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Integrated pharmacological actions of *Baccharis genistelloides* subsp. *crispa* on the digestive system: An updated review

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ABSTRACT

Baccharis genistelloides subsp. *crispa*, better known as carqueja, is a phytotherapeutic and ethnomedicinal resource widely used in South America, mainly for the treatment of gastrointestinal and hepatic disorders. In recent years, the species has been the target of scientific research on its pharmacological activities on the digestive system; however, due to the emergence of recent studies and the lack of a critical, integrated, and specific discussion on its action on the digestive system, there is a need for a review on this subject. For this purpose, an extensive and updated search was carried out on documents that discussed the effect of carqueja on the digestive system so that 19 articles were selected and included in the main section of the article. The compilation and discussion of these studies highlighted the activity of carqueja in improving gastric ulcers, acting as an inhibitor of the histaminergic and cholinergic pathways that stimulate gastric acid production, as well as its effect as a liver and intestinal regulator, preventing harmful effects on hepatocytes, promoting bile synthesis and acting as a relaxant of the jejunal muscles, in addition to inhibiting digestive enzymes. Furthermore, flavonoids, phenolic derivatives, clerodane diterpenes, and components of its essential oil are reported as its main active phytochemicals and are involved in a specific and synergistic way with the mechanisms of action described for the species.

1. INTRODUCTION

1.1. Botanical and Taxonomical Notes

Baccharis genistelloides subsp. *crispa* (Spreng.) Joch. Müll (Main synonyms: *Baccharis trimera* (Less.) DC.; *Baccharis crispa* Spreng.) commonly known as "carqueja," "carquejinha," "carqueja-amarga," "charara," "Mano-de-Dios," and "samba," among other denominations, is a native species from South America, being found in Brazil, Argentina, Uruguay, Paraguay, Bolivia, Peru, and Ecuador, and considered an important medicinal resource in these countries, mainly used for the treatment of ailments of the gastrointestinal system, as well as a hepatoprotective agent [1-4]. Carqueja is also indicated as an antidiabetic, anti-inflammatory, analgesic, and antiseptic medicine, among others uses, whether through professional prescriptions or popular use, characterized by its bitter taste [2,5]. *B. genistelloides* subsp. *crispa* is a perennial, erect, highly branched subshrub up to 1 m tall, with stems 80–100 cm long, trialate throughout, leafless or with reduced leaves, with its inflorescences being capitate, whitish, arranged along the branches, with some plants having male flowers and others, female flowers, which appear in the interruptions of the stem. Its fruit is an achene type, glabrous, and white pappus [Figure 1]. Microscopically, The difference from other species lies in its epidermis with straight, thick walls, and homogeneous mesophyll [5,6].

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1.2. Chemical Information

Its main phytochemical components include the monoterpenes and sesquiterpenes of its essential oil, among them, carquejyl acetate (commonly its chemical marker), carquejol, β-pinene, ledol, germacrene D, and bicyclogermacrene, which can vary significantly according to the region, where the species is found, as well as the presence of other terpenoids in extracts, such as hawtriwaic acid, in addition to those with clerodane and neo-clerodane nuclei, also including diterpene lactones [7,8]. The presence of phenolic and

Figure 1: *Baccharis genistelloides* subsp. *crispa*. Left: taxon of the species (Source: https://powo.science.kew.org); Center: plant with inflorescences (Author: Stéphane Bello); Right: inflorescences in detail (Source: https://www.biodiversity4all.org).

polyphenolic compounds, such as the flavonoids quercetin, rutin, quercitrin, kaempferol, luteolin, and apigenin glycosides, in addition to coumaric, caffeic, chlorogenic, ellagic, and gallic acids, as well as their derivatives, is also notable. Furthermore, some phytosterols, such as 3, 16-dihydroxyolean-12-en-28-oic acid, and its saponins, in addition to caffeine, were also found in carqueja [8].

1.3. Pharmacological Importance

The diversity of active components is responsible for the different pharmacological activities of carqueja, such as antiulcer, anti-inflammatory and antiparasitic activity, as well as its uses in folk medicine, among which the most notable are antidyspeptic use, treatment of stomach pain, diabetes, as well as a liver protectant and weight-reducing agent [2,4]. At the same time, substances isolated from carqueja have also demonstrated high pharmacological potency, such as carquejol congeners and some clerodane diterpenes, specifically acting on gastrointestinal mechanisms [9,10]. However, due to the emergence of new studies in recent years and the need for a global understanding of the action of carqueja in the digestive system, this review aims to compile and discuss studies related to its digestive pharmacological activities, providing the reader with an integrated, critical and updated view.

2. METHODOLOGY

The study employed specific search terms such as "*Baccharis genistelloides*" or "*Baccharis trimera*" and "activity" or "chemical" to identify relevant articles. Three research databases were utilized, including Science Direct (602 documents), Scopus (226 documents), and Google Scholar (8573 documents). The initial selection excluded most of the documents since they were not related to the topic or were only citations. In the second selection step, duplicate articles or those citing pharmacological studies that were not related to digestive activity were eliminated. Moreover, studies that described only basic chemical assays were also excluded. Following meticulous selection, 19 documents relating directly to the central theme and 11 describing relevant chemical identification were included and discussed in the succeeding review sections.

3. CHEMICALS FROM *B. GENISTELLOIDES* SUBSP. *CRISPA*

3.1. Essential Oil

The percentage of major compounds from the whole fresh flowering branches varied between male and female specimens. While the (E)-caryophyllene (16.1%), α-cadinene (11.6%), and α-cadinol (8.5%) are the main constituents in females, the α-humulene (20.3%), germacrene D (12.9%), and (E)-caryophyllene (10.2%) are the major substances in males. Furthermore, the hydrocarbon sesquiterpenes total composition is enhanced in male specimens, whereas oxygenated sesquiterpenes were predominant in females [11]. In other study, the GC/MS analyses of volatile compounds from cladodes and inflorescences from male and female specimens of *B. genistelloides* var. *crispa* collected in the states of Paraná and Santa Catarina, Brazil, showed that carquejyl acetate was the main substance (38–73%), while carquejol and ledol were present in lower concentrations [12]. On the other side, the monoterpenoid carquejol was found to be the major constituent of the essential oil from aerial parts collected in Estación Porvenír, Paysandú, Uruguay [13]. Furthermore, the carquejyl acetate (52.7%) and limonene (18.6%) were the main constituents in aerial parts of samples collected in Piraquara, Paraná, Brazil [14], whereas Bueno *et al*. [15] informed that the carquejyl acetate (65.1%), ledol (7.7%), and carquejol (6.2%) were the main substances in fresh aerial parts essential oil collected in Ponta Grossa, Paraná [Table 1].

3.2. Fatty Acids

Palmitic acid (68.93%) was the major fatty acid constituent of leaf extract, obtained by supercritical fluid extraction [16] [Table 1].

3.3. Phenolic and polyphenolic constituents

The glycosylated flavone rutin, the phenolic monomer ellagic acid, and the phenolic chlorogenic acid were the main constituents of the phenolic and polyphenolic classes in samples collected in the Rio Grande do Sul state, Brazil. In this study, it was also observed that the specimens collected in coal industry region produced lower phenolic content [17]. Besides chlorogenic acid, other caffeoyl and dicaffeoylquinic acids were also identified for this species [10]. Moreover, gallic acid, caffeic acid and the flavonoids quercetin, quercitrin, kaempferol, luteolin, catechin, and epicatechin are also found in significant concentrations, mainly when referred to the first three flavonoids mentioned [18]. In other studies, the flavonoids genkwanin, cirsimaritin, eupatorin, nepetin, apigenin, and hispidulin were also found [19-22] [Table 1].

3.4. Saccharides

The monosaccharide composition of *B. genistelloides* subsp. *crispa* showed fructose (93%), uronic acids (5%) and arabinose (2%),

Table 1: Main phytoconstituents of *Baccharis genistelloides* subsp. *crispa*.

Metabolite class	Main substances	References
Essential Oil (mono and sesquiterpenoids)	(E)-caryophyllene (16.1%), α-cadinene (11.6%) and α-cadinol (8.5%) (female); α-humulene (20.3%), germacrene D (12.9%), and (E)-caryophyllene (10.2%) (male)	[11]
	Carquejyl acetate (38–73%), carquejol and ledol	[12]
	Carquejol (main) and carquejyl acetate	[13]
	Carquejyl acetate (52.7%) and limonene (18.6%)	[14]
	Carquejyl acetate (65.1%), ledol (7.7%), and carquejol (6.2%)	[15]
Fatty acids	Palmitic acid (68.93%)	[16]
Phenolic and polyphenolics	Phenolic acids: Ellagic acid, gallic acid, chlorogenic acid, caffeic acid, 5′-O-caffeoylquinic acid, 1′-5′-O-dicaffeoylquinic acid; Flavonoids: Rutin, quercitrin, quercetin, kaempferol, luteolin, catechin, epicatechin, cirsimaritin, eupatorin, nepetin, apigenin, and hispidulin	[10,17-22]
Saccharides	Fructose, uronic acids, arabinose, and inulin-type fructan	[23]
Saponins	Glycosides of echinocystic acid or its enantiomer	[24]
Terpenoids	Clerodane and labdane type diterpenoids, triterpenoids	[10,25]

with polysaccharides presenting an inulin-type fructan as the main representant [23] [Table 1].

3.5. Saponins

Glycosides of echinocystic acid or its enantiomer are the main saponins of *B. genistelloides* var. *crispa* [24] [Table 1].

3.6. Terpenoids

The diterpenoids from clerodane and labdane types, as well as triterpenoids are found in leaves of *B. genistelloides* subsp. *crispa* [10,22,25] [Table 1].

4. EFFECTS OF *B. GENISTELLOIDES* SUBSP. *CRISPA* ON DIGESTIVE SYSTEM

4.1. Gastric Function

Regarding the gastric function, several studies have been carried out to investigate the effect of carqueja in the treatment of stomach ulcers, including the recent work of Bueno *et al*. [15], in which they indicated that the *B. genistelloides* subsp. *crispa* essential oil (EOBG) was effective in preventing ulcer and accelerating healing by decreasing myeloperoxidase (MPO) activity, increasing vascular endothelial growth factor (VEGF) expression, and decreasing metalloproteinase matrix metalloproteinase (MMP)-2 activity. The pretreatment with EOBG (100 and 200 mg/kg) significantly reduced the severity of ethanol-induced gastric lesions and decreased MPO activity in gastric tissue, promoting 94% and 98% recovery of the lesion area respectively, so that the EOBG treated group showed better resolution the positive-control group (carbenoxolone 100 mg/kg). Furthermore, in an acetic acid-induced ulceration model, after 10 and 14 days of daily EOBG treatment (100 mg/kg), the lesion area was reduced by 61% and 65.5%, respectively, compared to the control group. The healing effect of EOBG was associated with a decrease in cyclooxygenase-1 expression and an increase in VEGF expression, surpassing the effect of the anti-ulcer drug lansoprazole. Analysis of MMP-2 and MMP-9 activities confirmed the accelerated healing effect, with a significant reduction in pro-MMP-2 activity. Furthermore, no signs of toxicity were observed after 14 days of EOBG treatment, which suggests the safety of carqueja use [15].

Previously, antiulcer/antiacid activity studies, as well as gastroprotective effect of carqueja were evaluated through different models. Días *et al*. [26] demonstrated that the oral administration of raw extract (400 mg/kg) decreased the damaged stomachic area to only 10% [26], whereas Biondo *et al*. [10] showed that the intraduodenal administration of the aqueous extract (2 g/kg) caused inhibition of the histaminergic pathway and proton pump and that its oral administration at the same concentration culminated in the reduction of acid secretion by 50%, reduction of lesions by 41% and the number of ulcers by 52%. When tested with secretagogues, carqueja treatment reduced histamine-induced [14C]-AP uptake, suggesting that certain constituents of carqueja inhibit the histaminergic pathway regulating acid secretion, contributing to its anti-ulcer properties. The plant also lowered [14C]-AP accumulation triggered by carbachol (agonist cholinomimetic drug), indicating inhibition of the cholinergic regulatory pathway. These findings suggest that the inhibition of [14C]-AP uptake by both cholinergic and histaminergic stimuli, likely due to the flavonoid and diterpene fractions, points to a potential inhibition of H^+/K^+-ATPase, the final step in acid secretion [10]. The gastroprotective effect was also observed for the hydroethanolic and aqueous extracts of carqueja, which, at the dosage of 600 mg/kg, were able to promote the reduction of hemorrhage, inflammation, and hyperemia [27], whereas the hydroethanolic extract at 30 mg/kg reduced the oxidative stress and injuries in the stomach tissue. However, intraduodenal, intraperitoneal, and oral administration of the hydroethanolic extract did not alter the volume, total acidity, or peptic activity of gastric contents in rats with hypersecretion induced by a 4-h pylorus ligation. These findings contradict previous reports indicating that *B. genistelloides* subsp. *crispa* reduces acid secretion, thereby enhancing gastric protection [28].

Lately, another study demonstrated that intraperitoneal and intraduodenal administrations of *B. genistelloides* subsp. *crispa* aqueous extract (1 g/kg) in rats led to a reduction of gastric secretion collected 4 h after pylorus ligation (93% and 85%, respectively). The treatment lowered total acid secretion and increased gastric pH to levels comparable to ranitidine, an H_2 receptor antagonist used as a positive control. In addition, the extract caused smooth muscle relaxation in isolated jejunum samples. Pretreatment with *B. genistelloides* subsp. *crispa* 1–2 g/kg decreased the ulceration index (76% and 84%, respectively) and reduced the number of ulcers (40% and 60%) caused by immobilization at 4°C for 2 h, although it did not protect against indomethacin-induced gastric ulcers [29].

4.2. Hepatoprotective Action

The ethnobotanical reports on the use of carqueja in the treatment of liver diseases allowed the development of several studies on this topic, mainly focused on the determination of liver markers such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In this sense, the study of Mendes *et al*. [30] revealed that the treatment with 30 mg/kg *B. genistelloides* subsp. *crispa* ethanolic extract completely

reversed the increase in ALT, AST, albumin, urea, and creatinine levels that were altered by risk factors in rats (hypertension, dyslipidemia, and smoking). Interestingly, the dosage of 100 and 300 mg/kg extract and simvastatin + enalapril only partially reversed these changes. On the other hand, the globulin levels were not altered by the administration of carqueja extracts. Moreover, the results were corroborated by histological evaluation, since the doses of 30 and 100 mg/kg exerted minor lesions in liver tissue, whereas the 300 mg/kg concentration caused moderate lesions [30]. This effect was consistent with previous work conducted by Rabelo *et al*. [8,31] and Barbosa *et al*. [32], which confirmed that the ethanolic (30 and 100 mg/kg), hydroethanolic (600 mg/kg), and aqueous (600 mg/kg) extracts decreased the ALT and AST levels by oral administration so that it was also demonstrated the hepatoprotective activity in both acute and chronic intoxications models provoked by ethanol administration [8,31,32]. Still, the administration of carqueja tincture for 28 days induced a significant decrease in the AST levels in rats of both sexes in different doses (100, 200, and 400 mg/kg). The ALT activity also decreased in the rats treated with 200 and 400 mg/kg of tincture [33]. Moreover, Pádua *et al*. [34] also observed the inhibition of these transaminases by 3–5 fold in animals poisoned with paracetamol, when used the hydroethanolic extract at the concentration of 600 mg/kg [34]. In other study, the same type of extract, using the concentration of 30 mg/kg, also decreased AST and ALT levels by 92.88% and increased triglyceride excretion by 35.73%, also reducing the reactive oxygen species [35]. However, the work of Grance *et al*. [36] demonstrated that the administration of carqueja hydroethanolic extract at 8.4 mg/kg in pregnant rats produced diffuse cell swelling, leading to vacuolar degeneration, mainly in periportal hepatocytes, revealing to be toxic to maternal kidney and liver cells, although such alterations are reversible if the administration is discontinued [36]. Moreover, Torres *et al*. [37] informed that the hydroethanolic extract of carqueja (1–3.3%) potentiated the activity of glutathione-transferases, which, in turn, are important actors in the hepatic detoxification function [37]. Still, it was observed that the essential oils from carqueja, especially that are rich in carquejol derivatives, enhance the bile production and avoid the membrane lipid peroxidation (LPO) in hepatocytes [38]. Finally, the soluble polysaccharide fraction of the infusion (1 mg/kg), rich in inulin-type fructans, caused a decrease in plasma levels of ALT, AST, alkaline phosphatase, and LPO [23].

4.3. Intestinal Function

Some ethnomedicinal studies report the use of carqueja for intestinal ailments, such as the treatment of diarrhea and abdominal pain [4]. In this sense, the work of Gamberini *et al*. [29] revealed that the aqueous extract of carqueja (0.5–8 mg/mL) provided a reduction of jejunum smooth muscle contraction, which was dependent on the administrated dose and the extension of previous organ tonus, although it did not alter the maximum contractile response caused by acetylcholine. Furthermore, the pretreatment with 2 g/kg extract, 30 min before the administration of charcoal, reduced its small bowel presence by 20% in comparison to the control [29]. Likely, the hydroethanolic extract from leaves and stalks was also effective as an antispasmodic in isolated rat jejunum with an optimal concentration of 1 mg/mL, which, in turn, produced an antispasmodic effect of 79.65%, being significantly more active than atropine and hyoscine, the positive controls [39]. Besides this, the methanolic extract (100 mg/mL) produced an *in vitro* inhibition of both pancreatic lipase and α- and β-glycosidases, which, in turn, catalyze triglycerides and carbohydrates, respectively, decreasing their absorption by the small bowel, a mechanism known to contribute to weight loss [19].

5. CRITICAL DISCUSSION

Studies of the pharmacological effects of *B. genistelloides* subsp. *crispa* on the digestive system have focused on its activities of inhibiting gastric secretion, treating ulcers, regulating markers of hepatic toxicity, and relaxing the intestinal smooth muscle [Figure 2]. Although all the effects investigated have been observed, ethnomedicinal reports include other types of use for gastrointestinal disorders that have not yet been clarified, such as abdominal pain, diarrhea, and indigestion, although mechanisms inherent to the phytochemicals present in its extracts may be suggested.

The use of carqueja for the treatment of gastric ulcers has been based on its effects of inhibiting the histaminergic and cholinergic pathways of stomach acid production, in addition to its anti-inflammatory properties, already mentioned in other studies with the species [20,24,40,41]. However, the preference or purpose of use varied extensively in ethnomedicinal reports. This fact, besides ethnobotanical reasons, probably is also linked to the variation in the

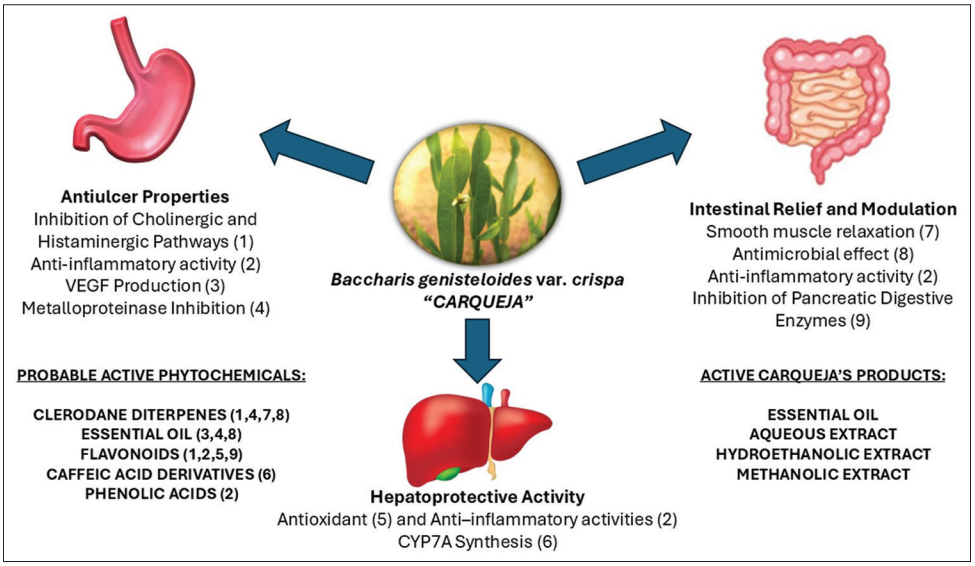

Figure 2: Effects of *Baccharis genistelloides* subsp. *crispa* on the digestive system and probable-related mechanisms of action.

chemical composition of products derived from carqueja [Table 1], as seen in some studies, which may depend on the place where the plant was collected, occurrence of varieties, extraction techniques, and storage. On the other hand, convergences and complementation are observed in several pharmacological studies that described the action of carqueja on digestive system. The study by Bueno *et al*. [15] reported that the main constituents of the essential oil were carquejyl acetate (65.1%), ledol (7.7%), and carquejol (6.2%), and that the essential oil was able to increase VEGF production and inhibit the activity of metalloproteinases [15], while the study conducted by Biondo *et al*. [10] suggests that the inhibition of the gastric cholinergic and histaminergic pathways by the aqueous extract is related to the presence of flavonoids and, mainly, a clerodane diterpene [10]. In its turn, Lívero *et al*. [28] reports that the hydroethanolic extract did not show gastric acid secretion inhibition activity, despite observing gastroprotective effects, which may be explained by the administration of lower doses when compared to the aforementioned works and even by a probable difference in their chemical compositions [28].

On the other hand, the hepatoprotective activity of carqueja is probably related to its high concentration of flavonoids and phenolic derivatives, which, in turn, have recognized antioxidant and anti-inflammatory activity [40,42]. In addition, essential oils and phenolic derivatives are involved in the increase in bile production and excretion, which facilitates the digestion of fats, which may explain its digestive use [43-45]. It was observed that caffeic acid derivatives from the genus *Baccharis* induced expression of cholesterol 7α-hydroxylase (CYP7A, the enzyme that initiates the classic bile acid biosynthetic pathway) [46]. Furthermore, the intestinal activity of carqueja appears to have several origins, including the relaxation of smooth muscle, which, together with its anti-inflammatory and antimicrobial activities, would be responsible for its use in the treatment of diarrhea, including those caused by enterotoxic bacteria, including *Salmonella typhi*, *Enterococcus faecalis,* and *Escherichia coli*, since its crude hydroethanolic extract showed action against them [47,48]. These biological activities probably contribute to a synergistic effect so that essential oils have potent antimicrobial activity, as well as the diterpenes, which also influence the intestinal muscle relaxation observed in the study by Gamberini *et al*. [29], whereas the anti-inflammatory activity is an important contributor factor for the intestinal regulation and closely related to the presence of flavonoids and phenolic derivatives in carqueja [29].

Furthermore, the treatment of intestinal and hepatic disorders caused by parasitosis is also related to the presence of clerodane diterpenes and essential oils, which have demonstrated high parasitic potential against *Schistosoma* so that epiisopiloturine and piplartine are essential oil components with high anti-*Schistosoma* activity, while clerodane diterpenes have also been related to this activity, specially against *Entamoeba histolytica* and *Giardia lamblia* [49-51]. In addition, flavonoids and phenolic acids are known inhibitors of some digestive enzymes, which may explain the action of the methanolic extract on pancreatic lipase and glycosidases [52-54].

It is also important to mention that the diversity and divergences of ethnomedicinal reports can be explained by the different chemical profiles found for carqueja [Table 1], such as in its essential oil, which has carquejyl acetate as one of its main markers. However, in some different studies, this substance and its structural congeners such as carquejol are not found, which can make a notable difference in terms of biological activity, since among these, there are substances that have high pharmacological potency [7,11,55,56].

According to the various pharmacological studies presented in this review, *B. genistelloides* subsp. *crispa* represents an effective and multi-target alternative for the treatment of gastric, hepatic, and intestinal disorders. It exhibits a broad diversity of uses within the digestive system, even when compared with other plant species that are globally recognized for their digestive applications, such as *Cynara scolymus*, *Taraxacum officinale*, *Mentha piperita*, *Zingiber officinale*, *Plantago ovata*, and *Silybum marianum* [57]. Even when compared with another species of the same genus with gastrointestinal applications, *Baccharis dracunculifolia*, carqueja demonstrates greater potency of its essential oil for the treatment of ulcers, as well as a broader range of pharmacological actions within the digestive system, although *B. dracunculifolia* has also been shown to be effective in the treatment of colitis [58]. However, despite the *in vivo* pharmacological studies conducted, some shortcomings can be identified, such as the absence of test groups using some isolated compounds from the species. This would provide greater robustness in explaining the mechanisms of action associated with each type of activity in the digestive system, as well as allowing a comparison with the administration of extracts, which would help assess the extent of the potential synergistic effect. Furthermore, to have a global understanding of the biological mechanisms of action of carqueja, pharmacological studies using ethnomedicinal preparations, such as infusions or decoctions, should be carried out. This could help to better understand the real properties of carqueja when used in a popular way, understanding how relevant are the effects observed with extracts, essential oils, and fractions, when administered together.

6. CONCLUSION

Carqueja (*B. genistelloides* subsp. *crispa*), a phytotherapeutic and ethnomedicinal resource widely used in the treatment of disorders in the digestive system, has demonstrated pharmacological actions through the improvement of gastric ulcers, as well as hepatoprotective and choleretic effects, relaxing intestinal smooth muscle, in addition to inhibiting digestive enzymes such as pancreatic lipases and glycosidases. Its effects are mainly related to the presence of flavonoids, phenolic derivatives, clerodane diterpenes, and components of its essential oil so that important mechanisms of action have been elucidated, such as the inhibition of gastric acid and metalloproteinase production and regulation of liver enzymes, and which, added to the already established anti-inflammatory and antimicrobial effects of carqueja, explain the diverse and potent use of this species in the treatment of digestive diseases.

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8. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data are available with the authors and shall be provided on request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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