Biological screening of extracts from leaf and stem bark of *Croton floribundus* Spreng. (Euphorbiaceae)

E. F. Barth\(^a,b\), L. S. Pinto\(^b\), P. Dileli\(^b\), D. C. Biavatti\(^b\), Y. L. Silva\(^b\), W. Bortolucci\(^b\), Z. C. Gazim\(^a,c\), O. S. Takeura\(^a\), M. B. Romagnolo\(^d\) and A. Laverde-Junior\(^a,b,e\)*

\(^a\)Programa de Pós-Graduação em Biotecnologia Aplicada à Agricultura, Instituto de Ciências Exatas, Agrárias, Tecnológicas e Geociências, Universidade Paranaense – UNIPAR, Praça Mascarenhas de Moraes, s/n, CP 224, CEP 87502-210, Umuarama, PR, Brazil

\(^b\)Laboratório de Química de Produtos Naturais, Instituto de Ciências Exatas, Agrárias, Tecnológicas e Geociências, Universidade Paranaense – UNIPAR, Praça Mascarenhas de Moraes, s/n, CP 224, CEP 87502-210, Umuarama, PR, Brazil

\(^c\)Laboratório de Farmacognosia, Instituto de Ciências Biológicas, Médicas e da Saúde, Universidade Paranaense – UNIPAR, Praça Mascarenhas de Moraes, s/n, CEP 87502-210, Umuarama, PR, Brazil

\(^d\)Laboratório de Vegetação Ripária, Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura – NUPELIA, Universidade Estadual de Maringá – UEM, Av. Colombo, nº 5790, CEP 87020-900, Maringá, PR, Brazil

\(^e\)Laboratório de Química Orgânica e Supramolecular – QuimiOS, Departamento de Química – DAQUI, Universidade Tecnológica Federal do Paraná – UTFPR, Av. dos Pioneiros, nº 3131, CEP 86036-370, Londrina, PR, Brazil

\(^*\)e-mail: aljunior@utfpr.edu.br

Received: July 12, 2016 – Accepted: July 14, 2017 – Distributed: November 30, 2018

(With 1 figure)

**Abstract**

This work describes the preliminary evaluation of cytotoxic, antimicrobial, molluscicidal, antioxidant and anticholinesterase activities from leaf (LECF) and stem bark alcoholic extracts (BECF) of the species *Croton floribundus* Spreng. (Euphorbiaceae), popularly known as capixingui or tapixingui. BECF presented significant toxicity (LC\(_{50}\) = 89.6 µg/ml) in the *Artemia salina* Leach, 1819 (Crustacea: Branchiopoda) bioassay, whereas LECF did not show activity (LC\(_{50}\) > 1000 µg/ml). From DPPH method, the values of IC\(_{50}\) for the LECF and BECF were 61.2 µg/ml and 62.2 µg/ml, respectively, showing that *C. floribundus* has an expressive antioxidant activity. Antimicrobial susceptibility was evaluated by microdilution technique and only BECF was active against *Staphylococcus aureus* (MIC = 39.6 µg/ml). The extracts did not present molluscicidal activity against snail *Biomphalaria glabrata* Say, 1818 (Gastropoda: Planorbidae). Both extracts revealed the presence of several components with an inhibiting capacity of acetylcholinesterase enzyme on the bioautographic assay. *C. floribundus* showed to be a promising species considering that it exhibited good biological activity in the most assays performed.

**Keywords:** anticholinesterase activity, antimicrobial activity, antioxidant activity, capixingui, *Croton*.

**Triagem biológica de extratos das folhas e caules de *Croton floribundus* Spreng. (Euphorbiaceae)**

**Resumo**

Este trabalho descreve a avaliação preliminar das atividades citotóxica, antimicrobiana, moluscidicial, antioxidante e anticolinesterásica de extratos alcoólicos das folhas (LECF) e das cascas do caule (BECF) da espécie *Croton floribundus* Spreng. (Euphorbiaceae), popularmente conhecida como capixingui ou tapixingui. O BECF apresentou toxicidade significante (LC\(_{50}\) = 89,6 µg/ml) no bioensaio com *Artemia salina* Leach, 1819 (Crustacea: Branchiopoda), enquanto que o LECF não apresentou atividade (LC\(_{50}\) > 1000 µg/ml). Da técnica de DPPH, os valores de IC\(_{50}\) para o LECF e BECF foram 61,2 µg/ml e 62,2 µg/ml, respectivamente, mostrando que *C. floribundus* tem uma expressiva atividade antioxidante. A pesquisa antimicrobiana foi realizada pela técnica de microdiluição e apenas BECF foi ativo contra *Staphylococcus aureus* (MIC = 39,6 µg/ml). Os extratos não apresentaram atividade moluscidial contra o caramujo *Biomphalaria glabrata* Say, 1818 (Gastropoda: Planorbidae). Ambos os extratos revelaram a presença de componentes com capacidade inibidora da enzima acetalcolinesterase no ensaio bioautográfico. *C. floribundus* mostrou ser uma espécie promissora considerando que exibiu boa atividade biológica no maior número de ensaios testados.

**Palavras-chave:** atividade acetalcolinesterase, atividade antimicrobiana, atividade antioxidante, capixingui, *Croton*.  

1. Introduction

Croton L., the second largest genus of family Euphorbiaceae, comprises ca. 1,300 species which are composed of shrubs and herbs distributed mainly in tropical and subtropical regions of the World of which nearly 316 species of Croton occur in Brazil, and 253 of them are endemic (Salatino et al., 2007). Several species of this genus also are found in Africa, Asia, and South America.

As most Euphorbiaceae, Croton species may contain latex, which is red-colored in some species (Dragon’s blood), a characteristic which is usually associated with medicinal properties (Gupta et al., 2008; Biscaro et al., 2013; Jura-Morawiec and Tulik, 2016). This latex has been used for centuries by indigenous communities of Amazon as a medicinal plant for several maladies. It is utilized in the regions of Colombia, Ecuador and Peru to treat wounds, ulcers, herpes lesions, cuts, burns, etc. (Gupta et al., 2008; Bailon-Moscoso et al., 2015). Popular uses of Croton spp. include treatment of cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain, ulcers and weight loss (Gupta et al., 2008).

This genus is rich in chemical constituents with biological activities, among them diterpenoids such as phorbol esters, clerodane, labdane, kaurane, grayanane, tigliane etc. (Liu et al., 2014; Wang et al., 2015; Zhang et al., 2015; Jang et al., 2016; Qi et al., 2016). Croton species are also rich in active alkaloids (Queiroz et al., 2014; Cordeiro et al., 2016). Furthermore, various species of the genus are aromatic, indicating the presence of volatile oil constituents (Santos et al., 2014; Donati et al., 2015). In recent reviews on the medicinal use, chemistry and pharmacology of this species, it is evident the potential of this genus (Gupta et al., 2008; Bailon-Moscoso et al., 2015).

Croton floribundus Spreng., popularly known as capixingui or tapixingui, is a latessant bush plant with reddish colour latex. In Brazil, it is found in the remains of the Atlantic Forest, mainly in the states of Rio de Janeiro, São Paulo, Mato Grosso do Sul, Minas Gerais, and Paraná, especially in the deciduous broadleaf forest (Lorenzi, 1992). In the folk medicine, the barks of the trunk of this species are used as a tea against syphilis and hemorrhoids. The leaves are used for ulcers, like cathartic ones. The fruits are considered as a characteristic which is usually associated with medicinal properties. The latex, which is red-colored in some species (Dragon’s blood), is utilized in the regions of Colombia, Ecuador and Peru to treat wounds, ulcers, herpes lesions, cuts, burns, etc. (Gupta et al., 2008; Bailon-Moscoso et al., 2015). Popular uses of Croton floribundus Spreng. include treatment of cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain, ulcers and weight loss (Gupta et al., 2008).

2. Materials and Methods

2.1. Plant material

The leaves and stem barks of Croton floribundus Spreng. were collected in May 2009 from the semi-deciduous Forest (Estação Ecológica do Caiuá) of the municipality of Diamante do Norte, Paraná State, Brazil, at 270 m above sea level (S22°35’17” and W52°53’44”). The plant was identified in the herbarium of the Universidade Estadual de Maringá where a voucher specimen was deposited according to exsiccate HUEM 16285.

2.2. Extracts preparation

The leaves and stem barks were separated, dried (ca. 37 °C) and were exhaustively extracted by maceration with ethanol (300 g of plant to 1000 mL of ethanol) at room temperature. The filtrates were individually concentrated under reduced pressure to give crude alcoholic extracts of the leaf (LECF) and stem bark (BECF).

2.3. Determination of cytotoxicity activity: brine shrimp lethality assay

Cytotoxicity evaluation of LECF and BECF extracts was carried out using the brine shrimp Artemia salina Leach, 1819 (Crustacea: Branchiopoda) as a model. Brine shrimp eggs were placed in seawater for 48 h before use. The eggs were placed in a two-compartment tank. One side was covered to keep the eggs in the dark while the other was illuminated to attract shrimps through perforations on the boundary plate. After 24 h, the phototropic brine shrimps (nauplii), which went to the illuminated compartment, were collected by pipette and incubated under illumination for 24 h at room temperature (Meyer et al., 1982). Shrimps were added in groups of ten nauplii in four vials with final seawater volume of 5 ml per tested concentration. The preliminary bioassay was carried out with 1000, 100, and 10 μg/ml after testing intermediate dosages. In order to verify the A. salina susceptibility, controls used only seawater. Potassium dichromate was used as a positive control in the bioassay. Each assay was performed in quadruplicate. The results were expressed as LC_{50} value (concentration able to kill 50% or more of the nauplii). LC_{50} was found by linear regression (square equation) using a significance level of 5% (p < 0.05) and reliability index of 95%. For this, the Probitos Statistical Methods/Micrcocal Origin 6.0 software was used. When found value of LC_{50} < 1000 μg/ml the assayed product was regarded as a toxic bioactive compound; on the other hand, when found value of LC_{50} ≥ 1000 μg/ml the assayed compound was regarded as non-toxic.
2.4. Determination of antioxidant activity: DPPH method

Scavenging activities of the extracts on the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) were assayed using a modified Blois method (Molyneux, 2004) by which the bleaching rate of DPPH is monitored at a characteristic wavelength in the presence of the sample. A volume of 0.1 ml of different concentrations of extracts from 1000 to 6.25 μg/ml, with 2.9 ml of a 60 μM DPPH (Sigma-Aldrich) solution in ethanol. These solution mixtures were kept in dark for 20 min and optical density was measured at 517 nm using Femto Spectrophotometer (700 plus model). Quercetin and butylated hydroxyanisole (BHA) solutions of different concentrations were used as positive controls for antioxidant activity while methanol with DPPH solution (60 μM) was the negative control. The percentage decrease of DPPH was calculated applying the following Equation 1:

\[
\text{% of inhibition} = \left[ 1 - \frac{(A_s / A_b)}{A} \right] \times 100
\]

Where \(A_s\) is the absorbance of the sample and \(A_b\) is the absorbance of the DPPH solution. The IC\(_{50}\) values denoted the concentration of each sample required to give 50% of the optical density shown by the control. The IC\(_{50}\) values were calculated from data obtained graphically. The results were expressed as the mean of three determinations made by duplicate.

2.5. Determination of molluscicidal activity

Molluscicidal evaluation of the \textit{C. floribundus} extracts was performed according to Bulletin World Health Organization guidelines (WHO, 1983) and adaptations (Silva et al., 2008) by a rapid screening procedure. The ethanolic extracts were dissolved in 100 μl DMSO at the concentration of 200, 100, 50 and 25 μg/ml and then added to glass beakers containing 100 ml of water from the aquaria. Two adult \textit{Biomphalaria glabrata} Say, 1818 (Gastropoda: Planorbidae) snails were placed in each container and maintained in a well-aerated place at room temperature. After 24 h the snails were placed on a Petri disc and their heartbeats were checked using a stereomicroscope. To confirm mortality the snails were transferred to vessels containing only deionized water and their condition was re-evaluated 24 h later. Control experiments were performed with deionized and dechlorinated water alone (negative control) or with niclosamide (0.5 μg/ml; Atenase®, Uci-farma) (positive control). Molluscicidal test with each plant extract dose was separately repeated five times.

2.6. Determination of antimicrobial activity

2.6.1. Microorganisms used and growth conditions

The test microorganisms included the bacteria \textit{Staphylococcus aureus} ATCC 6538, \textit{Escherichia coli} ATCC 8739 and \textit{Pseudomonas aeruginosa} ATCC 9027 and the yeast \textit{Candida albicans} ATCC 10231. The bacteria were grown in nutrient broth (Difco Laboratories) at 37 °C and maintained on nutrient agar slants at 4 °C. The yeast was grown and maintained on Sabouraud-dextrose agar (Merck).

2.6.2. Antimicrobial susceptibility testing

The minimal inhibitory concentrations (MIC) of all extracts were determined by microdilution techniques in Müeller-Hinton broth for bacteria (NCCLS, 2004) and Sabouraud broth for yeast (NCCLS, 1997). Each extract (10 mg/ml) was aseptically mixed with inoculums prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard [10° colony-forming units (CFU)/ml for bacteria and 106 CFU/ml for yeasts], and diluted 1:10 for the broth microdilution procedure. Microtiter plates were incubated at 37 °C and the MICs were recorded after 24 h of incubation. Two susceptibility endpoints were recorded for each isolated. The MIC was defined as the lowest concentration of compounds that the microorganism tested did not demonstrate visible growth compared with control. The antimicrobial activity of samples and reference antibiotics was established in accordance with the MIC values: MIC > 500 μg/ml (inactive), MIC = 250 and 125 μg/ml (moderately active), MIC = 62.5 and 31.2 μg/ml (active), MIC = 15.6 μg/ml (more active), MIC = 7.8 μg/ml (highly active) (Tona et al., 1999).

2.7. Determination of anticholinesterase activity: bioautographic method

Anticholinesterase activity was measured using a bioautographic assay adapted (Silveira et al., 2011) from Yang et al. (2009). Acetylcholinesterase (AChE - EC 3.1.1.7, Sigma-Aldrich) was dissolved in 0.05 M Tris-hydrochloric acid buffer at pH 7.8 with 1 mg/ml bovine serum albumin (BSA, 98%, Sigma-Aldrich). The stock solution was kept at 4 °C. LECF and BECF extracts were weighed, and made up into two stock solutions of 20 mg/ml with methanol. Then different volumes of LECF and BECF stock solutions (equivalent to 600, 400, 200, 150, 100, 50 and 25 μg dry mass) were loaded on two TLC F24 plates (10 × 10 cm, 0.2 mm thickness; Merck) and eluted in dichloromethane: methanol (9:1 v/v) for separation of compounds. The dried TLC plates were sprayed with AChE enzyme solution (1U/ml) and incubated at 37 °C for 20 minutes. For detection of the enzymes inhibitors, solutions of 1-naphthyl acetate (150 mg; Sigma-Aldrich) in ethanol 40% solution (100 ml) and Fast Blue B salt (50 mg; 95%, Sigma-Aldrich) in MilliQ® water (100 ml) were prepared immediately before use. After incubation of TLC plates, the 1-naphthyl acetate and Fast Blue B salt solutions were sprayed on the plates to give a purple coloration after 1-2 minutes. The inhibition of AChE was observed from the white spots on the purple colored dye background of the TLC plates.

2.8. Phytochemical screening

Phytochemical screening for major constituents (alkaloids, saponins, tannins, anthraquinones, coumarins, flavonoids, and cardiac glycosides) was undertaken using standard qualitative methods (Harborne, 1998). The test for tannins was carried out by subjecting 0.3 g of each extract in
6 ml of distilled water, filtered and ferric chloride reagents added to the filtrate. For cardiac glycosides, Keller Kiliani test was adopted (0.5 g of extract was added to 2 ml acetic anhydride plus H$_2$SO$_4$). The test for alkaloids was carried out bysubjecting 0.5 g extract in 5 ml 1% HCl, boiled, filtered and Mayer’s reagent was added to one portion and Dragendorff’s reagent to the other. The extract was subjected to frothing test for the identification of saponin. The extract was also tested for free glycoside bound anthraquinones (Borntraeger’s method): 0.5 g of extract was added to 10 ml benzene, filtered and ammonia solution added. The presence of flavonoids was determined using the Shinoda reaction: 1% aluminum chloride solution in methanol concentrated HCl, magnesium turnins, and potassium hydroxide solution.

3. Results and Discussion

The cytotoxic activity of LECF and BECF were verified preliminarily by the lethality assay on brine shrimp A. salina. This bioassay allows the evaluation preliminary of general toxicity of extracts and compounds. According to Meyer et al. (1982), this bioassay has shown good correlation with cytotoxicity on 9K and 9PS cells (leukemia), corroborating its usefulness as a tool for the preliminary determination of antitumor activity. Some studies have sought to correlate A. salina toxicity with other biological activities like antimicrobial, parasiticidal, virucidal, and molluscicidal activities (Arcanjo et al., 2012).

Based on the classification of toxicity (Meyer et al., 1982) in the present study, only BECF presented significant toxicity ($LC_{50} = 89.6 \mu g/ml$) whereas the LECF did not show relevant activity ($LC_{50} > 1000 \mu g/ml$). As significant toxicity was observed for BECF, it should be considered as an interesting characteristic to utilize this vegetal extract in further studies involving cytotoxicity.

This is the first report of activity against A. salina for C. floribundus, however, the same activity has already been described for essential oils of other Croton species: fresh leaves of C. hirtus (Lima et al., 2012), the bark of C. niveus and C. monteverdensis (Werka et al., 2007). Moreover, several studies have reported a series of Croton species and their secondary metabolites with cytotoxicity on cancer cell lines (Liu et al., 2014; Wang et al., 2015; Zhang et al., 2015; Qiu et al., 2016), including C. floribundus (Uchoa et al., 2013), here it was observed that a compound (ent-kaur-16-en-6α,19-diol) isolated from hexane extract of root bark showed moderate effect against three cancer cell lines: MDA-MB-435 (melanoma), HCT-8 (colorectal adenocarcinoma) and HCT-116 (colorectal adenocarcinoma). Thus, ours results for C. floribundus corroborate with those observed by Uchoa et al. (2013) and also contribute with others reported in the literature for this genus.

Several methods are used in order to determine antioxidant activity of extracts and isolated compounds. One of the most used in vitro method consists of the evaluation of the scavenging activity of DPPH free radical (Molyneux, 2004). This method is based on the electron transfer from one antioxidant compound to a DPPH free radical which loses the purple color when reduced, a process monitored by a spectrophotometer (Molyneux, 2004).

In the Figure 1 was analyzed the scavenging activity of LECF and BECF extracts on DPPH free radical when compared with the standard substances (quercetin and BHA) at varying concentrations.

Analyzing the obtained results, it was observed that the evaluated extracts presented good capacity to scavenge DPPH free radical, once the necessary concentration to inhibit half of the free radicals in solution ($IC_{50}$) was lower than 100 $\mu$g/ml. The values of $IC_{50}$ for LECF and BECF were 63.1 $\mu$g/ml and 62.2 $\mu$g/ml, respectively, demonstrating that C. floribundus presents compounds with a significant antioxidant activity. The quercetin and BHA standards presented $IC_{50}$ = 1.1 $\mu$g/ml and $IC_{50}$ = 1.9 $\mu$g/ml, respectively.

Considering the lack of studies concerning the chemical composition of C. floribundus, tests for phytochemical characterization of tannins, anthraquinones, cardiotonics, alkaloids, coumarins, saponins, and flavonoids were done. According to the tests carried out, there was only the indication of the presence of flavonoids and hydrolysable tannins. The presence of coumarins, anthraquinones, saponins, alkaloids and cardiotonics were not observed. According to our results, because the tests were positive for tannins and flavonoids, the observed antioxidant properties may be attributed to the presence of phenolic compounds (Takao et al., 2015).

This is the first report of the antioxidant activity of C. floribundus. Other species of Croton have been related to possessing an antioxidant activity (Aderogba et al., 2011; Donati et al., 2015; Shahwar et al., 2015). The popular use of these Croton species is enhanced by the verification of its antioxidant action (Morais et al., 2006).

The antimicrobial activity of C. floribundus extracts was also the target of our survey. LECF and BECF
Table 1. Antimicrobial activity of crude alcoholic extracts of leaves (LECF) and stem barks (BECF) of C. floribundus represented as MIC.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>BECF (μg/ml)</th>
<th>LECF (μg/ml)</th>
<th>Levofloxacin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> ATCC 8739</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>0.01</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 9027</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 6538</td>
<td>39.6</td>
<td>&gt;10,000</td>
<td>0.11</td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC 10231</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>0.24</td>
</tr>
</tbody>
</table>
may be active at concentrations well below those tested in this work.

Finally, TLC-bioautography can not only be used for screening of the components with anticholinesterase inhibitor potential but also for the purpose of quality evaluation of interest extracts at the same time. The studied extracts showed significant positive results at the bioautographic tests, revealing a promising source of natural agents with pharmacological potential.

4. Conclusions

The alcoholic extracts of stem bark and leaf of Croton floribundus Spreng. showed biological potential observed at different assays performed. The stem bark alcoholic extract of C. floribundus showed significant antioxidant, anticholinesterase, cytotoxic and antimicrobial activities. Whereas, the leaf alcoholic extract presented considerable antioxidant and anticholinesterase activities. All these activities have already been observed in other species of genus Croton L.; therefore, the present study corroborates information found in the literature for this genus. However, all these observed activities for this native species are being reported for the first time.

Preventive and symptomatic treatment of Alzheimer’s disease requires multitarget drug strategy. Therefore, it is suitable to explore a crude extract having both antioxidant and anticholinesterase activities. The obtained results could, therefore, form a good basis for selection of plant species for further investigation in the potential discovery of new natural bioactive compounds.

Acknowledgements

This project was supported financially by the Fundação de Apoio à Pesquisa do Estado do Paraná (Fundação Araucária - 14/2009-18804; conv. 503/2010) and Universidade Paranaense (UNIPAR - Proj.: 18005/2010 and 20152/2011). E.F. Barth thanks Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for providing a master fellowship (PROSUP/CAPES). P. Dileli, D. C. Biavatti, and Y.L. Silva thank Fundação Araucária and DEGPP/UNIPAR for the scholarships.

References


Table 2. Inhibition of acetylcholinesterase enzyme in the presence of different concentrations (dry mass) of crude alcoholic extracts of leaves (LECF) and stem barks (BECF) of *C. floribundus* by HPTLC bioautographic analyses.

<table>
<thead>
<tr>
<th>Observed spot</th>
<th>Rf</th>
<th>BECF (drymass)</th>
<th>LECF (drymass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>600 µg</td>
<td>400 µg</td>
</tr>
<tr>
<td>A1</td>
<td>0.85</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>B1</td>
<td>0.78</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>C1</td>
<td>0.41</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>D1</td>
<td>0.27</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>E1</td>
<td>0.11</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Legend: (-) no activity; (+) low; (+++) moderate; (++++) high activity.

607


