ARTIGO ORIGINAL

Safety of Boldo phytomedicines following systemic administration: A systematic review of pre-clinical studies

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ABSTRACT

Several species known in the Brazilian folk medicine as Boldo have been used in the form of infusions or other beverages, due to their therapeutic potential. However, academic concerns regarding the safety of Boldo extract have arisen due to reports of hepatotoxicity, teratogenicity, adverse pregnancy and abortive effects. We performed a systematic review of the systemic toxicity of different species of Boldo in preclinical studies. The Preferred Reporting Items for Systematic Reviews and Meta-Analyzes (PRISMA) statement was followed. The searches were performed in Pubmed, Science Direct, Scopus, Web of Science, Virtual Health Library and Google Academic databases, without language restrictions, until August 2023. Sixteen articles have been included for qualitative synthesis. The selected studies dealt with the species *Plectranthus barbatus* Andrews (Lamiaceae), *Peumus boldus* Molina (Monimiaceae), *Vernonia condensata* Backer (Asteraceae) and their botanical synonyms: *Coleus forskohlii* Briq. and *Coleus barbatus* (Andrews) Benth. ex G.Don for *P. barbatus;* and *Vernonia amygdalina* for *V. condensata*. Relevant changes in hepatocytes, liver weight, and fat deposition were reported. Furthermore, teratogenic potential, impaired motor activity and an important reduction in dopaminergic neurons were reported. The impaired methodological quality of the studies reinforces the need for further research to support the rationale use of Boldo phytomedicines in Brazilian complementary medicine.

Keywords: Folk Remedy; Medicinal Plants; Evidence-Based Pharmacy Practice.

INTRODUCTION

Historically, the search for plants with therapeutic activities is a very relevant axis in medicine. The use of medicinal plants has become an increasingly significant resource, which may be associated with the search for less aggressive and affordable alternative therapies, cultural aspects, the mega botanical diversity of some countries, market trends, and scientific advances in the validation of biological activities and clinical efficacy (Lopes et al. 2018). Most medicinal herbs are consumed as infusions because they are easier and quicker to prepare and use (Rocha et al. 2020), but herbal beverages also display an important market share (Chandrasekara and Shahidi 2018).

Boldo are among the native and exotic adapted species most used in complementary medicine worldwide, being of great relevance in several countries due to its ethnobotanical and pharmacological importance (Alasbahi and Melzig 2010a, 2010b). Considering the great variety of Boldo species, some are more frequently described in ethnopharmacological studies, i.e., *Peumus boldus* Molina (Monimiaceae), *Vernonia condensata* Baker (Asteraceae), *Plectranthus barbatus* Andrews (Lamiaceae), *P. ornatus* Codd (Lamiaceae) and *P. neochilus* Schltr. (Lamiaceae) (Monteiro et al. 2001; Mejía-dolores et al. 2014; Fernandes et al. 2021). Despite sharing the same popular name depending on the country, these species present distinct anatomical and mainly phytochemical characteristics, which may also vary in terms of efficacy and toxicity (Akowuah et al. 2015; Brito et al. 2016; Umegaki et al. 2019).

Owing to its therapeutic value, nineteen phytopharmaceutical preparations containing Bol-

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do are registered in the Brazilian National Health Surveillance Agency (Anvisa), which are often indicated owing to its cholagogue, antispasmodic, cathartic, and possible hepatoprotective activity (Carvalho et al. 2018). These bioactivities have been associated with their diverse phytochemical composition e.g., benzoquinoline alkaloids such as boldine, isocordine, secoboldine, and *N*-methylaurotetanine; flavonoids, particularly catechins and quercetin; and essential oils found in its leaves and bark (Schmeda-Hirschmann et al. 2003). Paradoxically, the safety of preparations based on Boldo extracts have been questioned by the academic community, especially due to reported cases of hepatotoxicity following oral administration (Piscaglia et al. 2005; Ribeiro et al. 2017; Ribeiro et al. 2017).

In Brazil, homemade beverages containing Boldo such as infusions (Tôrres et al. 2005; Tomchinsky et al. 2017) and the "bottled preparations" are widely consumed. Typically, for obtaining such products the herbal raw material is soaked in an alcoholic vehicle such as white wine, sugarcane brandy ("cachaça"), or grain alcohol (Agra et al. 2008). This process yields very bitter beverages which are easily marketed without regular sanitary inspection, boosting the indiscriminate use without any professional guidance, thereby posing the user's health at risk (Souza et al. 2017; Ribeiro et al. 2017).

Seeking to bring evidence that contributes to the rational use of Boldo species for therapeutic purposes, henceforth we present a systematic review of the toxicity of Boldo following systemic administration in pre-clinical studies.

METHOD

Study design

This systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Page et al. 2021). The systematic review protocol was registered in the International prospective register of systematic reviews (PROSPERO – CRD42020212281).

Guiding question and definition of "PECOS"

The following guiding question was determined: Is there a difference in toxicity regarding the internal use in animals of the various types of Boldo used in Brazilian herbal medicine? For the construction of the systematic review and guiding question the PECOS strategy was used, as follows: "P" (*problem situation*): herbal derivatives from different species popularly known as Boldo in Brazil; "E" (*exposition*): oral and intraperitoneal administration; "C" (*comparator*): placebos (negative controls), drugs (positive controls) or no intervention; "O" (*outcome*): toxicity or adverse effects; "S" (*study-design*): preclinical animal studies (e.g, randomized controlled, *cross-over*, non-randomized controlled).

Database and search strategy

The following databases were accessed: PubMed (MEDLINE), Scopus, Science Direct, Web of Science (Science Citation Index), Google Scholar, and Virtual Health Library (VHL), searching for relevant articles published until August 2023, without restricting the date of publication or language. The definition of descriptors was performed using Medical Subject Headings (MeSH) and Health Sciences Descriptors (DeCS). The search strategies used in each of the databases are presented in Table S1 of the Supplementary Material.

The authors who had unavailable articles were contacted twice via e-mail, where access to these articles was requested. The remaining articles were obtained in full by means of a Google search by name or Digital Object Identifier System (DOI).

Additional Analyzes

The reference list of the included articles was also analyzed for eligible publications, since they could not be identified in the selection of the studies (gray literature).

Eligibility criteria

Inclusion criteria were: i) animals of all sexes, ages and species/lines; ii) experimental models of oral and/or intraperitoneal intoxication in animals treated with vehicle/placebo or control; iii) treatment with Boldo in any dosage, administered at any time and frequency; iv) original published articles.

Exclusion criteria were: (i) studies in humans or animals with any comorbidity; (ii) *in vitro*, *in vivo* and *in silico* study designs, before-after studies without a control group, observational studies; (iii) animal models of oral and intraperitoneal intoxication treated with any other drug/or treatment with polyherbal preparation of Boldo/or isolated pure compounds/or Boldo combined with other toxic agents; (iv) case reports, review articles, editorials and letters to the editor, as well as papers presented at scientific events, news, commentaries, dissertations and theses.

Studies selection

The search and selection of articles was performed independently by two reviewers (GABS/

VMO). Research data was extracted from the database and exported to Rayyan QCRI web platform (Ouzzani et al. 2016) to facilitate the selection of potentially eligible studies. Duplicate articles were excluded, then the titles related to the theme were evaluated and those that were not related to the subject were excluded. Subsequently, a detailed reading of the abstracts of the included articles was performed, with the objective of selecting those that addressed the proposed subject, as well as excluding the abstracts that did not address the topic of interest. Of the articles selected, all were read in full, and once they met the inclusion criteria, they were included as results of the search. Throughout the study, each reviewer was blind to the decisions of the other, and any discrepancies have been discussed until consensus was achieved, or, if this is not possible, a third researcher was consulted.

Data extraction

The data of the included papers was extracted into a pre-piloted standardized data extraction form (electronic spreadsheet). We recovered information regarding the animal model was collected (animal species/lines used; sex of animals; number of animals per group; age and weight of animals); study design (number of experimental groups; duration of follow-up; method of oral toxicity evaluation); characteristics of the intervention (taxonomic identification of the species; preparation method of the Boldo extract; chemical composition; dose; dosage frequency; time and route of oral or intraperitoneal administration) and identification of the study (authors; year of publication; country where the research was performed).

The main outcomes evaluated were: LD_{50}; signs of intoxication or adverse effects, and survival. The secondary outcomes evaluated were: absolute body weight; liver and other organ weight; histopathological analysis; fetal morphological analysis; abortifacient capacity; teratogenicity; hematological analysis of red blood cell count (RBC); hemoglobin (Hb); hematocrit (Hct); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); platelet number; white blood cell (WBC) count and differential WBC count; biochemical analysis of alkaline phosphatase (ALP); alanine aminotransferase (ALT); aspartate aminotransferase (AST); bilirubin; cholesterol (total; LDL; HDL); uric acid (AUR); creatinine (CRE); total protein (PT) for liver function; cytochromes (CYP's); brain activity and motor coordination.

Taxonomical assessment

The taxonomy and nomenclature of the species used in the studies selected for review, were compared with taxonomic information available in existing standards in a botanical database The Flora on Line (http://www.worldfloraonline.org/) and the articles were classified according to the available information, enabling the analysis of possible taxonomic errors (Rivera et al. 2014).

Risk of bias and quality assessment

Two reviewers (GABS, VMO) independently assessed the risk of bias using the Center for Systematic Review for Experimentation with Laboratory Animals (SYRCLE) tool (Hooijmans et al. 2014). This tool allowed them to assess: i) selection bias; ii) performance bias; iii) detection bias; iv) attrition bias; v) reporting bias; and iv) other types of bias. Subsequently, two independent investigators (GABS, VMO) performed the quality assessment of all included studies using the CAMARADES checklist for study quality (Macleod et al. 2004; Auboire et al. 2018).

The checklist consists of ten "Yes or No" questions related to: publication in a peer-reviewed journal; statement of temperature control; randomization of treatment or control; allocation concealment; blinded outcome assessment; avoiding anesthetics with marked intrinsic properties; use of animals with hypertension or diabetes; sample size calculation; statement of meeting regulatory requirements; and statement of possible conflict of interest. Each item received a "Yes" score on the checklist according to its disclosure in the respective article. At the end, the "Yes" score was collected and given a total "Yes" score for each article. In total, each article can score up to 10 points, with the scores being averaged for each article. Disagreements were resolved by consensus.

Data synthesis and statistical analysis

The data that were taken from the included articles were described in a narrative synthesis, in addition to presentations in tables and figures to assess the possible sweeps reported. Signs were used to indicate statistically significant increase (↑), decrease (↓) or equality (↔) between the effects observed in the treatment group compared to the control. The Kappa coefficient (Landis and Koch 1977) was determined to assess the degree of agreement between the two raters (GABS, VMO). In this search, we considered a 95% confidence interval and used the Stata 11.0 software package.

RESULTS

Results of the search

As depicted in Figure 1, we have identified 7432 articles by electronic and manual search. After removing the duplicates (n=1273), 6158 articles were submitted to a screening of their titles and abstracts. Twenty-three (23) articles were read in full and submitted to evaluation according to the inclusion and exclusion criteria, and sixteen articles (16) were selected for qualitative synthesis. Seven articles were excluded due to the following reasons: one of them included animals with comorbidity (Fischman et al. 1991); three worked with isolated cells or organs (Owu et al. 2008; Falé et al. 2012; Hebbani Nagarajappa et al. 2016); and three articles did not configure an intoxication model (Awe et al. 1999; Falé et al. 2011; Matthew and Osime 2019). Throughout the eligibility evaluation, the degree of agreement between the two researchers was evaluated using the Kappa coefficient, which was 0.8944, considered practically perfect (Landis and Koch 1977).

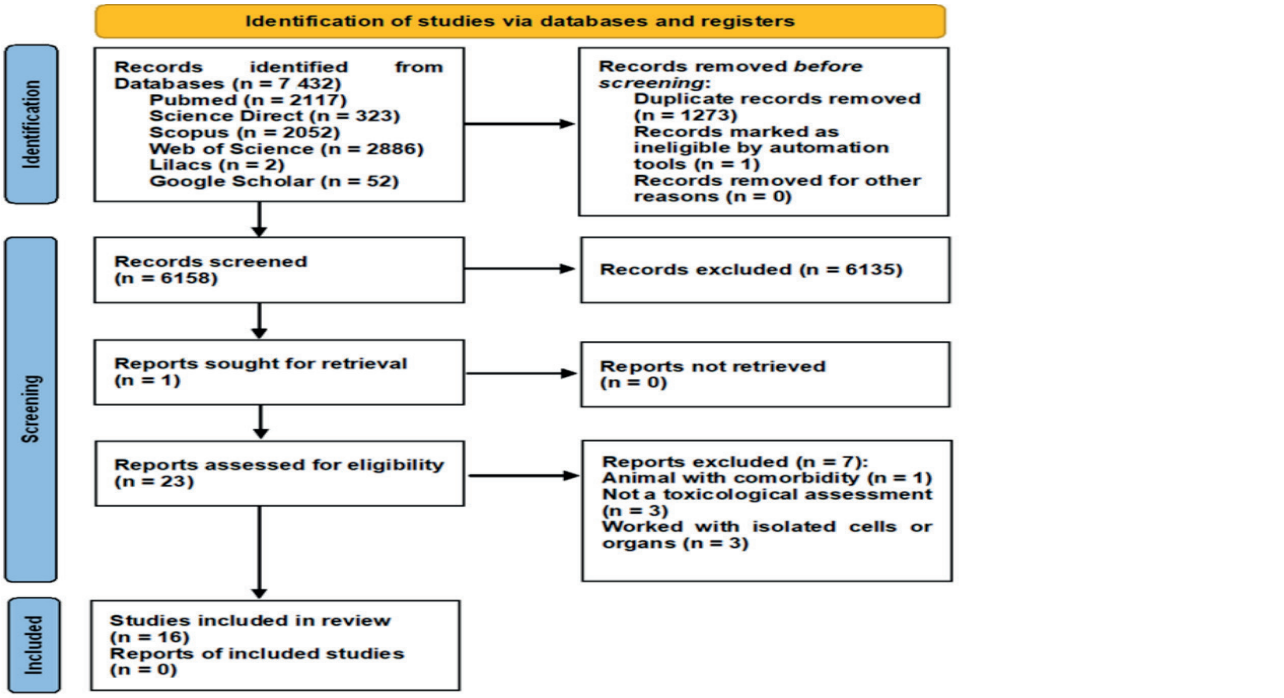
Description of included studies

The general features of the studies included in this review are summarized in Tables 1, 2, and 3. We found reports from different countries, Japan being the country with the greater number of publications (n=5), followed by Brazil (n=4), Nigeria (n=4), Malaysia (n=1), Peru (n=1) and Uganda (n=1). Three species of Boldo were reported in the included papers, i.e., *V. condensata*, *P. barbatus* and *P. boldus*.

It is noteworthy that some studies used botanical synonyms to refer to these species, such as *V. amygdalina* Del., botanical synonym of *V. condensata*, and *Coleus forskohlii* Briq. or *C. barbatus* (Andrews) Benth. ex G.Don which are synonyms of *P. barbatus*. Such information was considered, however, when referring to a particular article, we have decided to maintain the name of the species adopted by the author.

Regarding the animals used in the studies, the most recurrent species were ICR mice (n=5) and Wistar rats (n=5), followed by Swiss albino mice (n=2), Sprague-Dawley rats (n=2), Holztman rats (n=1) and c57bl/6 mice (n=1). Most experimental models used only male animals (n=8), while five studies were performed using animals of both sexes and three used only females.

As shown in Table 1, the age and the initial weight of the animals were assessed; seven articles did not indicate the age of the animals and six did not indicate the initial weight. Another point evaluated was the type of extract administered in the treatment group (TG), The majority (43.75%) of the selected articles used the aqueous extract of the leaves (AEL), thus ratifying the relevance of the in-



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For more information, visit: http://www.prisma-statement.org/

Figure 1. PRISMA flow diagram for screened included and excluded studies.

fusion on the Boldo´s ethnopharmacology; 31.25% used extract of the powdered root (PRE), 6.25% dry crude extract (DCE) and powdered leaves (PL), 6.25% ethanolic extract of leaves (EEL, that corresponds to the tincture or "bottled" preparations), 6.25% methanol leaves extract (MLE) and 6.25% , hydro-alcohol extract of the dry leaves (HAEDL).

All studies used a comparison group, i.e., a control group (CG), thus configuring controlled pre-clinical trials; and the CGs were saline solution, standard diet, or distilled water. As for the route of administration, some studies used more than one type, and it could be oral, intragastric or intraperitoneal. The oral route (O) had the greater prevalence (93.7%; n=15), followed by the intraperitoneal route (I.P; n=3) and intragastric (I.G; n=2).

Daily administration and repeated doses presented the greatest recurrence, corresponding to 87.5% (n=14) of the selected articles, while single administration of different doses of the extract was present in 12.5% (n=2) of the studies. The treatment time ranged from 30 min to 1 h for the single administration groups (acute intoxication model), and from 3 to 90 days for the daily administration groups. Herein we considered treatment time span ranging from 1 up to 28 days as subacute toxicity models, and greater than 28 days as subchronic toxicity models. Therefore, most of the studies fitted to subacute toxicity models, featuring only four cases of subchronic toxicity (De Almeida et al. 2000; Saalu et al. 2013; Akowuah et al. 2015; Oyinleye et al. 2021)showed abortive and teratogenic action and changes in the blood levels of bilirubin, cholesterol, glucose, alanine aminotransferase (ALT.

These rendered fundamental evaluations of the degree of toxicity and possible physiological alterations. The dosage regimen of TG ranged from 0.6 mg/kg to 5000 mg/kg, with 600 mg/kg being the most frequent dose, followed by 1000 mg/kg.

Table 1. General features of the included studies in the systematic review of the toxicity of Boldo following systemic administration in animals

Study/Country	Plant specie	Species/ Strain	Sex	Age (weeks)/ Weight (g)	N° of groups	Animal per group	Treatment	Doses (mg/kg)	Route of administration/ Frequencies	Duration of treatment (days)
Akowuah et al. (2015) Malaysia	*V. amygdalina*	Rat / Sprague– Dawley	F	N.I/ 170-190	4	6	MLE	300, 600, and 1200	O/Once daily	28
Almeida and Lemonica (2000) Brazil	*C. barbatus*	Rat / Wistar albino	F	N.I/ 200-220	4	12	AEL	220, 440, and 880	O/Once daily	15
Brandolt et al. (2007) Brazil	*P. barbatus*	Rat / Wistar albino	Both	N.I/ 200-300	3	12	AEL	22 and 66	O/Once daily	21
De Almeida et al. (2000) Brazil	*P. boldus*	Rat/Wistar albino	Both	N.I/ 160-220	14	20	HAEDL	50, 200, 500, and 800	O/Once daily	12-90
Igile et al. (1995) Nigeria	*V. amygdalina*	Mice/ ICR	Both	2/ 15–18.5	10	5-6	DCE and PL	8 % and 25 %	O/ Once Daily	14
Mejía-Dolores et al. (2014) Perú	*P. boldus*	Rat/Hortsmen	M	13/ 250-265	4	5	AEL	200	O/Once daily	21
Monteiro et al. (2001) Brazil	*V. condensata*	Mice/Swiss albino	Both	N.I/ 26-31	N.I	N.I	AEL	0, 300, 450, 500, 670, 1000, 1500, 2000, 2250, 3400, and 5000	O and I.P/ Once and daily	3-14
Njan et al. (2008) Uganda	*V. amygdalina*	Rat/Wistar albino and Mice/Swiss albino	Both	N.I / 130-150 and 18-26	11	4	AEL	10, 100, 200, 400, 500, 600, 800, 1.000, 1.500, 2.000, 3.000, 4.000, and 5.000	O, I.P and I.G/ Once and Daily	1-14
Ojiako and Nwanjo (2006) Nigeria	*V. amygdalina*	Rat/Wistar albino	M	N.I / N.I	8	6	AEL	50 or 100	I.P / Once or twice daily	1-4
Oyinleye et al. (2020) Nigeria	*V. amygdalina*	Mice/Swiss albino	M	11-15 / 28-30	7	5	AEL	62.5, 125, 250, 500, and 1000	O / Daily	35
Saalu et al. (2013) Nigeria	*V. amygdalina*	Rat/Sprague– Dawley	M	10-11/ 175-180	4	10	EEL	100, 300, and 600	O/ Daily	56

Study/Country	Plant specie	Species/ Strain	Sex	Age (weeks)/ Weight (g)	N° of groups	Animal per group	Treatment	Doses (mg/kg)	Route of administration/ Frequencies	Duration of treatment (days)
Umegaki et al. (2019) Japan	*C. forskohlii*	Mice/ C57BL/6	M	5 / N.I	7	4 – 5	PRE	0.5% (w/w)	O / Daily	7
Virgona et al. (2012) Japan	*C. forskohlii*	Mice/ ICR	M	5 /N.I	8	5-6	SPD + PRE	0; 6, 60, 600, 740, and 6000	O/ Daily	14-21
Virgona et al. (2013) Japan	*C. forskohlii*	Mice/ ICR	M	5 / N.I	8	6	SPD + PRE	0; 6, 60, 600, and 6000	O/ Daily	21
Yokotani et al. (2013) Japan	*C. forskohlii*	Mice/ ICR	M	4 / N.I	16	5 – 6	PRE	36, 60, and 750	O and I.G/ Daily	14-18
Yokotani et al. (2020) Japan	*C. forskohlii*	Mice/ ICR	Both	4 / N.I	12	5	SPD + PRE	7.2, 69.7, 82, 83, 697, 792 794, and 799	O/Daily	14

Abbreviations: AEL, aqueous extract from leaves; DCE, dry crude extract; EEAP, ethanolic extract from aerial parts; F, female; HAEDL, hydro-alcohol extract of the dry leaves; I.G, intragastric; I.P, intraperitoneal; M, male; MLE, methanol leaves extract; NA, non-applicable; NI, non-informed; O, oral administration; PL, powdered leaves; PRE, powdered root extract; SPD, semi purified diet AIN93G.

Table 2. Preparation methods, yield, and chemical composition of the extracts described in the systematic review of the toxicity of Boldo following systemic administration in animals

Study ID	Extraction method	Solvent	Plant: solvent ratio (w/v)	Extraction time	Processing	Extract Yield (% w/w)	Identified compounds / analytical method
Akowuah et al. (2015)	Dynamic maceration	Methanol	N.I	72 h	Filtration, rotaevaporation (40 ºC) and lyophilization	N.I	phenolic acids and flavonoids / FTIR
Almeida and Lemonica (2000)	Static maceration	70% ethanol	N.I	48 h	Rotaevaporation (< 40 ºC)	31.0±3.9	N.I
Brandolt et al. (2007)	Infusion	Distilled H_2O	0.98:50 and 2.88:50	N.I	N.I	N.I	N.I
De almeida et al. (2000)	Static maceration	92.8 ºGL ethanol	N.I	N.I	N.I	N.I	steroids, triterpenoids, mono and sesquiterpenes, flavonoids, alkaloids, and reducing sugars / standard screening tests
Igile et al. (1995)	Soxhlet extraction	Methanol	600 g	60 h	Vacuum drying, lyophilization and semi-purification	28.2 DCE; 2.8 crude saponin	0.23% vemonioside d / NMR
Mejía-dolores et al. (2014)	Static maceration	Distilled H_2O	N.I	6 h	Filtration and evaporation (37 °C)	N.I	alkaloids (Dragendorf and Mayer reagents), and flavonoids (Shinoda reagent)
Monteiro et al. (2001)	Decoction	Distilled H_2O	1:10	30 min (60 ºC)	Filtration and lyophilization	3	N.I

Study ID	Extraction method	Solvent	Plant: solvent ratio (w/v)	Extraction time	Processing	Extract Yield (% w/w)	Identified compounds / analytical method
Njan et al. (2008)	Dynamic maceration followed of soxhlet extraction	Distilled H_2O	1.5:20	24 h	Filtration and evaporation (water bath)	21.53	tannins, saponins, phenols, flavonoids, steroids, and alkaloids / standard screening tests
Ojiako and nwanjo (2006)	Dynamic maceration	Distilled H_2O	1:10	24 h	Filtration and rotaevaporation	9.6	N.I
Oyinleye et al. (2020)	Infusion	Distilled H_2O	1:1	N.I	Filtration	N.I	N.I
Saalu et al. (2013)	Static maceration	95% ethanol	1:3	72 h	Filtration and concentration (vacum)	3.77	N.I
Umegaki et al. (2019)	Powdered standardized extract (purchased)	Methanol and hexane (subfractions)	N.I	N.I	Purification by column chromatography and rotaevaporation	N.I	1,9-dideoxyphorskolin (1.99%), forskolin (9.98%), 7-deacetyl-1,9-dideoxyforskolin, 14-deoxycoleon U, and crocetin dialdehyde / HPTLC - NMR
Virgona et al. (2012)	Supercritical fluid extraction	CO_2	N.I	N.I	N.I	N.I	forskolin (10%) / N.I
Virgona et al. (2013)	Supercritical fluid extraction	CO_2	N.I	N.I	N.I	N.I	forskolin (10%) / HPLC - ELSD
Yokotani et al. (2013)	Supercritical fluid extraction	CO_2	N.I	N.I	N.I	N.I	forskolin (10%)
Yokotani et al. (2020)	Powdered standardized extract (purchased)	N.I	N.I	N.I	N.I	N.I	forskolin (10.37%) and 1,9-di-desoxyforskolin (1.71%) / HPLC – UV

DAD, diode array detector; DCE, dried crude extract; ELSD, evaporative light scattering detector; N.I, no informed; FTIR, Fourier transform infrared spectroscopy; HPLC, High Performance Liquid Cromatography; MS, mass spectrometer; NMR, nuclear magnectic ressonance; TLC, Thin layer chromatography; DCE, dry crude extract; PL, powdered leaves; UV, ultraviolet detector.

Table 3. Summary of the main outcomes in the systematic review of the toxicity of Boldo following systemic administration in animals assessing

Study ID	Lethal dose/ death reports	Biochemical parameters	Physiological changes	Histopathology/Anatomopathological Analysis	Behavioral tests
Akowuah et al. (2015)	LD_{50}: >1200 mg/kg	↔AST; ↔ALT; ↔ALP; ↔total bilirubin; ↔urea; ↔creatinine ↔cholesterol; ↔triacylglycerol	No significant changes were observed neither in body weight or relative organ weight (heart, lung, liver, kidney, and spleen)	N.D	N.D
Almeida and Lemonica (2000)	N.D / No deaths were observed along the follow-up	N.D	TG 880 mg/kg: ↓n° of implantation sites; ↓ n° of live fetuses per litter; ↑ of preimplantation loss rate; ↑ placental weight; ↓ maternal weight; ↓ fetal weight. TG 440 mg/kg: ↑ fetal weight; ↑ of skeletal variations and ↓ of the n° of fetal ossification centers.	N.D	TG 880 mg/kg: decrease in food and water intake.

Study ID	Lethal dose/ death reports	Biochemical parameters	Physiological changes	Histopathology/Anatomopathological Analysis	Behavioral tests
Brandolt et al. (2007)	N.D / No deaths were observed along the follow-up	N.D	Congenital abnormalities were not observed.	There was not interference with the embryonic development of any group.	No reduced water and food consumption.
De Almeida et al. (2000)	N.D / No deaths were observed along the follow-up	↑cholesterol* (60 days); ↑AST* (60 days); ↑ALT* (60 days); ↓cholesterol* (90 days); ↓AST* (90 days); ↓total bilirubin*; ↔direct bilirubin; ↔indirect bilirubin; ↓glucose*; ↔creatinine; ↓urea	Anatomical changes were observed in the fetus of TG (800 mg/kg). Also, reduction in body weight for the malformed fetus, as well as incidents of blastocystotoxic-antizygotic action, and a few cases of abortive activity have been reported.	TG (800 mg/kg) showed a discrete histological change in the liver and one such change, steatosis, observed in only two animals. No histological changes were detected in heart or kidney tissues.	N.D
Igile et al. (1995)	N.D / Groups 1 and 5 each lost a mouse on the 11th day, while two mice died on the 14th day in group 8.	↓liver cholesterol*; ↓plasma cholesterol*	Animals treated with PL and DCE significantly decreased the body weight gain and showcased drastic decrease in the liver weight.	N.D	No dietary changes were reported.
Mejía--Dolores et al. (2014)	N.D / No deaths were observed along the follow-up	↔uric acid	N.D	Significant loss of dopaminergic neurons in the substantia nigra.	The rotarod test showed a reduction in latency time, indicating impaired motor coordination after administration of the extract in TG.
Monteiro et al. (2001)	ALD (I.P): 3400 mg/kg for ♂ and as high as 5000 mg/kg for ♀	N.D	A slight reduction in fetal body weight was observed with 2000 mg/kg TG, in addition to a retardation of prenatal growth	N.D	N.D
Njan et al. (2008)	LD_{50}: 560±1.21 mg/kg (I.P) 3320±150 mg/kg (O)	↑Hb; ↔PCV; ↔MCHC; ↓RBCs*; ↓platelets*; ↓WBCs*; ↑neutrophils*; ↑lymphocytes*; ↔total protein; ↔albumin; ↑direct bilirubin *; ↑total bilirubin *; ↔ALT; ↔AST; ↔ALP ↔cholesterol; ↔HDL; ↔LDL; ↔VLDL; ↔K^+ ↑Na^+* ↑Cl^-*; ↔HCO_2^-; ↑uric acid*; ↓urea*; ↔creatinine.	All animals showed a progressive increase in body weight during exposure. Hematological results showed a significant (P+-0.05) decrease in the red blood cell count at the 2000 mg/kg dose compared to the control.	N.D.	In the acute toxicity evaluation, gait abnormalities were observed in the higher doses.
Ojiako and Nwanjo (2006)	LD_{50}: 500 mg/kg (I.P)	↔total bilirubin; ↔conjugated bilirubin; ↔unconjugated bilirubin; ↔ALT; ↑AST*; ↔ALP	N.D	N.D	N.D

Study ID	Lethal dose/ death reports	Biochemical parameters	Physiological changes	Histopathology/Anatomopathological Analysis	Behavioral tests
Oyinleye et al. (2020)	N.D / No deaths were observed along the follow-up	N.D	None of the extracts induced adverse effects on body weight	No histological lesions were observed in the TG; it significantly reduced the frequency of aberrant spermatogenesis and improved spermatogenesis.	No changes in diet intake were reported
Saalu et al. (2013)	N.D / No deaths were observed along the follow-up	N.D	No changes in body weight have been reported	The spermatogenesis process may have been affected in the TGs at average and high dose (300 and 600 mg/kg, respectively). Furthermore, there were a reduction in the tubular diameter, cross-sectional area of the tubules, number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of TGs (300 and 600 mg/kg).	No changes in diet intake were reported
Umegaki et al. (2019)	N.D / No deaths were observed along the follow-up	↑CYP; ↑CYP1A1; ↑CYP1A2; ↑CYP2C; ↑CYP2C; ↑CYP3A*; ↑liver; ↔liver phospholipid; ↔liver cholesterol	TGs significantly increased both absolute and relative liver weight, but not the final body weight	N.D	Food intake did not differ among the TGs
Virgona et al. (2012)	N.D / No deaths were observed along the follow-up	↑CYP*; ↑CYP1A1; ↑CYP1A2; ↑CYP2; ↑CYP2C; ↑CY3A*; ↑GST	TGs significantly increased relative liver weight, but not the final body weight; TGs markedly induced hepatic drug metabolizing enzymes in a dose and time-dependent manner;	N.D	Food intake did not differ among the TGs
Virgona et al. (2013)	N.D / No deaths were observed along the follow-up	↑AST*; ↑ALT*; ↑ALP *	Significant dose-dependent increase on relative liver weight; Reduction in both body fat and visceral fat weight; No significant differences in the relative kidney weight and serum electrolytes.	Hepatocyte hypertrophy, unicellular hepatocyte necrosis, fat deposition and cellular infiltration	Food intake significantly decreased in the greater dose
Yokotani et al. (2013)	N.D / No deaths were observed along the follow-up	↑CYP content*; ↑GST activity*; ↑CYP2B activity*; ↑CYP2C activity*; ↑CYP3A activity*	TGs significantly increased the final body weight, absolute liver weight and relative liver weight.	N.D	Food intake did not differ among the TGs
Yokotani et al. (2020)	N.D / No deaths were observed along the follow-up	↑CYP1A1*; ↑CYP1A2*; ↑CYP2C*; ↑CYP3A*; ↔CYP1B1; ↑CYP2B10*; ↔CYP2E1; ↑CYP1C29*; ↑CYP3A1*,	TGs did not affect neither the body weight nor the fat tissue; Relative liver weight significantly increased with the greater dose both in male and female;	N.D	Food intake did not differ among the TG's

N.D, not determited; TG, treatment group; CG, control group; I.P, intraperitoneal; ALD, Approximate Lethal Dose; LD_{50}, Mean Lethal Dose; * Statistically significant differences at $p < 0.05$ compared to control; ↑,increase; ↔,no change; ↓,reduction.

Taxonomical assessment of included studies

All the included articles used the scientific name, and all scientific names presented agreed with the references provided in the botanical database Tropicos.org. Nine (56.3%) of the sixteen studies presented complete taxonomic information on the plant name, specimen identification, and voucher specimens deposited. However, the other seven (43.7%) articles presented only information about the name of the plant, being absent information regarding taxonomic identification.

Phytochemical standardization of the extracts

Information regarding the preparation methods, yield, and chemical composition of the Boldo extracts included in this systematic review are presented in Table 2. The solvents and techniques used in the preparation methods varied widely. Moreover, only 6 studies provided quantitative data of the phytochemicals (chemical markers) contained in the extracts (Igile et al. 1995; Virgona et al. 2012, 2013; Yakotani et al. 2013; Umegaki et al. 2019; Yokotani et al. 2020). The saponin vemonioside d was the chemical marker assessed for *V. amygdalina* extracts (Igile et al. 1995) and its derivatives were the main phytochemicals reported to *C. forskohlii* (Virgona et al. 2012, 2013; Yakotani et al. 2013; Umegaki et al. 2019; Yokotani et al. 2020).

Primary outcomes

Table 3 summarizes the main safety concerns reported in the included articles. Three addressed the Mean Lethal Dose, in which the LD_{50} values were between 500 mg/kg and 3320 mg/kg (Ojiako and Nwanjo 2006; Njan et al. 2008; Akowuah et al. 2015). One of the studies also evaluated the lethality through the Approximate Lethal Dose (ALD), defining the values of 3400 mg/kg for males and 5000 mg/kg for females, after the intraperitoneal administration of the aqueous extract (Monteiro et al. 2001). Noteworthy, these studies were performed with the same species, i.e., *V. condensata*; however, some authors referred to it by its botanical synonym *V. amygdalina*.

One of the articles also defined the No Observed Adverse Effect Level (NOAEL), that is the dose administered in which no adverse effects are observed. Herein, it was 1200 mg/kg for the animal model used (Akowuah et al. 2015).The LD_{50} for oral administration of the dried crude *V. amygdalina* extract was greater than 1200 mg/kg (Akowuah et al. 2015), while for the aqueous extract of the aerial parts a LD_{50} of 3320 ± 0.15 mg/kg was established (Njan et al. 2008). For intraperitoneal administration of the aqueous extract of the aerial parts of *V. amygdalina*, LD_{50} values were between 500 mg/kg (Ojiako and Nwanjo 2006) and 560 mg/kg (Njan et al. 2008). Moreover, beside the studies reporting the LD_{50} or ALD, one investigation reported 4 deaths in the TG on days 11 and 14 of follow-up (Igile et al. 1995).

Secondary outcomes

Concerning the other toxicity indications, behavioral changes were observed in three studies (Almeida and Lemonica 2000; Virgona et al. 2013; Mejía-dolores et al. 2014). By using the Rotarod test, one study revealed possible change in motor coordination and response to stimuli after administration of the AEAP of *P. boldus*. Such changes were confirmed with oral and daily administration of 200 mg/kg of the extract (Mejía-dolores et al. 2014).

Among the many other reported changes, liver weight showed significant variations in several studies drawing attention to possible signs of hepatotoxicity (Igile et al. 1995; De Almeida et al. 2000; Virgona et al. 2012; Virgona et al. 2013; Yakotani et al. 2013; Umegaki et al. 2019; Yokotani et al. 2020); in addition, the reduction in animal weight (Igile et al. 1995; Almeida and Lemonica, 2000; Njan et al. 2008; Virgona et al. 2013), fetal weight (Almeida and Lemonica 2000; De Almeida et al. 2000; Monteiro et al. 2001) testicular changes (Saalu et al. 2013) were also key to toxicity reports. It is noteworthy that only four studies addressed changes in food intake, showing significant reductions only in the group receiving 1000 mg/kg *V. amygdalina* extract and 6000 mg/kg *C. forskohlii* extract (Njan et al. 2008; Virgona et al. 2013; Yakotani et al. 2013; Umegaki et al. 2019; Yokotani et al. 2020).

Regarding the studies which evaluated fetal weight, a significant reduction in this parameter was remarkable; even for those articles using different species of Boldo, the decline in fetal weight was recorded by all, in which one of them described a 14% reduction in fetal weight when administering a dose of 880 mg/kg of *C. barbatus* extract, botanical synonymy of *P. barbatus* (Virgona et al. 2013). The two other studies also confirmed such weight reduction using the species *P. boldus* and *V. condensata* (Monteiro et al. 2001).

Of the selected articles, 37,5% (n=6) performed a histopathological analysis of a variety of animal organs, tissues or cells (De Almeida et al. 2000; Njan et al. 2008; Saaluet al. 2013; Virgona et al. 2013; Mejía-dolores et al. 2014; Oyinleye et al. 2021). One of them indicated minor changes in liver histology after daily oral administration of dried crude extract of *P. boldus*, with steatosis present in only two animals, even so, no histological changes in cardiac or renal tissues were observed (De Almeida et al. 2000).

This study also reported important anatomical changes in fetuses of pregnant rats treated with 800 mg/kg of the crude extract, indicating blastocystotoxic-antizygotic and abortive activity. In addition, hypertrophy, necrosis, and significant fat deposition in hepatocytes were demonstrated after administration of doses above 600 mg/kg of *C. forskohlii* extract. In this same article serum levels of alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) showed significant increases when compared to GC (Virgona et al. 2013). Another important factor observed was a significant decrease in red blood cell count after administration of a 2,000 mg/kg dose of the aqueous extract of *V. amygdalina* (Njan et al. 2008).

Testicular alterations were observed in the selected studies that used males in their experimental models, where upon oral administration of *V. amygdalina* extract at doses of 300 and 600 mg/kg, mean seminiferous tubular diameters showed significant decline, as well as disparity in the cross-sectional area of the tubules, which may indicate possible testicular toxicity (Saalu et al. 2013) the effects of varying doses of ethanolic leaf extract of Vernonia amygdalina on the rat testis histo-morphometry were investigated. Forty male wistar rats were divided into groups of four. Group A, as the control was given 10 ml/kg/day/oral distilled water while Group B, C and D subsequently treated with 100, 300 and 600 mg/kg/day/oral route Vernonia amygdalina leaves extract respectively for 56 days. Results showed that Group B rats had normal testis histology comparable to the control group. However, rats in Group C and D exhibited dose-dependent poor testes histo-morphometric profiles, with the higher dosage-group (D.

Risk of bias and methodological quality

Figure 2 displays the results of the risk of bias assessment for each study according to the SYRCLE tool (Hooijmans et al. 2014). All studies showed low risk of bias in four domains. However, all studies showed high risk of bias in the same five domains related to selection, performance, and detection issues. This raises issues regarding how the studies were performed, directly interferes with internal validity, and may impair the integrity of the results and conclusions presented, which is the major limitation of this systematic review.

Figure 3 reports the methodological quality assessed by the CAMARADES checklist (Macleod et al. 2004; Auboire et al. 2018) for the included pre-clinical trials. All studies were published in peer-reviewed journals; avoided the use of anesthetics that could interfere with the outcomes assessed; did not use animals with metabolic disorders; and reported no conflicts of interest.

Similarly, most of the studies that claimed to control environmental conditions during the experimental period, claimed to follow regulatory requirements regarding the use of animals in experiments, and the study reported randomization for both WT and CG. However, none of the included studies provided complete information on sample size calculation, allocation concealment, and

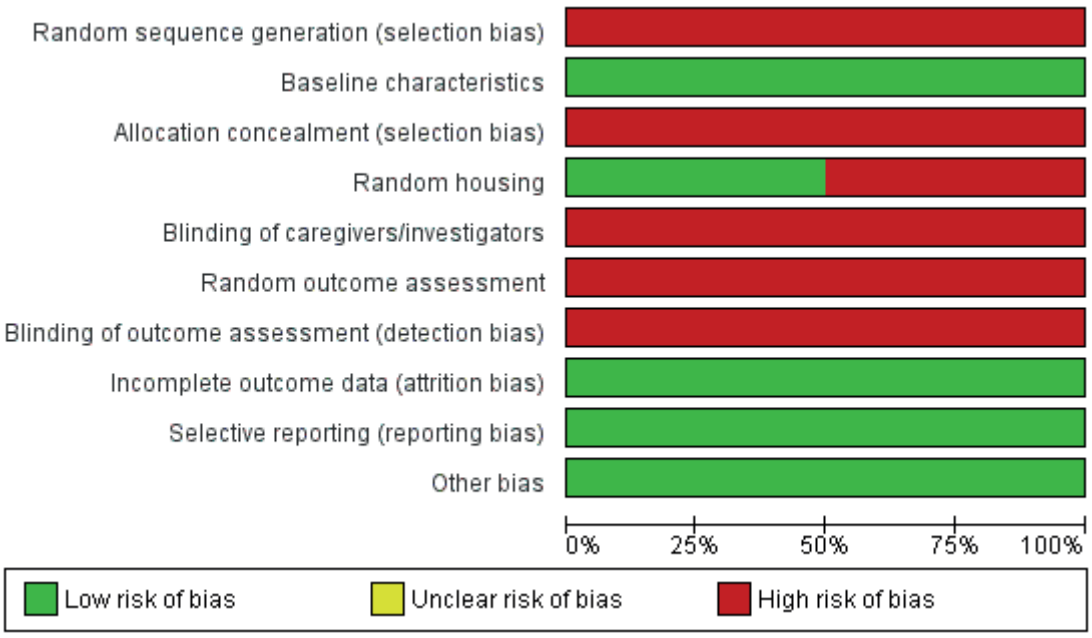

Figure 2. Reporting of risk of bias for the studies assessing the toxic potential of Boldo extracts following systemic administration in animals.

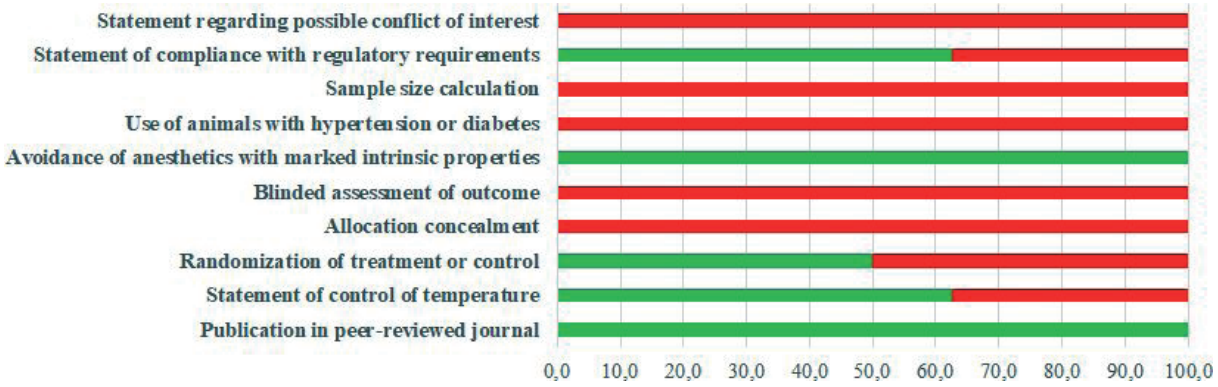

Figure 3. Reporting of quality indicators for the studies assessing the toxic potential of Boldo extracts following systemic administration in animals.

blinding during outcome assessment. The methodological quality scores of the studies ranged from four to seven with a mean of 5.8, thus indicating low methodological quality (Peter et al. 2021).

DISCUSSION

The use of various species of Boldo is common in several regions of the world, demonstrating the importance of evaluating the evidence that proves the safety of the use of preparations containing such species. Such importance is justified, for example, by the Normative Instruction in n° 86, of March 12, 2021, which defines the list of drugs exempt from prescription, being contained in this, four herbal medicines that have *P. boldus* in their composition (Brasil, 2022).

Assuming the importance of Boldo for Brazilian herbal medicine, this systematic review evaluated the toxicity features that have been reported following the systemic administration of different species of Boldo in animal models. During this study, important signs of toxicity reported in the selected articles were observed, such as the reduction or increased liver weight of treated animals when compared to the control group, being *C. forskohlii* and *V. amygdalina* the species used in these studies (Igile et al. 1995; Virgona et al. 2012; Virgona et al. 2013; Yakotani et al. 2013; Umegaki et al. 2019; Yokotani et al. 2020).

As shown in Table 2, some of the phytochemicals found in the Boldo species investigated have been reported. Flavonoids have been frequently mentioned, thus ratifying the relevance of these secondary metabolites for the analysis of the toxicity of the different species of Boldo, since the exacerbated and chronic administration of this phytochemicals may be related to allergic reactions in humans, as well as changes in the hepatocyte membrane, necrosis, mutagenicity and clastogenicity in the bone marrow and even death when administered to animals (Silva et al. 2015)

Another important group are the terpenes, mainly forskolin, a diterpene, which has antihypertensive activity, in addition to activating the adenylate cyclase enzyme (Passos et al. 2009).

Boldine is an alkaloid found in the leaves and bark of boldo, and has important activities such as attracting dopaminergic receptors, competitively inhibiting human 5-HT3 receptors, in addition to acting as a cardiovascular α-adrenergic blocker (Ivorra et al. 1993; Schwanz et al. 2008; Walstab et al. 2014).The histopathological findings of hepatocyte hypertrophy, necrosis, and significant fat deposition of the liver drive our attention towards a remarkable hepatotoxic activity (De Almeida et al. 2000; Virgona et al. 2013). These results point to a dose-dependent hepatotoxicity with fatty liver, as changes were observed at administrations starting at 0.5% powdered extract of *C. forskohlii* root in the diet, corresponding to approximately 600 mg/kg (Virgona et al. 2013).

A case of hepatotoxicity induced by *P. boldus* was reported by Ribeiro et al. (2016). A 72-year-old patient with a medical history of cholecystectomy (10 years ago) regularly used a leaf infusion for 2 weeks. After this period, she developed jaundice, increased eosinophils, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase, alkaline phosphatase, and total bilirubin. All parameters returned to their pre-usage levels after discontinuation of the infusion. In other articles reviewed in this present study, some of the parameters evaluated in the case report also showed alterations (De Almeida et al. 2000; Ojiako and Nwanjo 2006; Njan et al. 2008; Virgona et al. 2013).

However, only the study by De Almeida et al. (2000) evaluated the same species in question (*P. boldus*). In other case reports involving the

consumption of *P. boldus*, either as an infusion or in capsule form, alterations in liver enzymes were also reported (Piscaglia et al. 2005; Nortadas and Barata 2011; Sá et al. 2020).

Plectranthus barbatus is used to treat various digestive problems, including nausea. This can lead several pregnant women to mistakenly use it. Its use should be approached with extreme caution, considering the results that have shown alterations in fetal weight (Almeida and Lemonica 2000; Lukhoba et al. 2006). *Vernonia amygdalina* is also mentioned in the literature in several studies, being used during pregnancy for issues such as nausea and vomiting (Ahmed et al. 2018). However, its use is also associated with reduced fetal weight and prenatal growth retardation (Monteiro et al. 2001). In relation to the use of *P. boldus*, extreme caution must be exercised, due to the results that have shown fetal alterations and some cases of abortive activity (De Almeida et al. 2000).

Both *P. barbatus* and *V. condensata*, a synonym of *V. amygdalina*, are listed in the National List of Medicinal Plants of Interest to the SUS (RENISUS) (Brasil. 2009). *P. boldus*, on the other hand, is included in the List of Non-Prescription Medications, according to Normative Instruction No. 120, dated March 9, 2022. As a result, they are used as references for various treatments and indications without requiring professional supervision, due to their safety and effectiveness. However, it has been observed that in terms of safety, there is a need for monitoring hepatotoxicity and nephrotoxicity through the supervision of qualified professionals and laboratory tests (Brasil 2022).

In the Phytomedicines Formulary of the Brazilian Pharmacopoeia, both *P. barbatus* and *P. boldus* are recommended for use through the infusion of leaves in water, alcohol, or dry leaf extract. However, there are contraindications for pregnant women, lactating women, children, individuals with liver diseases, and other specific groups. Regarding *V. amygdalina*, it was removed from the second edition of the Formulary, indicating that its use is not recommended or supported for medicinal purposes in this context (Brasil 2021).

The mean lethal dose was addressed in a few selected studies and is of great importance for the knowledge of the toxic potential of the administered species. Further studies are needed to determine the LD_{50} of different species and methods of extraction and preparation in order to obtain more information regarding the safety of Boldo.

According to the World Health Organization guidelines, continuous human use for 7 days is equivalent to rodent use for 4 weeks (WHO 2000; Akowuah et al. 2015). This information is extremely necessary when evaluating potential sub-acute toxicity in the population.

The extraction system used to obtain compounds from Boldo can have a direct impact on its pharmacological activity and acute toxicity. Regarding the potential difference in toxicity between species and extraction and processing methods, it is difficult to categorically state which form of plant consumption is more harmful, due to the variety of extraction methods, solvents, and processes employed (Risso et al. 2010).

The reports of toxicity of *C. forskohli*i or *C. barbatus*, synonymous with *P. barbatus*, were more significant in animals that received treatment with powdered root extract compared to those that received the aqueous extract from leaves (Almeida and Lemonica 2000; De Almeida et al. 2000; Brandolt et al. 2007; Njan et al. 2008; Saalu et al. 2013; Umegaki et al. 2019; Oyinleye et al. 2021). The main active compound with medicinal properties found in the roots of *P. barbatus*, forskolin, did not demonstrate toxicity when isolated. However, the use of the root extract in the population should be closely monitored (Costa 2006; Virgona et al. 2013).

It also can be observed that *C. barbatu*s and *V. amygdalina* did not show physiological, histopathological, anatomopathological, and behavioral changes in animals that consumed the aqueous extract of the leaves extracted by infusion in distilled water (Brandolt et al. 2007; Oyinleye et al. 2021). The methanolic extract of *V. amygdalina* also showed less toxicity than alcoholic, aqueous, crude, and powdered leaf extracts (Igile et al. 1995; Monteiro et al. 2001; Ojiako and Nwanjo 2006; Njan et al. 2008; Saalu et al. 2013; Akowuah et al. 2015; Oyinleye et al. 2021).

After the administration of the aqueous extract of *P. barbatus*, no interference or abnormality was observed in the treated animals, however, low concentrations (22 and 66 mg/kg) were used in the study when compared to other studies (Brandolt et al. 2007). According to the authors, the purpose was to simulate the usual preparation and ingestion carried out by the population. It is not possible to assert that the AEL obtained by infusion has lower toxic potential than other species or forms of extraction and administration.

Owing to the limitations related to the methodological quality of the included articles, the safety following systemic administration of phytopharmaceuticals based on these species of Boldo needs further clarification. Regardless of the species and type of herbal preparation whether aqueous or alcoholic, the internal use or systemic administration of Boldo derivatives should be guided by qualified professionals. Moreover, patients might be encouraged to periodically evaluate liver function when

opting for the regular intake of these Boldo derivatives.

To the best of our knowledge this is the first systematic review gathering, organizing and critically analyzing scientific evidences concerning the toxicity of Boldo species used in Brazilian complementary medicine. Furthermore, other strengths of this work are the replicability, transparency and accuracy of the methodology used, which can dwindle the risk of bias when reporting and discussing our results.

In conclusion the hepatotoxicity and teratogenic potential of herbal derivatives obtained from *V. condensata*, *P. barbatus* or *P. boldus* is noteworthy. Therefore, the intake of beverages (e.g., tisanes, infusions or bottled preparations) based on these species should be avoided by patients with liver failure and pregnant women. So far it is still not possible to assert which Boldo species showcase a greater toxic potential compared to the others. Further studies involving different species and extracts of Boldo, evaluating the same parameters, should be conducted to assess differences in toxicity regarding their use. The extraction process should be standardized in research and commercial applications to ensure consistent and reliable results.

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AUTHOR´S CONTRIBUITIONS

G.A.B.S and V.M.O: investigation, data curation, formal analysis, and writing - original draft; L.S.S validation and writing - review & editing; F.M.D.C and N.R.B: methodology, software, writing - review & editing. R.O.C conceptualization, project administration, funding acquisition, supervision, and writing - review & editing. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

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