, due to a displacement of the control curve to the right and reduction of the maximum contraction effect (Emax). Afterward, the participation of the calcium and/or potassium channels was evaluated by increasing the extracellular potassium, and it was observed that the EH-FAB relaxed the rat jejunum, suggesting the participation of the Ca2+ channels. To corroborate that hypothesis, the EH-FAB was tested against cumulative curves to Ca2+ in a free depolarizing solution of Ca2+, and it was observed that there was a shift of the curve to the right with a reduction in Emax. Conclusions: EH-FAB presents antispasmodic activity in isolated rat jejunum and it is suggested to block the influx of Ca2+ through voltage-gated calcium channels, signaling the therapeutic potential for the treatment of colic and/or diarrhea.

Keywords: Medicinal Pant. Bignoniacea. Smooth Muscle. Leaf. Bowel. *Rattus norvegicus*.

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1 INTRODUCTION

*Arrabidaea brachypoda* (DC) Bureau (synonym: *Fridericia platyphylla* (Cham.) LG Lohmann), belonging to the Bignoniacea Family and to the genus *Arrabidaea*, it is a native bush of the Brazilian Cerrado, with 1.0–2.0 m height, popularly known as “cervejinha do campo”, and used in the southeast and northeast of Brazil for the treatment of kidney stones and painful joints.
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arthritic). This species has been studied from a chemical and pharmacological point of view (Yanagizawa & Maimoni-Rodella, 2007; Rocha et al., 2011 and 2014; Rodrigues et al., 2017; Serpeloni et al., 2020; Bertanha et al., 2020).

For example, the leaves of *A. brachypoda* showed the presence of flavonoids (cirsimaritine, cirsiliol, hispidulin and 3’, 4’-dihydroxy-5,6,7-trimethoxyflavone) with antifungal activity (Alcerito et al., 2002; Patel & Patel, 2017); flavonols (arrabidoside A and B, rutin and isoquercitrin) with antioxidant, analgesic and anti-inflammatory potential (Garcia, 2008; Da Rocha, 2010, 2013), among other things. In addition, the literature highlights the obtaining of some patents with this species (Da Rocha et al., 2015a, b, c).

The pharmacological study of plants constitutes a field of new scientific knowledge and generators of wealth. In addition, it interests the Ministry of Health, as well as the National Health Surveillance Agency (“ANVISA”), to collect pharmacological information about the plants used in Brazil (Balbino & Dias, 2010; De Figueredo et al., 2014; Souza et al., 2016).

In order to contribute to the evaluation of the pharmacological potential of plants in Brazil and to propose new herbal medicines for popular uses with efficacy and safety (Resende et al., 2017), this study aimed to determine the antispasmodic activity of *A. brachypoda*, because, by the chemotaxonomic criterion, it was observed that other species of the Bignoniaceae presented this activity (Gharib et al., 2007; Cavalcante et al., 2008, 2010). In addition, typical plants of the Brazilian Cerrado, such as those of the genus *Arrabidaea*, are known to be valuable sources of bioactive compounds (Castro et al., 1999).

The antispasmodic activity is related to the ability of certain medications (extracts of plants, fractions, or isolated substances) to prevent or interrupt the painful and involuntary contraction (spasm) of the intestinal smooth muscle (Forster et al., 1980; Hani, 2014; Har & Croffie, 2017; Monteiro et al., 2018). To study that activity and its likely mechanism of action in intestinal smooth muscle, segments of isolated jejunum from rats can be used.

The preparation of isolated tissues is easy to handle and the presence or absence of the antispasmodic effect can be fully assessed. Smooth muscle is present in several hollow organs in the body systems of animals and humans controlling various physiological processes, such as intestinal peristalsis, among other things, whose deregulations are implicated in diseases such as, dysentery and intestinal colic (Webb, 2003; Kim et al., 2008; Sweeney & Hammers, 2018; Monteiro et al., 2020).

Thus, this work aims to contribute to the pharmacology of the Bignoniaceae family and as an alternative for obtaining herbal medicines accessible to the neediest population (Brandão et al., 2006; Shakya, 2016; Jamshidi-Kia et al., 2018). Therefore, the investigation of plants with antispasmodic activity is essential from a socio-economic point of view, and the investigation of the mechanism of action is one of the most interesting points of Pharmacology and may guide research in order to obtain drugs safely and effectively.

2. MATERIAL AND METHODS

2.1 Material

2.1.1 Botanical material

Samples of *A. brachypoda* leaves were collected in April 2016 at Santa Ana da Serra farm in João Pinheiro, Minas Gerais, Brazil (Location: 17°44'45" S, 46°10'44" W). A voucher specimen (Nº. 17935) was deposited at the Herbarium of the Federal University of Ouro Preto, Minas Gerais, Brazil. The plant was collected according to Brazilian laws concerning the protection of biodiversity (SISGEN n° A451DE4).
2.1.2 Animals

Rats of the species *Rattus norvegicus* were used, Wistar lineage, adults, male, healthy at the clinical examination, approximately 80 days old (250 – 350 g), provided by Central Biotery of UFMA. The animals were kept in polyethylene cages, lined with xylan, with food and water ad libitum and under a 12-hour light / dark cycle, at a temperature of 22 °C. All procedures described in the present study were approved by the Animal Research Ethics Committee of UFMA, Brazil (process No. 23115.004440/2017-09).

2.1.3 Devices and drugs

Kymographs whose cylinders are used to register phasic contractions, using frontal registration isotonic levers (DTF, Brazil). Sensitive Isometric Transducer (Model n°: MLT0202, ADInstruments, Inc., Colorado Springs, CO) for measuring tension (0.0 and 25.0 g) coupled to Power Lab 8/35 data acquisition system (Model No PL3508/P, ADInstruments Pty Ltd, Castle Hill, Australia) which was connected to a computer. The drugs used to generate contractions were purchased from SigmaAldrich (St. Louis, MO, USA): Carbamylcholine chloride (Carbachol or CCh); Potassium chloride (KCl); Calcium chloride (CaCl₂). All buffer salts were purchased from Vetec (Rio de Janeiro, RJ, Brazil): Normal Tyrode’s Solution (mM): NaCl (135.0); KCl (5.0); CaCl₂ (2.0); MgCl₂ (1.0); NaHCO₃ (15.0); NaH₂PO₄ (1.0); Glucose (11.1) (Udia et al., 2009). Depolarizing Tyrode’s Solution (KCl, 70 mM; Ca²⁺-free): (mM): NaCl (65.0); KCl (70.0); CaCl₂ (2.0); MgCl₂ (1.0); NaHCO₃ (15.0); NaH₂PO₄ (1.0); Glucose (11.1).

2.2 Method

2.2.1 Preparation of crude extract

The leaves were dried in an oven-controlled temperature of 60 °C, then they were crushed with a knife mill equipped with mechanical stirring to obtain a fine powder (1.5 kg). The extraction was performed by means of exhaustive percolation using 70% v/v ethyl alcohol. After extraction, the evaporation of the solvent was carried out in a rotary evaporator with reduced pressure and maximum temperature of 40 °C, and later lyophilized, obtaining about 20 g (yield of approximately 1.3%) of a concentrated hydroalcoholic extract, which was called EH-FAB (Da Rocha, 2013). This was solubilized in distilled water to a concentration of 10 mg/mL (stock solution), preserved at 0 °C and diluted in distilled water according to the need for each experimental protocol in the day of the experiment. EH-FAB concentrations were used in multiples of three, with the maximum concentration being 729 µg/mL since it is the maximum concentration used in experiments with isolated organs. When the observed effect was larger than 50% at 729 µg/mL, it was sought the concentration that would provide a 0% effect (Monteiro et al., 2018, 2020).

2.2.2 Analysis of hydroalcoholic extract of leaves of *Arrabidaea brachypoda* by FIA-ESI-IT-MS^n

The hydroalcoholic extract of *Arrabidaea brachypoda* was analyzed by a mass spectrometer LCQ Fleet, Thermo Scientific, equipped with apparatus for direct insertion of sample by flow injection analysis (FIA). The sample’s ionization was made by electrospray (ESI) and the fragmentations in multiple stage (MS^n) were performed in ion-trap (IT). Negative mode was used for analysis of first order and multiple stage mass spectra. Analysis conditions: capillary voltage of -4 V and -5 kV for spray, capillary temperature of 280 °C, carrier gas (N₂) with flow of 60 units. Acquisition range of m/z 50-1000, with two or more events scans performed simultaneously in MS LCQ. The first event was a complete scan (full-scan) to get ions in acquisition range. Other events were the MS^n experiment with collision induced dissociation energy between 20 and 35. The software Xcalibur software was used (Thermo Scientific^n) for acquisition and data processing.
2.2.3 Investigation of the effect of EH-FAB in isolated rat jejunum

All rats were euthanized with CO₂ gas following the principles of laboratory animal care based on the guidelines of the bioethics committee. The jejunum was isolated from rats that remained fasting for 18 h, with water at will. The isolated tissues were cleaned, under a Petri dish containing adequate nutrient solution and aerated with oxygen. After removing the fat, the tissue was sectioned (1.5 cm) and suspended in glass vats (05 or 10 mL) containing physiological solution, maintained at 37 °C. According to each experimental protocol, the jejunum tissue remained under tension (1 g) for 30 or 60 min, with intervals of 15 min of washing with nutrient solution to avoid the interference of metabolites (Altura & Altura, 1970; Brito et al., 2018).

After the initial procedures, as described above, the jejunum segment is tensioned (30 min) by means of a frontal registration lever on a kymograph cylinder to assess the phase component of the contraction. Posteriorly, two curves of similar amplitudes were induced by carbachol (CCh) 10⁻⁶ M in rat jejunum. In the presence of different concentrations of the EH-FAB extract, a third contraction was induced to assess the inhibitory effect. The concentration of EH-FAB that produced the maximum inhibitory effect (E_{max}) was expressed as mean ± S.E.M. In another experiment, the jejunum segment is tensioned (60 min) through force transducers assessing the tonic component of contraction. Posteriorly, the tissue of rat jejunum was contracted by CCh 10⁻⁶ M and when a stable contraction was attained (15-20 min), EH-FAB was cumulatively added. The relaxing effect induced by EH-FAB was expressed as the reverse percentage of the initial contraction force elicited by the agonist. The concentration of EH-FAB that produced the maximum relaxing effect (E_{max}) was expressed as mean ± S.E.M. (Monteiro et al., 2018).

2.2.4 Investigation of the antispasmodic mechanism of action of the EH-FAB

2.2.4.1 Determination of the type of antagonism of EH-FAB in isolated rat jejunum

The jejunum was set up as described in item 2.2.2. The responses were recorded on a Kymograph paper through an isotonic frontal writing lever. After the stabilization during 30 min, two cumulative concentration-response curves were obtained by cumulatively adding CCh (10⁻⁹ up until 3 x 10⁻⁵ M) in rat jejunum; then, in the absence of CCh, the EH-FAB (27, 81, 243 and 729 µg/mL) was incubated for 15 min in different tests and preparations. After that time, a new cumulative CCh response curve was displayed in the presence of the EH-FAB. The type of antagonism was evaluated by comparing the values of E_{max} of contraction in the absence (control) and in the presence of EH-FAB (Van Rossum, 1963; Ali et al., 2020).

2.2.4.2 Evaluation of the participation of calcium or potassium channels of EH-FAB in isolated rat jejunum

The initial procedures were described in item 2.2.2. Posteriorly, the tissue of rat jejunum was contracted by KCl 75 mM and when a stable contraction was attained (15-20 min), EH-FAB was cumulatively added. The relaxing effect induced by EH-FAB was expressed as the reverse percentage of the initial contraction force elicited by the spasmogenic agent. The E_{max} of relaxation was expressed as mean ± S.E.M. (Hamilton & Weston, 1989).

In another experiment, the following experimental protocol was performed: the jejunum was mounted as described in item 2.2.2. The responses and the profile of the experimental protocol were recorded as described in item 2.2.3.1. After, the external calcium of the normal Tyrode's solution was eliminated with depolarizing Tyrode's solution (KCl, 70 mM; Ca²⁺-free). Two cumulative concentration-response curves of Ca²⁺ were obtained by cumulatively adding CaCl₂ (3 x 10⁻⁸ up until 3 x 10⁻⁵ M) in the absence and presence of EH-FAB (27, 81 and 243 µg/mL), which were added to the bath 10 min before the addition of Ca²⁺. This curve was compared with those obtained in the absence of EH-FAB and the results were expressed as a percentage of the maximal response to CaCl₂ alone (Van Rossum, 1963; Ali et al., 2020).
2.2.5 Statistical analysis

All the results obtained were expressed as a percentage of the mean ± standard error of the mean (S.E.M.) and analyzed statistically using the "t" test or analysis of variance (ANOVA) "one-way" followed by the Bonferroni test, where P values less than 0.05 were considered significant. The values of E_max were calculated by non-linear regression for all experiments performed (Jenkinson et al., 1995; Arifin & Zahiruddin, 2017). All data were analyzed using the Graphpad Prism program version 5.01 (Graphpad Software Inc., San Diego CA, USA).

3. RESULTS

3.1 Chemical characterization of EH-FAB

The chemical characterization of EH-FAB, obtained by FIA-ESI-IT/MS showed the presence of important metabolites, such as flavonoids. It was possible to identify 10 of these components, mainly rutin (Figure 1 and Table 1).

![Fig. 1: Typical direct flow injection analysis FIA-ESI-IT-MS fingerprint spectra obtained in negative ion mode of the 70% EtOH from the leaves of Arrabidaea brachypoda.](image)

| Compound          | Fórmula molecular (Weight Molecular) | [M-H] | MS^a     |
|-------------------|-------------------------------------|-------|---------|
| Apigenin          | C_{15}H_{10}O_5 (270)               | 269   | 151 = [M-11 18-H]- |
| Luteolin          | C_{15}H_{10}O_6 (286)               | 285   | 267 = [M-18-H], 243 = [M-42-H]- |
| Hispidulin        | C_{16}H_{12}O_6 (300)               | 299   | 284 = [M-15-H], 117 = [M-15-167-H]- |
| Cirsiliol         | C_{17}H_{12}O_7 (330)               | 329   | 314 = [M-15-H]- |
| 7-metoxipigenina-6-C-hexose | C_{20}H_{24}O_{10} (446) | 445   | 401 = [M-44-H], 269 = [M-132-H]. |
| 7-methoxyluteolin-6-C-hexose | C_{21}H_{26}O_{12} (464) | 461   | 443 = [M-18-H], 371 = [M-90-H], 341 = [M-120-H], 313 = [M-120-28-H], 298 = [M-120-28-15-H] |
| Isoquercitrin     | C_{17}H_{20}O_{12} (464)            | 463   | 445 = [M-132-H], 301 = [M-132-H]- |
| Apigenin-6-C-hexose, 8-C-hexose | C_{22}H_{20}O_{15} (594) | 593   | 575 = [M-18-H], 503 = [M-90-H], 473 = [M-120-H], 383 = [M-120-90-H], 485 = [M-90-18-H]- |
| Rutin            | C_{22}H_{24}O_{16} (610)            | 609   | 463 = [M-146-H], 301 = [M-146-162-H]- |
| Arrabidoside A    | C_{23}H_{26}O_{19} (786)            | 785   | 609 = [M-176-H], 301 = [M-308-H]- |

3.2 Investigation of the effect of EH-FAB in isolated rat jejunum

EH-FAB inhibit phasic contractions induced by CCh 10^{-6} M (E_max = 100%) in isolated rat jejunum (Figure 2). In addition, EH-FAB relax rat jejunum pre-contracted by CCh 10^{-6} M of manner concentration-dependent (E_{max} = 90.8 ± 5.7%) (Figure 3).
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Fig. 2. Effect of EH-FAB against phasic contractions induced by CCh 10^{-6} M in isolated rat jejunum. The vertical columns and bars represent the mean and standard error of the mean of three experiments, respectively. One-way ANOVA followed by Bonferroni's post-test, *** \( p < 0.001 \) (control vs. EH-FAB), (n = 4).

Fig. 3. Effect of EH-FAB against tonic contractions induced by CCh 10^{-6} M in isolated rat jejunum. The symbols and vertical bars represent the percentage of the average and the e.p.m., respectively, (n = 3).

3.3 Investigation of the antispasmodic mechanism of action of the EH-FAB

3.3.1 Determination of the type of antagonism of EH-FAB in isolated rat jejunum

EH-FAB (27, 81, 243 and 729 µg/mL) antagonized the cumulative concentration-response curves to CCh (10^{-9} to 3 \times 10^{-5} M), shifting it to the right and reducing \( E_{\text{max}} \) (100%) of the CCh to 80.2 ± 6.9; 62.9 ± 9.8; 19.9 ± 4.8 and 3.0 ± 1.4% (Figure 4).

Fig. 4. Effect of EH-FAB against cumulative CCh contractions in isolated rat jejunum. The symbols represent the mean and standard error of the mean, respectively. One-way ANOVA followed by the Bonferroni post-test, ** \( p < 0.01 \) and *** \( p <0.001 \), (n = 5).
3.3.2 Evaluation of the participation of calcium or potassium channels of EH-FAB in isolated rat jejunum

EH-FAB (1, 3, 9, 27, 81, 243 and 729 µg/mL) relaxes the rat jejunum pre-contracted by KCl 75 mM in a concentration-dependent manner (E_{max} = 82.5 ± 3.8%) (Figure 5). In addition, EH-FAB (27, 81, 243 and 729 µg/mL) antagonized the cumulative concentration-response curves to CaCl$_2$ (3 x 10$^{-8}$ to 3 x 10$^{-5}$ M), shifting it to the right and with a reduction in the E_{max} (100%) of the CCh to 80.2 ± 6.9; 62.9 ± 9.8; 19.9 ± 4.8 and 3.0 ± 1.4% (Figure 6).

![Fig. 5. Effect of EH-FAB against tonic contractions induced by KCl 75 mM in isolated rat jejunum. The symbols and vertical bars represent the percentage of the average and the e.p.m., respectively; (n = 3).](image)

![Fig. 6. Effect of EH-FAB on isolated rat jejunum contractile response to CaCl$_2$. Symbols and vertical lines indicate means ± SEM, respectively. One-way ANOVA followed by Bonferroni’s test (Control vs EHF-SC), ***p < 0.001; (n = 3).](image)

4. DISCUSSION

The chemical and pharmacological study of the hydroalcoholic extract of the species *Arrabidaea brachypoda* (EH-FAB) is presented in this study. Chemical characterization is important to know which secondary metabolites are present in a given species since chemical compounds can undergo quantitative or qualitative variations influenced by the main factors: environmental, ontogenetic, and hereditary (Kyriacou et al., 2019).

Therefore, it was possible to demonstrate that the EH-FAB showed the presence mainly of flavonoids (Figure 1 and Table 1). Although the role of secondary metabolites is to defend the plant, they are extremely important for human health. Flavonoids, for example, can play an important role in preventing gastrointestinal disorders such as diarrhea and colic (Oteiza et al., 2018). In addition, many medicinal plants containing flavonoids show antispasmodic activity (Sadraei et al., 2018).

The antispasmodic activity can be investigated through simple experiments using isolated tissues of intestinal smooth muscle such as rat jejunum. In addition, it is possible to investigate
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the mechanism of action antispasmodic at a functional level. The contraction in the smooth muscle of the intestine in response to various agents is often composed of two phases: phasic, fast, and unsustainable component followed by a tonic, slow, and sustained component. The mechanism responsible for the phasic component is related to the activation of a metabotropic receptor coupled to protein G. On the other hand, the tonic component is mainly mediated by calcium influx through voltage-dependent calcium channels (CaV) (Bolton, 1979; van Breemen & Saida, 1989; Murthy, 2006; Sakamoto et al., 2007; Qin et al., 2017).

It was observed that EH-FAB has an effect on both phasic and tonic component of CCh-induced contraction, in other words, it inhibited contraction and relaxed the isolated rat jejunum, respectively, in a significant and concentration-dependent manner (Figures 2 and 3). In the literature, it is observed that atropine, have antispasmodic activity because they inhibit competitively muscarinic metabotropic M3 type receptors (Melchiorre et al., 1987; Montgomery et al., 2016). In isolated rat jejunum the M3 type metabotropic receptors are primarily responsible for the component phasic of the contraction (Suguro et al., 2010). Then the following came up question: would the EH-FAB be acting by competitive antagonism in isolated rat jejunum? To answer this question, it was decided to investigate which type of antagonism the EH-FAB acts. The result shows, at a functional level, non-competitive antagonism (Figure 4).

The non-competitive antagonism can be explained by the blocking of CaV or by activating the potassium channels, which are present in the plasma membrane of intestinal smooth muscle. The activation of the CaV is responsible for the sustained tonic component of the contraction, while the regulation of the contractile process is performed through the activation of the potassium channels (Bolton et al., 1981; Thorneloe & Nelson 2005; Mehmood et al., 2015).

The evaluation of the participation of the CaV or the potassium channel in the relaxing action mechanism of the EH-FAB can be done by analyzing Figure 5, where, it can be seen that the EH-FAB significantly relaxes the rat jejunum when pre-contraction by high concentrations of extracellular potassium (electromechanical coupling). As the main mechanism by which KCl induces contraction is the opening of the CaV by depolarization of the membrane (Long et al., 2005; Ratz et al., 2005; Hou et al., 2020), the CaV block hypothesis is accepted to explain the mechanism of action of EH-FAB in rat jejunum. To corroborate this hypothesis, Figure 6 shows that the EH-FAB shifts the CaCl2 curve to the right, with a reduction in the maximum effect, characteristic of CaV inhibition of calcium influx.

The same suggestion of blocking calcium influx through the CaV was observed with Arrabidaea chica in the smooth muscle of arteries (Cartagines et al., 2014). In addition, the effect reported for A. chica and now A. brachypoda may be due to the flavonoids present in the phytochemical composition of these two species (Takemura et al., 1995; Alcerito et al., 2002; Siraichi et al., 2013), since the literature shows some plants with flavonoids promoting the blockage of Ca2+ influx by CaV (Ghayur et al., 2006; Chen et al., 2009; Carvalho Correia et al., 2013; Basir, 2017; Patel & Patel, 2017).

The present study investigated the effect of the hydroalcoholic extract of the leaves of A. Brachypoda (EH-FAB) on isolated rat jejunum. The results obtained indicate that EH-FAB has antispasmodic activity by inhibiting Ca2+ influx through CaV. As a perspective, it will be used the (±)-Bay K8644 agonist to investigate voltage-sensitive calcium channels (Vissiennon et al., 2007; Kumar et al., 2019). Therefore, with the data presented here, a great contribution is made to the pharmacology of the genus Arrabidae. Although more studies need to be carried out to better characterize the antispasmodic activity presented by EH-FAB, this study contributed to show the potential of crude leaf extract in the development of bioproducts to treat disorders of contractility of the smooth muscle of the intestine.

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