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# Effects of Ingested *Baccharis dracunculifolia* D.C. (Asteraceae) Extract in the Liver of *Prochilodus lineatus* Fish

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## Abstract

*Baccharis dracunculifolia*, popularly known in Brazil as "alecrim-do-campo", is widely recognized for its therapeutic potential. The extract of its leaves is used for liver problems, stomach disorders and others. The objective of the present study was to perform a histochemical analysis of curimbata fish livers to evaluate the potential effects and risks of the ingestion of *B. dracunculifolia.* Thirty-two animals were divided into two experimental groups in duplicate: Control group (regular food) and *B. dracunculifolia* Treated group (food added with *B. dracunculifolia*). The fishes were collected on the 14th and 21st days after the treatment period of 21 days. The histological alterations were evaluated using the semiquantitative methods Mean Value of Alterations (MVA), Histopathological Alteration Index (HAI) and Image J®. HAI and MAV showed that the extract caused slight but statistically significant damages, widely distributed throughout the organ. The results showed significant hepatic alterations caused by the ingestion of *B. dracunculifolia* extract.

## Keywords

Asteraceae, Hepatotoxicity, Lipofuscin, Macrophages, *Prochilodus lineatus*

## 1. Introduction

Asteraceae is one of the largest angiosperm families, with more than 1535 genera, 23000 species and 17 tribes distributed throughout the world. Among them, the genus *Baccharis* comprises about 500 known species (Abreu and Onofre,

2010) distributed throughout the Americas, from Southern United States to Southern Argentina, and Brazil, with the greatest number of species—approximately 120 species [1].

The species *Baccharis dracunculifolia* (popularly known in Brazil as "alecrim-do-campo") has been intensively studied due to its therapeutic uses and potentialities. It has been used by the pharmaceutical industry in the production of green propolis—produced by *Apis mellifera L.* bees [2] [3], and in food industry, as a functional food product [4]. *B. dracunculifolia* and *B. trimera* have been widely used in popular medicine to treat stomach, liver and kidneys dysfunction, diabetes, prostate conditions, inflammations and detoxifications in general [5].

Studies have demonstrated that the essential oil of *B. dracunculifolia*is mainly constituted of mono and sesquiterpene, such as nerolidol (33.51%) and spathulenol (16.24%) [6]. Nerolidol has presented satisfactory results in several treatment models analyzing rats with induced ulcer, which confirms the indication of *B. dracunculifolia* essential oil to control the disease [7]. The main secondary metabolites identified in this species are the terpenoids, flavonoids and prenylated phenolic compounds derived from coumaric acid [5].

Studies have demonstrated that the essential oil of *B. dracunculifolia* has antiulcerogenic [8], antimicrobial [9], analgesic, antispasmodic, sedative, cytostatic [10] properties. Moreover, according to [11], the species *B. dracunculifolia* has anti-inflammatory, anti-protozoal, anthelmintic, antioxidant, anticancer, anticariogenic, cytotoxic, mutagenic (in high concentrations) and cicatrizing potential.

Most medicinal plants have not had their toxic and mutagenic potentials thoroughly investigated [12] [13] [14]; however, it is known that *Baccharis* plants present high toxicity levels [15] [16]. Therefore, this study performed the histochemistry of the livers of fish treated with *B. dracunculifolia* to evaluate the possible risks of the ingestion of this medicinal plant.

## 2. Material and Methods

### 2.1. Specimens

The *Prochilodus lineatus* juveniles used in this experiment (60.7 ± 1.3 g and 8.0 ± 1.5 cm) were purchased from Piscicultura Polettini, Mogi Mirim/SP, Braziland transported to the Histology Laboratory of UNESP, Campus Rio Claro, Sao Paulo, Brazil. The animals were previously climatized in polyethylene boxes (500 liters) with constant aeration and fed with appropriate commercial food (325 g of crude protein) once a day.

### 2.2. *B. dracunculifolia* Leaves

The *B. dracunculifolia* leaves used in this experiment were collected in Rio Claro-SP, Brazil (22˚22'30.0"S, 47˚28'31.5"W) and, after identification, exsiccates of the vegetal material were deposited and registered in the herbarium "Herbário Rioclarense", Botany Department, UNESP, Campus Rio Claro (number 58140).

### 2.3. Ethanolic Extract Preparation

The leaf compound extraction followed the protocol established by ANVISA—(Brazilian Health Surveillance Agency) for the *Preparation mother tinctures from dry plants through maceration*. The leaves were macerated with grain alcohol, 30% and 70% for nine days. The product of the 30% maceration was mixed to the 70% and vice-versa. After nine days, all the leaf compounds were obtained, those soluble in alcohol 70% and 30%. For each treatment group, 1.5 mL (amount ingested in treatment in folk medicine) of the extract was added to 1.2 g of commercial food (Poytara®). The material was kept in microbiological incubator at 37˚C for alcohol evaporation and stored in amber jars.

### 2.4. Control and Treatment Groups

Two experimental groups of 30 individuals were used, the experiment was made in duplicate. The control group (Ctrl) and *B. dracunculifolia* Treated group (BdT). The animals were randomly distributed into four 70-liter tanks (8 animals each) with air pumps, cooling, thermostat (to maintain constant temperature) and covered with UV blocking material to reduce stress. Both groups were fed for a maximum of 21 days: Control group with regular commercial food and *B. dracunculifolia* Treated group with the food added with *B. dracunculifolia* extract. The animals were collected 14 and 21 days after the experiment (21-day feeding period), 6 individuals per treatment were collected. The fish were kept in semi-static system (every day about 20% of the water was renewed) and the water physical and chemical parameters (pH, ammonia, hardness and temperature) were measured at each collection.

### 2.5. Histological Processing

Fragments of the liver were fixed in formalin 10%, transferred to sodium phosphate pH = 7.4, dehydrated in crescent ethanol series, included in Leica historesin and sectioned (6 μm thickness) using microtome Leica RM2245. The sections were subjected to specific reactions and mounted on slides. For lipofuscin, the slides were mounted using Entellan, without the need of specific reactions, once lipofuscin is fluorescent [17]. The material was analyzed using fluorescence microscope Olympus-BX51 and photographed using software DP-Controller, light filter 450 - 490 nm.

### 2.6. Hepatic Morphology Analysis

The morphological alterations were evaluated through semi-quantitative methods: Mean Value of Alterations (MVA) and Histopathological Alteration Index (HAI). The MVA was calculated based on the incidence of lesions, according to18where a numeric value is attributed to each animal according to the scale: 1 (absence of histopathological alterations), 2 (localized lesions), and 3 (widely distributed lesions) and the HAI was based on the severity of each lesion. [19]. The HAI value was calculated for each animal, according to the formula:

$$HAI = (1 \times \Sigma I) + (10 \times \Sigma II) + (100 \times \Sigma III)$$

where ΣI, II and III correspond to the number of the stages: I, II and III, respectively. The HAI values between 0 and 10 indicate normal tissue functioning; between 11 and 20 indicate mild damage to the organ; between 21 and 50 indicate moderate damage; from 51 to 99, severe damage and greater than 100 indicate irreversible tissue damage [18].

### 2.7. Hepatic Collagen Quantification

For collagen quantification, six liver sections of each individual were analyzed after Picrosirius red reaction, according to [20]. The collagen was isolated using software Image J® version 1.51p and the collagen total area was quantified.

### 2.8. Glycogen and Bile Stagnation Quantification

For the glycogen and bile stagnation quantification, six sections of each individual were analyzed following PAS reaction, according to the protocol stablished by [21]. Ten fields from each section were photographed, five for bile stagnation and five for glycogen. Glycogen was semi-quantitatively evaluated and bile stagnation was analyzed using software Image J®. The total bile stagnation area was quantified according to [22].

### 2.9. Lipofuscin Quantification

To analyze the amount of lipofuscin in the tissues, ten photographs of six liver fragments of each individual were taken, according to [23]. The images were analyzed using program ImageJ®, the lipofuscin granules were isolated and the total area was quantified.

### 2.10. Total Proteins and Macrophages

Total proteins detection was performed using six sections of each animal, which were subjected to Xylidine Ponceau reaction according to [24]. Macrophages detection was carried out subjecting the same number of sections to Gomori reaction [25]. Total proteins were analized using semi-quantitative method, and machophages were quantified using the software Image J® following [23].

### 2.11. Statistical Analysis

The data obtained through the analyses were submitted to Shapiro-Wilk to verify normality and to ANOVA/Tukey test to obtain parametric results. The groups that did not satisfy normality assumptions were submitted to Kruskal-Wallis/Dunn, with significance level $p < 0.05$. Statistic test was performed using software Bioestat 5.0® and Graph Pad Prism 5.0®.

### 2.12. Use of Experimental Animals

All experiments were performed in accordance with relevant guidelines and reg-

ulations and approved by the Ethics Committee on Animal Use (CEUA)—from Estadual University of São Pauolo, Rio Claro, license number 10/2017. Before the euthanize the animals were anesthetized with benzocaine solution (0.1 g of benzocaine in 1 mL of ethanol for each 100 mL of deionized water).

## 3. Results

### 3.1. Hepatic Morphology

The water parameters remained within the acceptable levels [23]. The liver of the specimens presented the following alterations: cytoplasmic vacuolation, nuclear hypertrophy, sinusoid capillary dilatation and congestion, and the relative frequency in which they occurred are displayed in Table 1. The most frequent alterations are displayed in Figure 1.

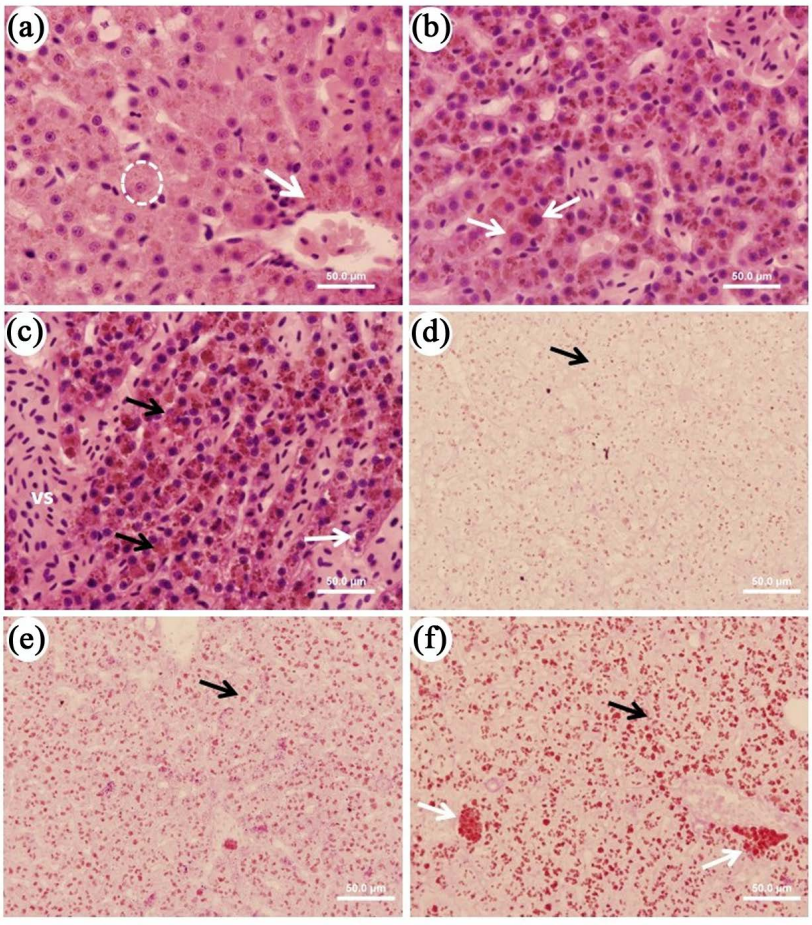


Figure 1. Most frequent alterations in the liver of *P. lineatus*. (a) Ctrl group 14 days: regular hepatic tissue, without significant alterations—central vein (arrow), hepatocyte (circle). (B) BdT group 14 days: nuclear hypertrophy (arrows). (C) BdT group 21 days: sinusoid capillary dilatation, congestion, bile stagnation (black arrow), cytoplasmic vacuolation (white arrow) and blood vessel (bv). HE technique. (D) Ctrl group 21 days: bile stagnation (arrow). (E) BdT group 14 days: bile stagnation (arrow). (F)BdT group 21 days: bile stagnation (black arrow) and melanomacrophage centers (white arrow)—note the increase in bile stagnation. PAS technique.

The MVA and HAI obtained for the hepatic alterations were significantly higher in comparison with the control in the 21-day feeding period—ANOVA/Tukey test (Figure 2).

### 3.2. Hepatic Collagen

Some collagen staining was observed in vessel walls, in insufficient amounts for quantification.

### 3.3. Bile Stagnation

The animals fed for 21 days presented significant increase in bile stagnation in comparison with the control group, with $p < 0.05$ for Kruskal-Wallis/Dunntest (Table 2).

### 3.4. Total and Proteins

The animals fed during both treatment periods presented significant difference of macrophage number, as well as of total proteins in comparison with the control group, with $p < 0.05$ for ANOVA/Tukey test.

### 3.5. Lipofuscin

Lipofuscin granules were identified as red punctuate cytoplasmic fluorescence. The number of granules in the liver increased in both treatments; however, not significantly in comparison with the control group (Figure 3), with $p < 0.05$ (Kruskal-Wallis/Dunn) (Table 2).

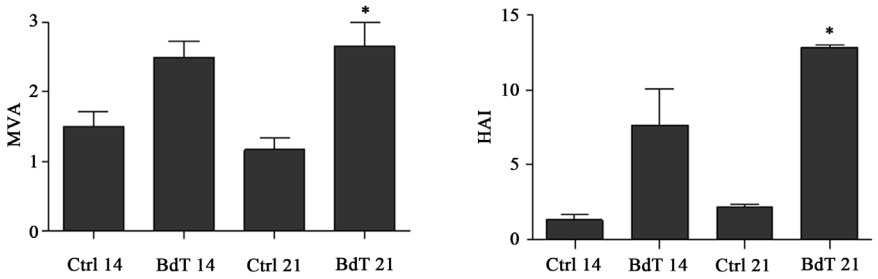


Figure 2. Mean MAV and HAI values of *P. lineatus* liver. Data expressed in mean ± standard error. Significant difference at $p < 0.05$. (*) significant difference in comparison with the control group.

Table 1. Frequency of histological alterations in the livers of *P. lineatus*. 0 = no alterations 0+ = rare alterations + = frequent ++ = very frequent +++ = extremely frequent.

| Liver | Stage | Ctrl 14 d | Ctrl 21 d | BdT 14 d | BdT 21 d |
|---|---|---|---|---|---|
| Cytoplasmic vacuolation | I | + | + | + | + |
| Nuclear hypertrophy | I | 0+ | 0+ | 0+ | 0+ |
| Sinusoidal capillary dilatation | I | 0+ | 0+ | + | ++ |
| Glycogen | I | + | + | + | + |
| Total proteins | I | + | + | ++ | ++ |
| Congestion | II | 0+ | 0+ | + | ++ |

Ctrl—Control group; BdT—*B. dracunculifolia* Treated group.

**Table 2.** Means in μm² of the area occupied by lipofuscin and bile stagnation. Means, standard deviation (S±) and significance (*). Note the increased levels of lipofuscin and bile stagnation in the BdT group.

| | LIPOFUSCIN | | | | BILE STAGNATION | | | |
|---|---|---|---|---|---|---|---|---|
| | Ctrl Group | | BdT Group | | Ctrl Group | | BdT Group | |
| | Mean | S± | Mean | S± | Mean | S± | Mean | S± |
| 14 days | 914041.2 | 472324.5 | 1066679.8 | 642860.6 | 3868.1482 | 2321.4600 | 4589.1319 | 1145.6950 |
| 21 days | 953439.2 | 402236.3 | 988093.9 | 530541.0 | 4834.8480 | 1158.5428 | 6479.3136* | 2205.9563 |

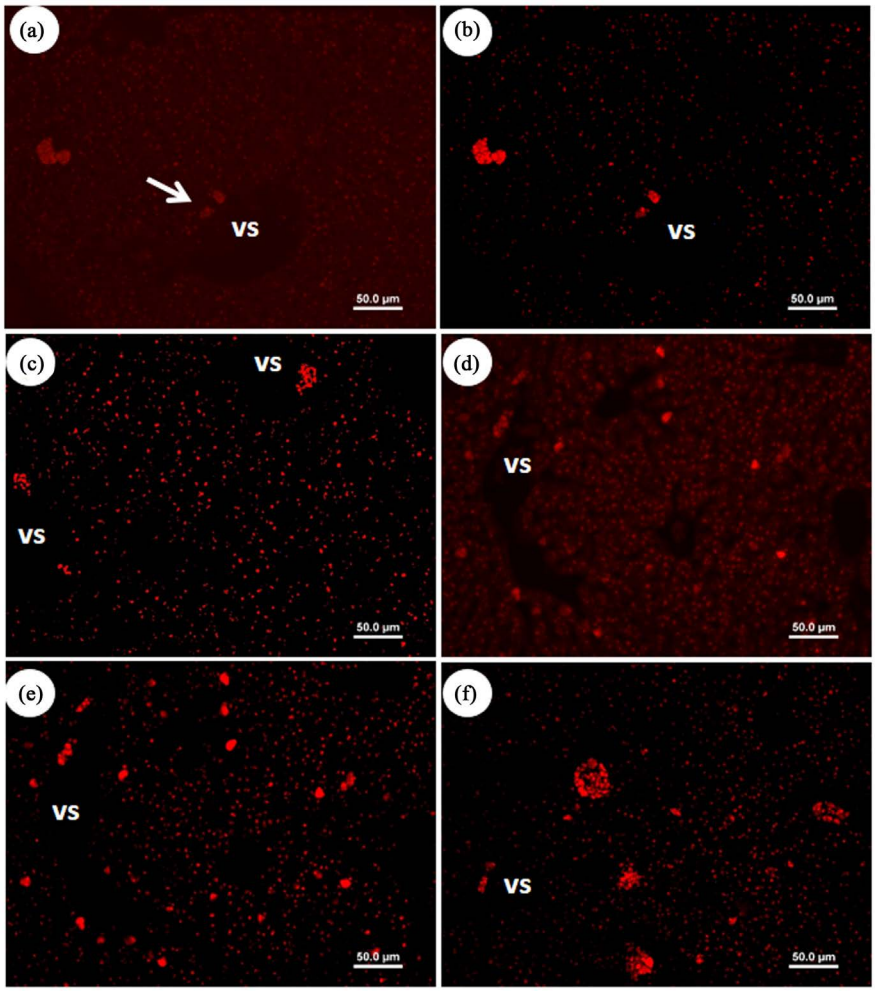


**Figure 3.** (a) and (b) Ctrl group liver 14 days after treatment—lipofuscin granule (arrow). (c) Liver of the Ctrl group on the 21st day after the experiment. (d) and (e) Liver of the BdT group, on the 14th day after the experiment. (f) Liver of the BdT group, on the 21st day after the experiment. Note the increase in lipofuscin in the BdT group. Fluorescence microscopy technique.

## 4. Discussion

Histopathological analyses are important to verify the sensitivity of the organs to toxic substances. Lesion severity is associated with the pathologic potential; therefore, how the lesion affects the organ functions and the survival of the animal is taken into consideration to analyze the importance of the lesion [26]. The

present study analyzed alterations stages I and II, cytoplasmic vacuolation, nuclear hypertrophy, sinusoid dilatation and congestion, the HAI revealed that the extract caused slight not statistically significant damages to the organ. The results suggest that *B. dracunculifolia* has toxic components, once some alterations were observed in more advanced stages, making the tissue recovery slower.

Several studies have reported an increase in the amount lipofuscin [27] [28] [29] [30]. Despite not statistically significant, the increase in the levels of lipofuscin observed in the present study can be associated with liver damages, once the lipofuscin is a product of lipid peroxidation and indicates oxidative lesion [31].

Alterations as nuclear hypertrophy and sinusoid dilatation, more frequent in the animals fed for 21 days, indicate an increase in the metabolic activity of the hepatocytes, probably representing a response to the presence of stressing agents. The presence of vessel congestion suggested that blood flow was obstructed, consequently causing blood to accumulate in the venous circulation. According to [32], this can be caused by physical obstruction of small or large vessels or by a failure in the regular flow.

One of the consequences of the exposure to toxic products is bile stagnation, characterized by the presence of brownish-yellow granules in the cytoplasm of the hepatocytes [33]. This alteration consists in the manifestation of a physiopathological condition caused by a lack of bile metabolism and excretion [34]. In the present study, the bile stagnation observed in the *P. lineatus* indicates that the animals were in contact with *B. dracunculifolia* metabolic products, which acted as toxic agents. Furthermore, the presence of melanomacrophage centers—which play a role in elimination of particles—may indicate inflammation [35], health problems and conditions of environmental stress [36].

Changes in the number of macrophages were also observed in the liver, gills and intestine of *P. lineatus* [23] [37] [38]. Alterations in total proteins levels in this study could be occurred due to the health conditions of the animals [39]. According to these authors, when total proteins are at high levels, it may represent a chronic liver disease, and when at low levels, it could be a result of liver failure and kidney disease.

The results of the present study are corroborated by [40], who submitted rats to high concentrations of *B. dracunculifolia*. Three days following exposure the animals presented behavioral alterations and the toxicity of the extract was confirmed by the decrease in polychromatic and monochromatic erythrocytes. [13] reported that the hydroethanolic extract of *Baccharistrimera* administered to pregnant rats at 8.4 mg/kg was toxic to maternal kidney and liver cells, although such alterations are reversible once administration is discontinued.

Studies have demonstrated that plant flavonoids, such as quercetin and rutin [41] can produce genotoxic effects in high concentrations [41] [42] [43]. The caffeic acid, a phenolic acid found in *B. dracunculifolia* extracts [44] [45], induced damage to the DNA of rats at 8 mg/kg [43]. The molecular mechanisms of mutagenicity caused by flavonoids have not been clarified; however, several stu-

dies have demonstrated that they can act as pro-oxidants, overcoming nuclear antioxidant defenses and leading the DNA to oxidative damage [41] [46]. Therefore, the liver alterations observed in the present study may have occurred due to the action of similar components present in the *B. dracunculifolia* extract.

## 5. Conclusion

In conclusion, the extract of *B. dracunculifolia* caused significant hepatic alterations in the species; moreover, the HAI and MAV demonstrated that the ingestion of the extract caused widely distributed damage to the liver.

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## Authors' Contribution

Wrote the main manuscript text:Jeffesson de Oliveira-Lima, Bruno Fiorelini Pereira, João Rodolfo Tuckumantel Valim.

Worked on graphics, statistics and figures: Thiago Gazoni, Dimitrius Leonardo Pitol, Flavio Henrique Caetano.

## Conflicts of Interest

The authors declare no conflict of interest.

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