

- 1 **The aqueous extract of *Bellucia dichotoma* Cong. has a protective effect on mice**
- 2 **spleens after envenomation by *Bothrops atrox*: a stereological study**
- 3

1
2
3 **ABSTRACT**

4 The aqueous extract of *Bellucia dichotoma* (AeBd) has traditionally been used in cases
5 of snakebites and has been shown to be promising against the effects of *Bothrops atrox*
6 venom (BaV). There is evidence of local venom blocking action by AeBd in
7 envenomed mice; however, studies of the systemic action of this plant against BaV are
8 lacking. Antithropic serum (ABS) is the conventional therapy after envenomation by
9 *B. atrox*. The association of AeBd and ABS can also be an alternative for the patient. As
10 an organ capable of initiating the innate and adaptive defense, the spleen is a sensor of
11 the body's immune status. Via stereology, this study evaluated the mouse spleen when
12 challenged with the venom of *B. atrox*, as well as the role of AeBd (alone or in
13 association with ABS) as an alternative treatment for *Bothrops* envenomations. In this
14 study, male Swiss mice were divided into five groups: control, BaV, AeBd, AeBd/ABS,
15 and ABS. After 24 h, the spleens were removed and set in plastic resin according to the
16 norms of stereology to produce 9-13 sections/organ. Red (RP) and white pulp (WP)
17 represented 67-75 % and 22-29 %, respectively, in all groups. Spleen volume was
18 reduced in BaV due to decreased RP and WP (PALS). The number of macrophages was
19 increased in BaV in relation to the other groups (from 2.289 ± 301 in the control to
20 3.807 ± 494 cells.mm⁻²). No changes were detected in the numbers of lymphocytes and
21 megakaryocytes. In conclusion, the aqueous extract of *B. dichotoma* administered orally
22 shortly after a *B. atrox* envenomation was effective in inhibiting morphological
23 alterations in the spleen of the mice. The association AeBd/ABS maintains the same
24 venom blocking effectiveness, thus showing that the use of this traditional therapy
25 (AeBd) by local populations can be effective in inhibiting the systemic effects caused
by *B. atrox* envenomations.

1 Keywords: *Bothrops atrox*, *Bellucia dichotoma*, aqueous extract, herbal medicine,
2 spleen, stereology.

3

4 **Introduction**

5 Bites caused by snakes are considered a serious public health problem and, every
6 year, they cause thousands of deaths or serious sequelae in the victims. In 2017, the
7 World Health Organization added snakebites to the list of neglected tropical diseases,
8 estimating that annually 1.8-2.7 million people are envenomated by snakes worldwide,
9 resulting in between 81,000 and 138,000 deaths and 400,000 survivors with permanent
10 sequelae (Longbottom et al., 2018). In Brazil, an estimated 26,000 cases of snakebite
11 envenoming occur every year. Many of the victims are rural workers, fishers,
12 extractivists, indigenous people or other groups who live in close proximity to the forest
13 (Schneider et al., 2021). The northern region of the country has the highest incidence of
14 cases per 100,000 inhabitants (Zemero et al., 2023).

15 Most of the envenomations that occur in the Amazon region are attributed to the
16 species *Bothrops atrox*, popularly known as the jararaca, white-tailed jararaca,
17 surucucurana and jipoboia (Cardoso et al., 2009; Waldez and Vogt, 2009; Wen et al.,
18 2015). The venom of this snake has a hemolytic, edematous and necrotizing effect. The
19 use of antiophidic serum is the conventional therapy for treating injured victims;
20 however, access to this type of treatment in most cases, is a privilege of the population
21 living close to urban centers. In the Amazon, great distances separate the native
22 population from this the centers that provide this type of treatment, and this is the
23 reason why the use of alternative treatments with natural or herbal products is so
24 widespread (Bochner et al., 2014; Waldez and Vogt, 2009).

25 Medicinal plants are frequently used in folk medicine around the world as pre-
26 and post-treatments against envenomations by snakes. *Bellucia dichotoma* Cogn. is a
27 species that is endemic to the Amazon region and, in Brazil, it is found in the states of
28 Acre, Amazonas, Amapá and Pará, and is popularly known as muúba or goiaba-de-anta
29 (Baumgratz, 2013). The population uses the bark to prepare a tea before entering the
30 forest, as a preventive measure and it is also given to victims after snakebite. The main
31 classes of substances found in the aqueous extract of *B. dichotoma* bark are fatty acids,
32 flavonoids, terpenes, hydrolyzable and condensed tannins (Moura et al., 2013). This

1 extract has already been shown to be effective in inhibiting the hemorrhagic,
2 phospholipasic, edematogenic and coagulant effects in rats after envenomation by
3 *Bothrops jararaca* and *B. atrox* snakes (Moura et al., 2015; Moura et al., 2013). In
4 mice, the aqueous extract of *B. dichotoma*, given orally as traditionally used,
5 significantly reduced paw edema in the first 30 minutes after *B. atrox* envenomation and
6 its full anti-edematogenic action was observed after 6 hours of treatment (Moura et al.,
7 2014). More positive results were obtained with the simultaneous use of the extract
8 (orally) and antiothropic serum (intravenously) (Moura et al., 2014). These results are
9 promising for the protective effect of the *B. dichotoma* extract in other sites, such as the
10 spleen, for example.

11 The spleen is the largest secondary lymphoid organ and is responsible for
12 immune surveillance, immune response to circulating antigens, hematopoiesis, and
13 blood filtration (Cesta, 2006; Mebius and Kraal, 2005; Steiniger, 2015). The splenic
14 parenchyma is divided into white and red pulp. The white pulp is responsible for
15 initiating immune responses to blood antigens, as it contains a quarter of the body's
16 lymphocytes and has other phagocytic cells that are important in the immune response
17 (*e.g.*, macrophages, neutrophils and dendritic cells). The white pulp is composed of a
18 periarteriolar lymphoid sheath that is rich in T lymphocytes (PALS), follicles (B
19 lymphocytes) and a marginal zone (B lymphocytes) (Cesta, 2006). Macrophages are
20 categorized into different subsets based on their location, function, and phenotype.
21 Blood arriving in the spleen via the splenic artery follows two parallel paths: the
22 closed/rapid circulation (inside vessels such as the central arterioles and their branches
23 surrounded by the PALS of the white pulp) and then follows through post-sinusoid
24 venules that converge in the splenic vein; the open/slow circulation goes through the
25 splenic cords and sinuses of the red pulp where macrophages and reticular cells filter the
26 blood cells before they return to the general circulation. Defective cells and antigens are
27 phagocytosed by macrophages or dendritic cells (Steiniger, 2015).

28 Several studies have shown that alterations occur in the spleen after snake
29 envenomations and the subsequent use of medicinal plants (Almeida et al., 2020;
30 Goorani et al., 2020; Zangeneh, 2020). Pathological alterations indicate congestion,
31 hemorrhage, extramedullary hematopoiesis and necrosis in the most severe cases
32 (Raposo et al., 2000; Silva et al., 2012; Venkatesan et al., 2014). Thus, this study
33 quantitatively evaluated the spleen morphology of mice challenged with *Bothrops atrox*

1 venom, as well as the role of the extract of *Bellucia dichotoma* (alone or in association
2 with antiothropic serum) in the preservation of splenic components.

3

4 **Materials and methods**

5 **Experimental animals**

6 Male Swiss mice (N=17, 34-41 g) obtained from the Animal Facility of the
7 Federal University of Oeste do Pará (UFOPA), Santarém, Pará, Brazil were used. This
8 study was approved by the Ethics Committee on the use of Animals at the University of
9 the State of Pará (UEPA/Protocol 43/11), Santarém, Pará, Brazil. The animals were
10 maintained in standard cages at a temperature of 22 ± 1 °C, in a 12 h light/12 h dark
11 cycle, with water and feed *ad libitum*.

12

13 **Acquisition of *Bothrops atrox* venom and antiothropic serum**

14 The venom was obtained by squeezing the glands of adult *Bothrops atrox*
15 (Linnaeus, 1758) (Viperidae) snakes obtained in the Tapajós National Forest (FLONA),
16 located at km 83 of the BR-163 highway, in Santarém, Pará, Brazil. Both the collection
17 of the snake and the extraction of the venom were permitted by the Chico Mendes
18 Institute for Biodiversity Conservation (ICMBio), via the Biodiversity Authorization
19 and Information System (SISBIO – No. 14018). The venom was collected, then
20 lyophilized and kept at -20 °C until use (permission to keep snakes in the vivarium of
21 Faculdades Integradas do Tapajós (FIT), Santarém, PA, was obtained from the National
22 Council for Control of Animal Experiments - CONCEA No. 26/2013). In this study, the
23 antiothropic serum (ABS) produced by Instituto Butantan, São Paulo, Brazil, (batch
24 number: 105113B) was used.

25

26 **Aqueous extract of the bark of *Bellucia dichotoma***

27 The aqueous extract of the bark of *B. dichotoma* (Melastomataceae) (AeBd) was
28 prepared in accordance with the methods used by the residents of the Eixo Forte
29 communities in the western region of Pará, Amazon, Brazil (Access to associated

1 traditional knowledge, process No. 01450.008934/2014-68-DPI/IPHAN). In summary,
2 50 g of powdered *B. dichotoma* bark was diluted in distilled water (1:10) maintained at
3 a constant temperature of 100 °C. After cooling, 150 mL was removed, which is the
4 equivalent to a cup of tea, as used locally. This was then lyophilized and the final dry
5 weight of the extract obtained was 2.9 g.

6

7 **Experimental design**

8 The mice were randomly distributed in standard cages and submitted to one of
9 the following treatments for 24 h: Control – subplantar injection of 0.9% saline solution
10 (n=3); BaV – *B. atrox* venom injection (5 µg, equivalent to two minimal edematogenic
11 doses). The venom was injected into the footpad of the right hind paw of the mice in a
12 constant volume of 50 µL (n=4); AeBd – oral administration of aqueous extract of *B.*
13 *dichotoma* (283.30 mg/kg), immediately after injection of *B. atrox* venom (n=3);
14 AeBd/ABS – AeBd + antiothropic serum (ABS) via ophthalmic venous plexus (100
15 µL), after injection of *B. atrox* venom (n=3); ABS – injection of antiothropic serum
16 (ABS) via ophthalmic venous plexus (100 µL), after injection of *B. atrox* venom (n=4).

17

18 **Spleen removal and volumetry**

19 After 24 hours, the animals were sacrificed by displacement of the cervical spine
20 and the spleen was removed, which was then weighed on an analytical balance
21 (Shimadzu AY220, Japan) and fixed in buffered formalin for 48 hours.

22 Two procedures were adopted to determine the absolute volume of the spleen:
23 (i) fluid displacement (Scherle, 1970) and (ii) serial sections according to the Cavalieri
24 principle (Howard and Reed, 2005). The Cavalieri principle is described in the
25 stereological section. Both approaches allow one to obtain the absolute volume, but they
26 differ in terms of precision and accuracy of results and in terms of their practicality of
27 application. In the first approach, the intact organs were individually submerged in a
28 container with the same fixative solution using a thin copper wire (Fig. 1A). The organ
29 was fully immersed in the solution, but without touching the bottom and sides of the
30 container. The set was then placed on an analytical balance (Shimadzu AY220, Japan).

1 Before immersion, the balance was reset and the value observed on the electronic panel
 2 after immersion represented the organ volume without fluid density correction (Scherle,
 3 1970). For density correction, the following equation was used: $V = \frac{W}{\rho}$, where V , organ
 4 volume; W , weight of the immersed organ and ρ is the density of the fluid
 5 (formaldehyde solution = 1.060 g.cm³).

6

7 **Histological processing and stereology**

8 The spleens were cut in half and the systematic, uniform and random orientation
 9 was obtained using the orientator (Mattfeldt et al., 1985). This procedure allows one to
 10 obtain sections with high variability in the arrangements of structure profiles, which
 11 ensures a reduction in the sampling bias. Then, the spleens were dehydrated in
 12 increasing concentrations of ethanol (70 and 96%), pre-infiltrated with 96%
 13 ethanol/plastic resin, placed in individual molds and infiltrated with hydroxyethyl-
 14 methacrylate plastic resin (Technovit 7100, K lzer-Heraues, Germany). The molds were
 15 left in a heated oven at 40  C for 24 h for the complete polymerization of the resin (Fig.
 16 1B). The total length of the two halves in the resin block was determined and this value
 17 divided by 9-13, depending on the length of the spleen (Fig. 1B). The microtomy was
 18 then performed (Leica RM 2145, Germany). The sections were stained with 0.5%
 19 Toluidine Blue (Toluidine Blue, 0.12 g; Na⁺ borate, 0.5g; distilled H₂O, 100 mL; for 30
 20 seconds) and Basic Fuchsin (Basic Fuchsin, 0.5 g and distilled H₂O, 100 mL; for 2
 21 seconds) and then photographed (magnification at 100x) under a light microscope
 22 (Leica DM4B, Germany). A grid was superimposed on each image containing points
 23 generated using the Imod software (version 4.7/stereology module) (Kremer et al.,
 24 1996). The analysis consists of counting random points that are overlaid on the image;
 25 those that touch the spleen are counted (Fig. 1C).

26 The volume of the spleen was determined by:
 27 $V_{spleen} = \sum_{i=1}^m Pi \times T \times \frac{a}{p}$, where, $\sum Pi$ is the total number of points on each spleen,
 28 $\frac{a}{p}$ is the area represented by each point (44,100  m²) and T (1,000  m) is the distance
 29 between each serial section. An error limit of 5% was considered acceptable (Gundersen
 30 and  sterby, 1981).

1 The percentage of internal components (volume density) was obtained using
 2 Delesse's principle (Howard and Reed, 2005). A magnification of 400x was used in the
 3 randomly selected fields of view, which were photographed under a light microscope
 4 (Leica DM4B, Germany) (Fig. 1D). The percentage of volume occupied by each
 5 component in relation to the reference space (splenic parenchyma) was calculated using
 6 following equation: $Vv = \frac{\sum_{i=1}^m P_{comp}}{\sum_{i=1}^m P_{ref}}$, where Vv is the volume density of a given
 7 component (red pulp, RP; periarteriolar lymphoid sheath, PALS; follicles; marginal
 8 zone, MZ; trabecula; capsule) and P_{ref} is the sum of points that touch the reference
 9 space. The white pulp (WP) represented the sum of PALS + follicles + MZ. The
 10 percentages obtained for each component were transformed into absolute volume by
 11 being multiplied using the Cavalieri volume:

$$\text{Absolute volume (mm}^3\text{)} = Vv \times \text{Cavalieri Volume (mm}^3\text{)}$$

12

13 **Number of cell profiles**

14 A two-dimensional quantification was used to determine the number of profiles
 15 of megakaryocytes, lymphocytes and macrophages in the marginal zone. For this, a grid
 16 containing four lines delimiting a counting frame was used (Fig. 2). The technique
 17 consists of counting the cell nuclei contained in the counting system. The following
 18 equation was used:

$$Q = \frac{\sum Q}{(\sum Nframes \times \sum Aframe)}$$

19 Where: $\sum Q$ is the number of cell profiles; $\sum Nframes$ is the sum of analyzed
 20 frames and $\sum Aframe$ is the frame area ($261 \times 10^{-6} \text{ mm}^2$).

21

22 **Statistical analysis**

23 The Prism program (GraphPad Software, Inc., CA, USA) was used for the
 24 statistical and graphical analysis of this study. Data were tested for normality using the
 25 Kolmogorov-Smirnov test and analyzed using one-way ANOVA. In cases in which the
 26 difference between groups is indicated, Tukey's parametric test of multiple comparisons
 27 was used to compare the mean values between the experimental groups. The confidence
 28 limit established for the tests was 5%. The stereological data evaluated for each animal
 29 and the variance estimator was determined using the coefficient of error according to
 30 **Cruz-Orive (1999)**:

1
$$CE = \left[0.0724 \times \frac{B}{\sqrt{A}} \times \frac{\sqrt{n}}{(\sum_{i=1}^m Pi)^{\frac{2}{3}}} \right]^{\frac{1}{2}}$$
, where: CE indicates the coefficient of
 2 error, $\frac{B}{\sqrt{A}}$ indicates the variance of the cross-sectional areas (shape coefficient) and
 3 depends on the complexity of the structures; n represents the number of evaluated
 4 sections and $\sum_{i=1}^m Pi$ is the number of points counted in the sections.

5

6 **Results**

7 There was no significant difference in the spleen volume when measured using
 8 the Cavalieri principle and when using fluid displacement (Fig. 3A). The higher values
 9 obtained using fluid displacement may be related to the presence of extra splenic tissues
 10 that increase the total volume of the organ. The RP was the most representative
 11 component in all the treatments (67-75%), followed by WP (22-29%), capsule (0.8-
 12 2.9%) and trabecula (0.7-1.0%) (Fig. 3B).

13 The spleen mass and the spleen/body mass ratio showed a non-significant
 14 decrease in the BaV group (Fig. 4A and B). The spleen volume (Cavalieri, $\text{mm}^3 \text{kg}^{-1}$)
 15 was reduced in the BaV group compared to the other treatments (Fig. 4C). The decrease
 16 in the volume of red pulp (Fig. 4D) in the BaV group compared to the control and white
 17 pulp (Fig. 4E), in relation to the other treatments, explains the reduction in splenic
 18 volume. PALS was reduced in the BaV group (Fig. 4F). No changes were observed in
 19 the follicles (Fig. 4G) or in the marginal zone (Fig. 4H). Neither were there any changes
 20 in the trabecular connective component (Fig. 4I), but the capsule was increased in
 21 AeBd/ABS group in relation to the BaV group (Fig. 4J).

22 The number of macrophages was increased in the marginal zone of the BaV
 23 group when compared to the other treatments (Fig. 5A). There was no significant
 24 difference in the number of lymphocytes in the same region (Fig. 5B) or in
 25 megakaryocytes in the red pulp (Fig. 5C). The structural morphology of the spleen was
 26 maintained in all treatments. The spleen presented a typical structure with a distinct red
 27 and white pulp (Fig. 5D). Splenic cords were seen bordering splenic sinusoids in the red
 28 pulp. The white pulp presented well-defined PALS delimited by a marginal zone
 29 bordering the red pulp (Fig. 5E). Megakaryocytes were frequently observed in the
 30 red pulp.

1 Figure 6 shows the interaction between the components of the spleen and their
2 relationship with the total volume of the organ. In this interpretation, it is possible to
3 observe the relative distance between the BaV group and the others. This treatment was
4 characterized by a smaller volume of white and red pulp, resulting in a smaller volume
5 of the spleen. The AeBd and AeBd/ABS groups showed comparable effect to the
6 control and the ABS.

7 Figure 7A-E shows the influence of the number of sections on the accuracy of
8 the volume determination using the Cavalieri principle. The spleen volume in the
9 different treatments starts to stabilize after the 12th serial section. However, a coefficient
10 of error below 5% can already be observed from the 6th section onwards. These results
11 indicate that efficiency and precision in this study were achieved with 12 histological
12 sections. Less is imprecise, more is inefficient.

13

14 **Discussion**

15 Antiophidic therapy based on popular knowledge is widely used to alleviate the
16 damage caused by snakebites in the Amazon. *Bellucia dichotoma* is traditionally used as
17 an antidote for snakebites and for various other ailments. Our study revealed that the
18 acute action of BaV reduced the volume of RP and WP, specifically by reducing PALS,
19 thus contributing to the reduction in spleen volume. Furthermore, snake venom
20 increased the number of macrophages in MZ. The extract of *B. dichotoma* inhibits the
21 effects of *Bothrops atrox* venom, and preserves the splenic microstructures and
22 probably the spleen's function. The combination of the extract of *B. dichotoma* and the
23 antithrotophic serum has the same splenic protective effect.

24 The spleen histology of the animals in this study is seen to be in agreement with
25 the literature (Cesta, 2006). The spleen volume of the mice (34-41 g), in this study
26 assessed by point counting on serial sections (40-58 mm³, Cavalieri volume) and by
27 fluid displacement (49-90 mm³), is in accordance with the volume specified by optical
28 contrast CT (44 -61 mm³) on six-week-old female Balb/c mice (McErlean et al., 2015)
29 and by stereology (35 mm³, Cavalieri volume) on Balb/c male mice (25-30 g) (Vojdani
30 et al., 2010).

1 Approximately 75% of the spleen is RP (Fig. 3B), which consists of connective
2 tissue, arterioles, capillaries, sinusoids, venules and blood cells (Steiniger, 2015). The
3 reduction of RP in the BaV group, and probably the impairment of the
4 filtering/hematopoietic function of the spleen, may be associated with (i) the hemolytic
5 action of metalloproteinases and serineproteases (Gutiérrez, 2002) associated with the
6 effect of an impairment of the filtering/hematopoietic function by the consumption of
7 endogenous fibrinogen that exacerbates the hemorrhagic condition (Gutiérrez, 2002;
8 Gutiérrez and Rucavado, 2000) and (ii) the direct action of the venom on the splenic
9 structure causing cell death. In the study by Navarro et al. (2014), *Crotalus atrox* venom
10 (0.5-1.0 µg, peritoneal injection) showed direct cytotoxic activity in the splenocytes of
11 CD-1 mice (females, 6-8 weeks old, 20-25 g), in addition to an increase in the
12 expression of inflammatory cytokines.

13 Snake venoms cause hemorrhage, congestion, autophagy, splenomegaly and,
14 rarely, necrosis of the spleen (Raposo et al., 2000; Silva et al., 2012; Venkatesan et al.,
15 2014). An analysis of the paws inoculated with the venom revealed a high percentage of
16 hemorrhage (unpublished data), which indicates the action of proteases in coagulopathy,
17 cytotoxicity (especially phospholipases A₂) and in the degradation of the components of
18 the extracellular matrix of the vessels, which promotes the extravasation of blood cells
19 to adjacent tissues. Under such circumstances, a state of splenomegaly (as mentioned
20 before), with increased red pulp as an indication of activation of extramedullary
21 hematopoiesis and antigen filtration, is common (Silva et al., 2012). Our results are in
22 opposition to this scenario possibly due to the acute aspect of the envenomation
23 protocol employed. These observations suggest that damage to the spleen is progressive
24 after venom inoculation and that in the first 24 h after the inoculation morphological
25 alterations are less severe. A similar condition was reported by Moreno and Gutiérrez
26 (1988), whereby the spleens of mice envenomed by *Bothrops asper* showed no
27 significant histological alterations in the RP after 24 h. More severe alterations such as
28 congestion and splenic hemorrhage have been reported in horses after *Bothrops sp*
29 envenomations (>24 h) (Raposo et al., 2000).

30 The *Bothrops* venom was responsible for the reduction in PALS and,
31 consequently, for the decrease in the volume of WP, possibly due to its cytotoxic action
32 (Fig. 4E-F). WP is responsible for initiating immune responses to blood antigens, as it
33 contains a quarter of the body's lymphocytes, in addition to other important phagocytic

1 cells (macrophages, neutrophils, and dendritic cells). These alterations can affect
2 lymphocyte trafficking and the plasma cell development that takes place in PALS. The
3 increase in BaV macrophages may be related to phagocytosis of toxic compounds from
4 the venom and cell debris from cell death. Macrophage activation is directly related to
5 the production of pro-inflammatory cytokines (Cesta, 2006). Increased numbers of
6 splenic follicular macrophages were also observed in rabbits after envenomation by
7 *Vipera raddei*, an indication of moderate inflammation and complement system
8 activation (Aznaurian and Amiryan, 2006).

9 Our results showed that the combination of AeBd/ABS promoted a significant
10 increase in the splenic capsule, possibly providing greater physical protection to the
11 organ against rupture. The dense fibroelastic nature and the presence of smooth muscle
12 cells in the capsule make it a tough yet flexible scaffold enabling contraction and release
13 of blood cells into the circulation (Kai et al., 2000). However, recent studies indicate
14 that, although mouse splenic capsules are extensively innervated, there is no evidence
15 that they can contract, as occurs in rats (Seifert and Offner, 2018) and humans (Lodin-
16 Sundström and Schagatay, 2010). The role of catecholaminergic innervation appears to
17 be related to controlling immune cell death (Lodin-Sundström and Schagatay, 2010). In
18 our study, the trabeculae in the AeBd/ABS group also showed an increase, although it
19 was not statistically significant. The lowest mean values for the capsule, seen in the
20 AeBd group, should be the focus of future investigations.

21 A number of extracts and their isolated compounds have been evaluated for their
22 antiophidic capacity (Félix-Silva et al., 2017; Fung et al., 2009; Gopi et al., 2015). In
23 our study, the AeBd and AeBd/ABS treatments were effective in preserving splenic
24 components. A possible explanation lies in the ability of the aqueous extract of the bark
25 of *B. dichotoma* to block the activity of phospholipases A₂ or PLA₂ (Moura et al., 2013)
26 and metalloproteinases Zn⁺⁺/dependent (Moura et al., 2014). The blocking action of the
27 extract are believed to be related to the presence of polyphenolic compounds and
28 tannins that precipitate the venom proteins and form a complex with Zn⁺⁺ and Ca⁺⁺,
29 which inhibits the enzymatic activity (Moura et al., 2014; Moura et al., 2013).

30 In several recent studies, stereology has been used to investigate the medicinal
31 value of plants traditionally used in folk medicine. The study by Jena et al. (2016)
32 showed the protective role of *Prunus africanus* in benign prostatic hyperplasia in rats.
33 *Allium sativum* and *Nigella sativa* also protected the duodenal mucosa of chickens

1 (Aydogan et al., 2020). CCl₄ hepatotoxicity was reduced in mice treated with *Sophora*
2 *alopecuroides* (Doorgard and Pournaghi, 2018). The use of stereology for investigating
3 the effect of plant extracts on the spleen has shown that *Spinacia oleracea*
4 (nanocapsules) reduces myeloblast infiltration in leukemic mice (Zangeneh, 2020),
5 *Allium eriophyllum* (nanocapsules) plays an immunoprotective role on the white and red
6 pulp during hemolytic anemia in mice (Goorani et al., 2020) and *Athenaea velutina*
7 increases red pulp (improving hematopoiesis) in cancer-induced mice (Almeida et al.,
8 2020). In our study, stereology was efficient and accurate for determining the total
9 volume of the spleen and its components. The use of 9-13 sections obtained throughout
10 the organ was sufficient in order to determine the volumes, with an error of below 5%.
11 These data support the precision of our analysis and make it clear that the use of few (<
12 6) and non-serial sections is highly risky and may compromise the study, leading the
13 researcher to draw erroneous conclusions based on the obtained quantifications.

14 Conclusion- This study showed that the aqueous extract of *B. dichotoma*,
15 administered orally soon after inoculation with *B. atrox* venom, had a splenic-protective
16 effect. The association of the extract with the antithrombotic serum maintains the same
17 blocking effectiveness, thus showing that the use of this therapy, which is used by local
18 populations, can be effective in inhibiting the local (de Moura et al., 2017; Moura et al.,
19 2014) and systemic effects caused by *B. atrox* venom.

20

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5

1 **Figure Legends**

2

3 **Figure 1. A.** Direct determination of volume using fluid displacement on an analytical
 4 balance. **B.** Application of the Cavalieri principle by counting points on the serial
 5 sections. Organs were sectioned in halves, set in plastic resin and oriented randomly.
 6 The total length of the two halves (L1 and L2) was added and divided by 9-13
 7 (equivalent to the number of sections to be obtained). **C.** Counting system containing
 8 crosses superimposed on each of the sections. A magnification of 100x was used to
 9 increase counting accuracy. For this, the counting system was moved along the entire
 10 section (in this example, 10 points are countable). The area denoted as x/y in the
 11 counting system indicates the area per point ($a/p = 44,100 \mu\text{m}^2$), an essential factor for
 12 transforming points into volume. **D.** Diagram of the randomly selected field seen at
 13 higher magnification with details of the counting system for determining the percentage
 14 of components in the spleen using the Delesse principle.

15 **Figure 2. A.** Diagram of the spleen region superimposed with a counting system
 16 containing a frame of defined dimensions. This system was used for counting
 17 megakaryocyte profiles (m1, m2 and m3), lymphocytes and macrophages. The system
 18 has solid lines (permitted line) and dashed lines (exclusion line). If the nucleus of the
 19 cell in question is seen within the frame or touching the continuous line, it is counted
 20 (*e.g.*, m2 and m3). However, if the nucleus is outside the frame or touches the dashed
 21 line, it is not counted (*e.g.*, m1). **B.** Counting system with defined area for counting
 22 lymphocytes and macrophages. Red pulp, RP; white pulp, WP; MZ, marginal zone.

23 **Figure 3. A.** Spleen volume determined using the Cavalieri principle (left) and fluid
 24 displacement (right). Data in mm^3 . **B.** Percentage of splenic components. Red pulp, RP;
 25 white pulp, WP; trabecula, TRA; capsule, CAP.

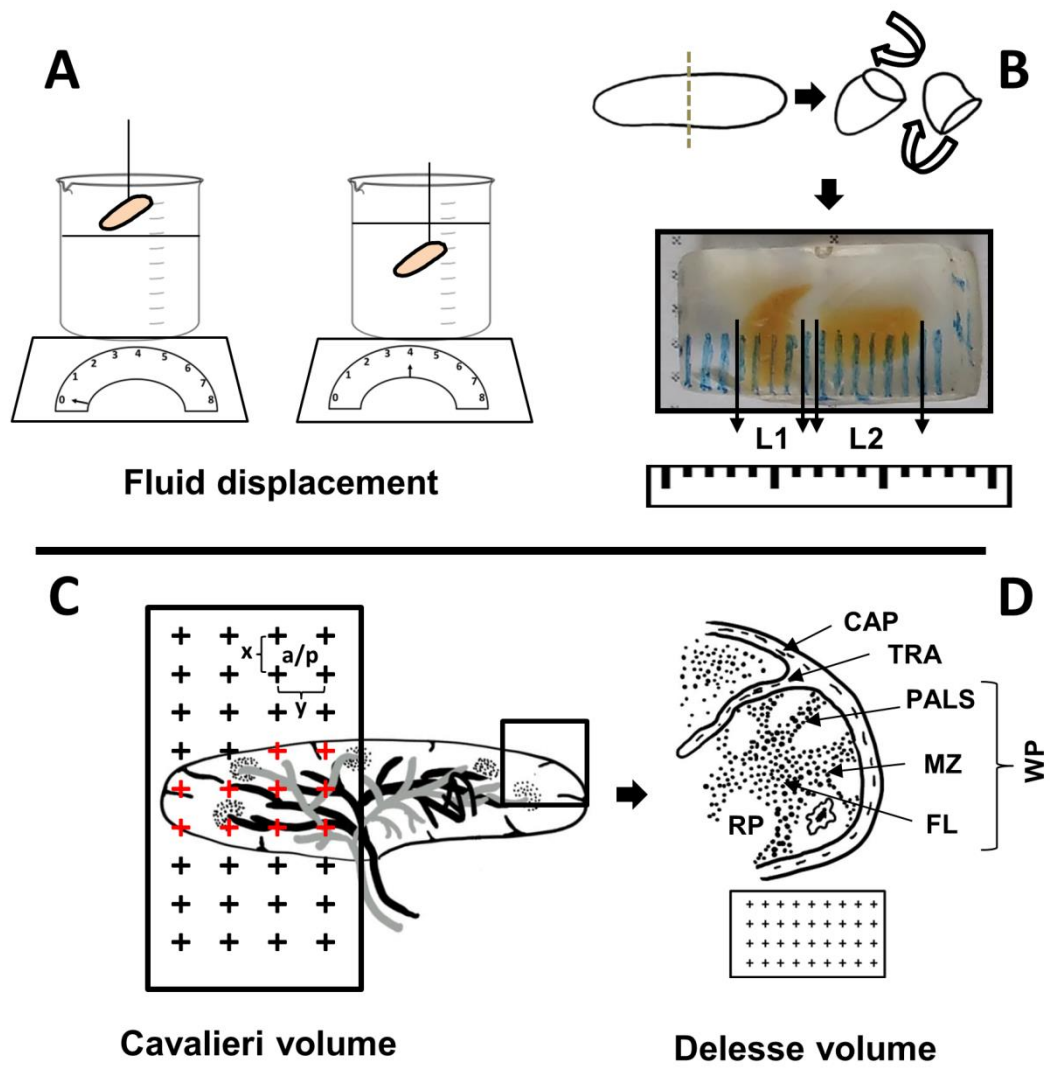
26 **Figure 4. Biometrics and stereology (absolute volumes). A.** Spleen mass. **B.** Spleen
 27 mass and body mass ratio. **C.** Cavalieri volume. **D.** Red pulp volume, RP. **E.** White pulp
 28 volume, WP. **F.** Periarteriolar lymphoid sheath volume, PALS. **G.** Follicle volume. **H.**
 29 Marginal zone volume, MZ. **I.** Trabecula volume. **J.** Capsule volume. Statistical
 30 difference indicated (One-way ANOVA, Tukey test).

1 **Figure 5.** Cell number and histology. **A.** Macrophages. **B.** Lymphocytes. **C.**
2 Megakaryocytes. **D.** Red pulp. Top detail: entire section of the spleen revealing its main
3 components. Lower detail: megakaryocytes. **E.** White pulp. CAP, capsule; TRA,
4 trabecula; RP, red pulp; WP, white pulp; FL, follicle; PALS, periarteriolar lymphoid
5 sheath; MZ, marginal zone; SC, splenic cords; SS, splenic sinusoids. Statistical
6 difference indicated (One-way ANOVA, Tukey test).

7 **Figure 6.** Trilinear diagram of the interaction between the major components of the
8 spleen. Note the separation between the BaV group and the other treatment groups.

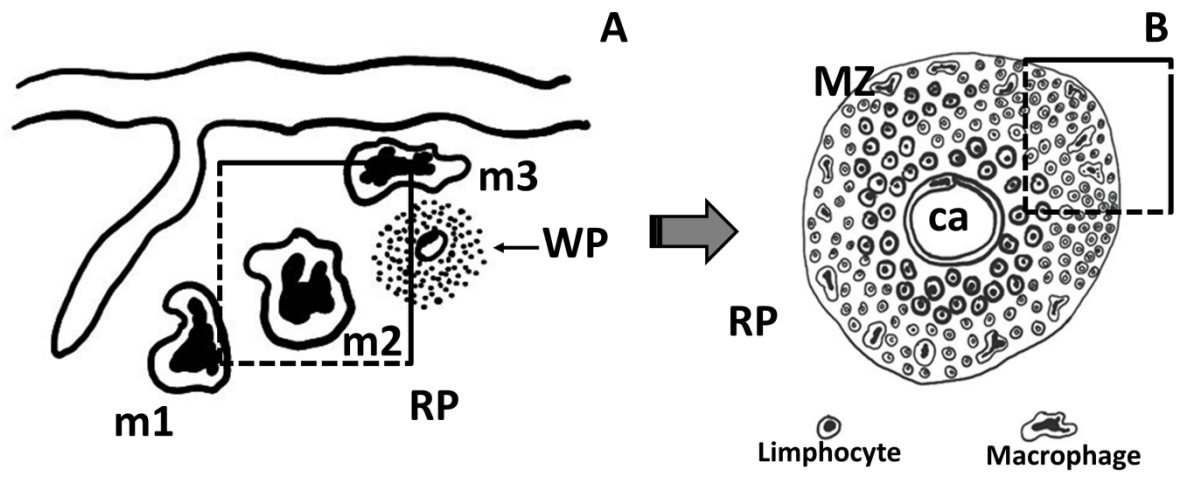
9 **Figure 7.** Effect of increasing the number of serial sections on Cavalieri volume and
10 coefficient of error. **A.** Control. **B.** BaV. **C.** AeBd. **D.** AeBd/ABS. **E.** ABS. Increasing
11 the number of sections reduces the CE of the analysis, bringing the volume closer to a
12 stability plateau. Solid lines = volume. Dashed lines = coefficient of error (CE).

1 **Figure 1.**



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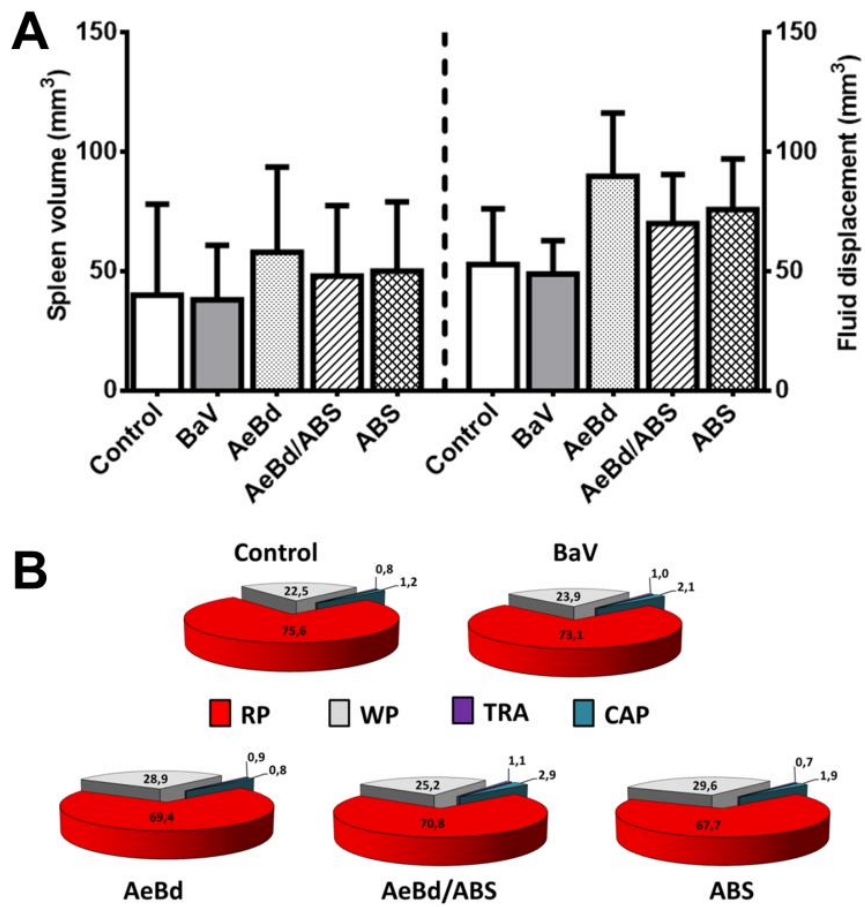
1 **Figure 2.**



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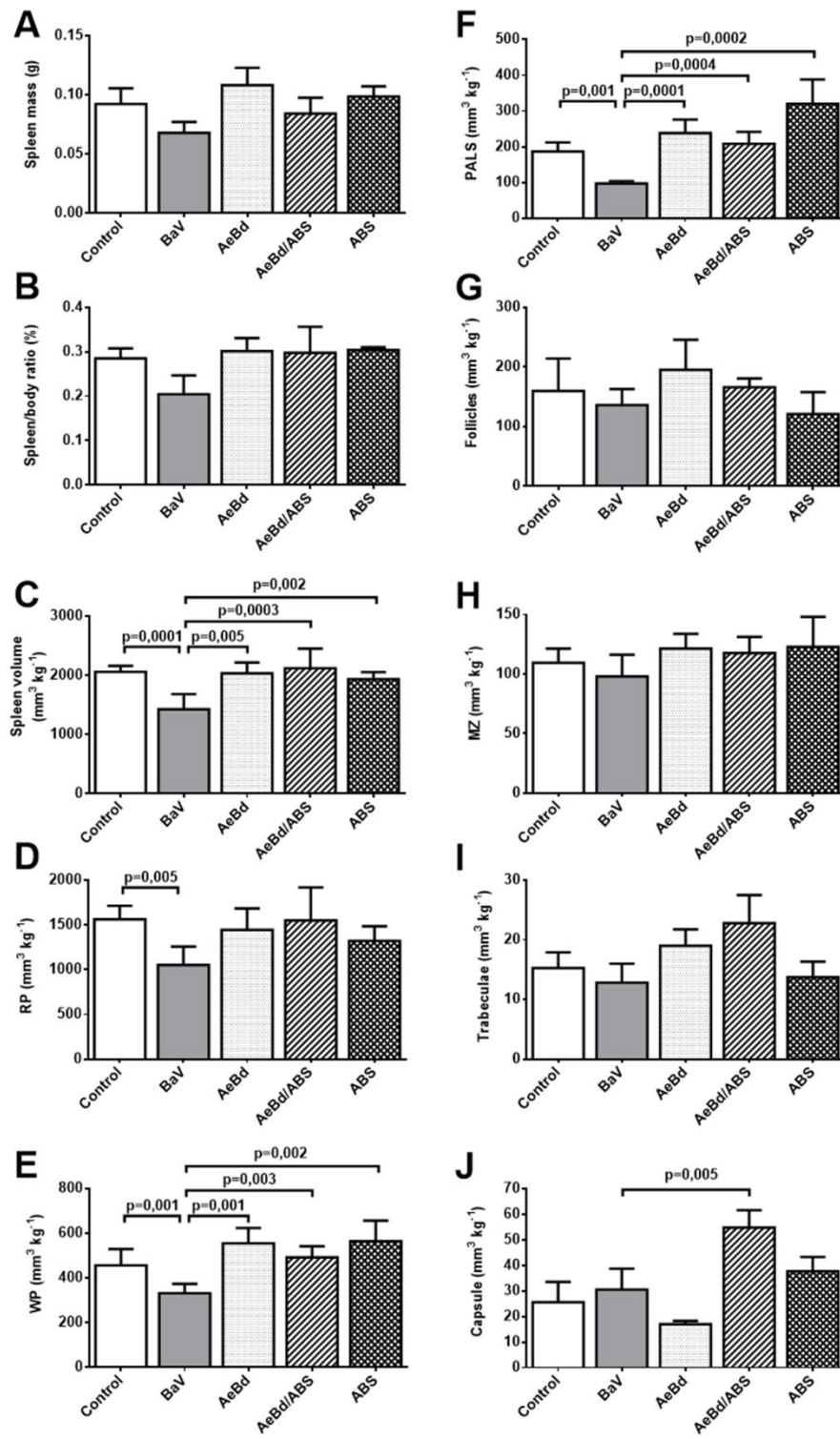
1 **Figure 3.**

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1 **Figure 4.**



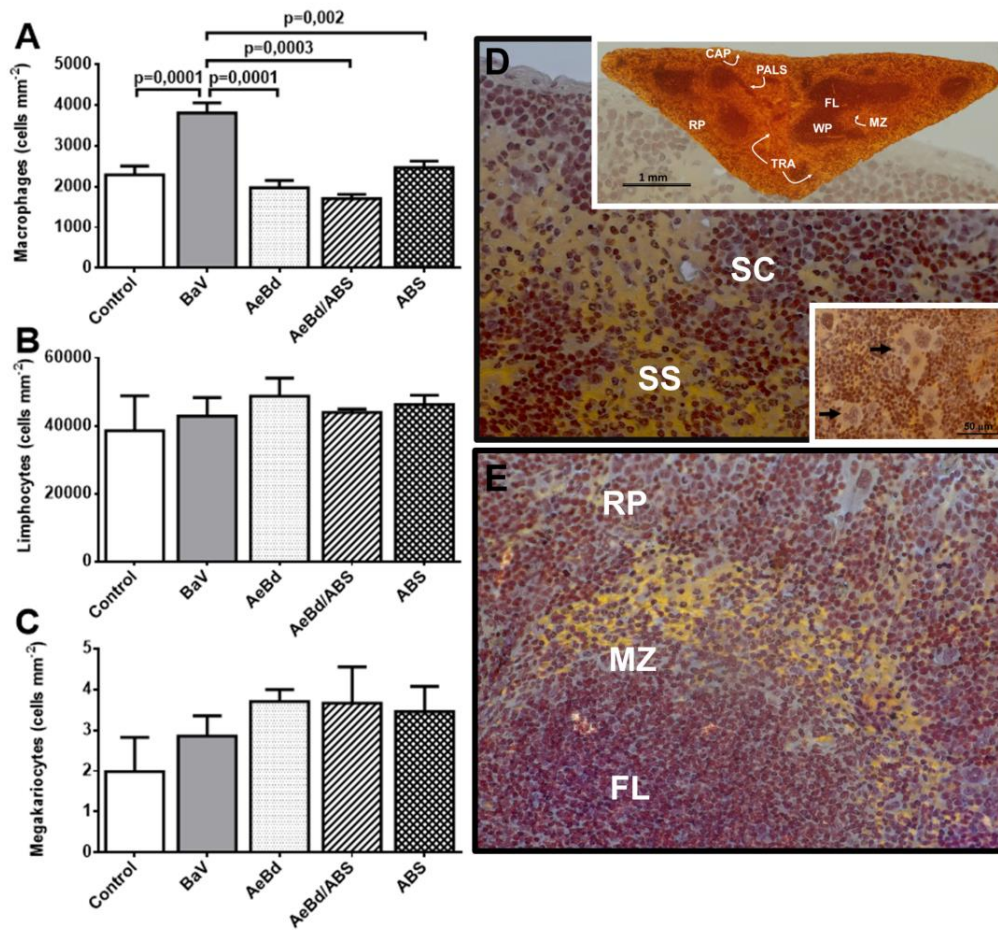
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1 **Figure 5.**

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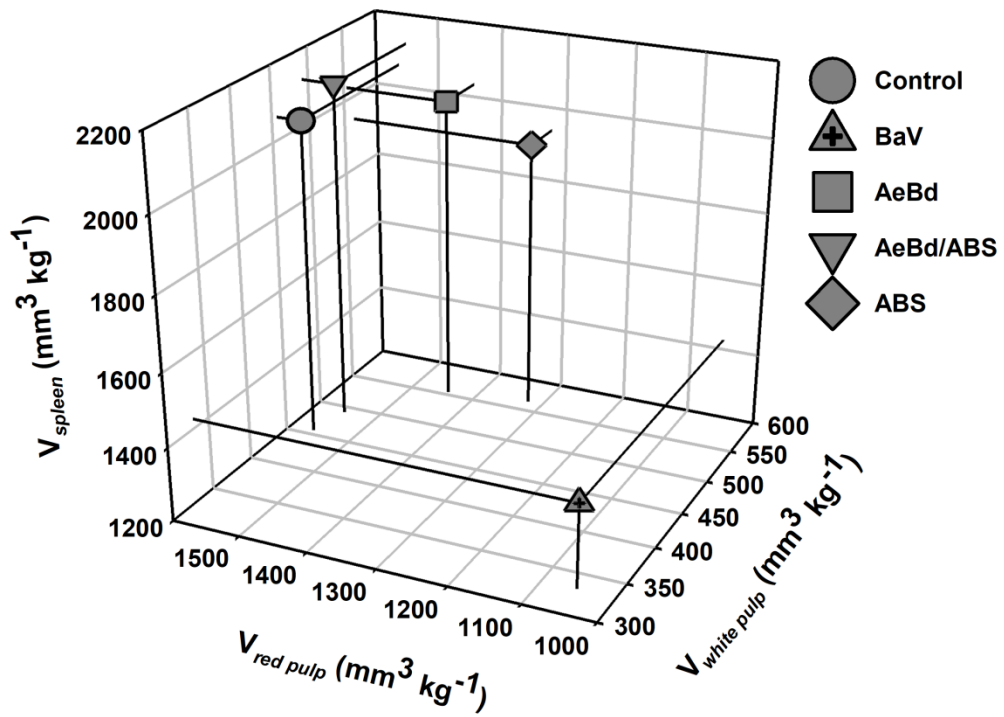


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1 **Figure 6.**

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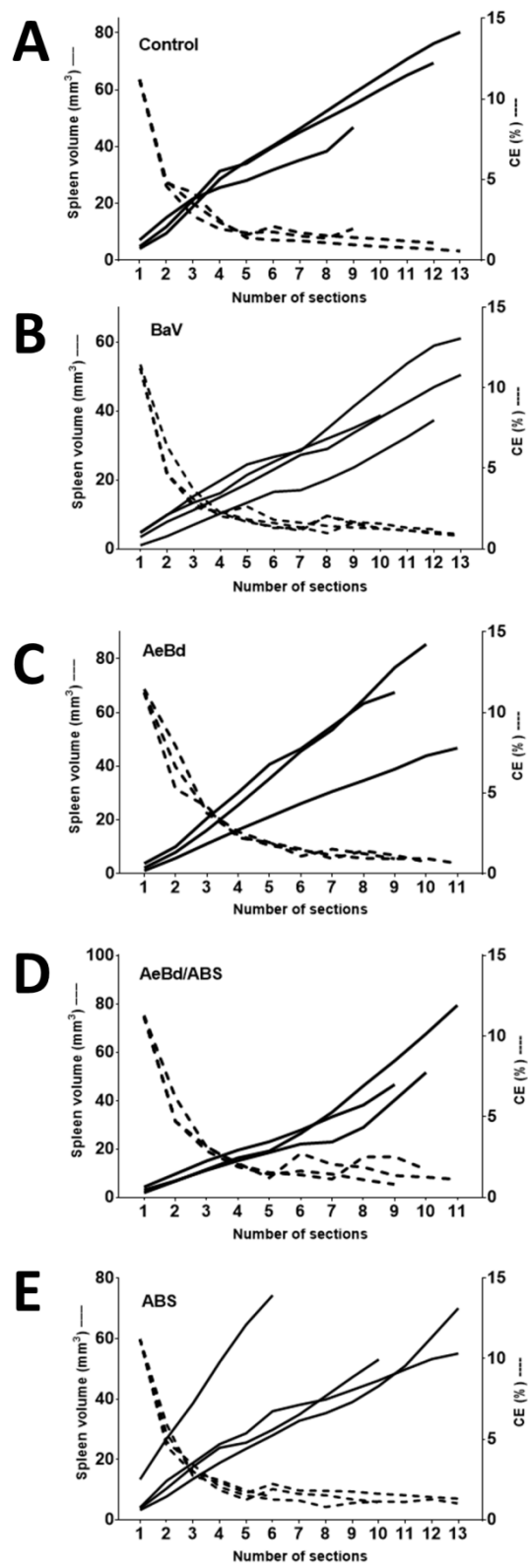
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1 **Figure 7.**



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