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Bocconia frutescens L. induces neurological defects in rat offspring

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Original Article

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Abstract

Nearly 80% of the world's population trusts traditional medicine and plant-based drug compounds to improve health, and more than 50% of women who participated in a study have used herbal remedies during pregnancy. *Bocconia frutescens* L. is a plant native to tropical America, where infusion of its leaves has been widely used for the treatment of several gastrointestinal disorders. We have already shown that orogastric consumption of *B. frutescens* L. during the organogenesis period at concentrations equivalent to human consumption produces teratogenic effects in rats, but effects on progeny development have not yet been studied. In this study, we aimed to investigate the possible association between the consumption of *B. frutescens* L. at a dose equivalent to that consumed by humans and the neurological development of rat progeny. Pregnant Wistar rats were administered lyophilized *B. frutescens* L. extract at 300 mg/kg/day or vehicle via the orogastric route during the organogenesis period (gestation days 7–13). The physical development and sensory and motor maturation of their offspring during lactation were analyzed with a battery of reflex and physical tests. *B. frutescens* L. produced a significant delay in physical development and sensorimotor maturation, compared to the control group. Proton nuclear magnetic resonance spectroscopy analysis showed signals for both flavonoids and alkaloids in the *B. frutescens* L. extract. We conclude that the delay in physical and neurological development could be interpreted as alterations in the maturation of some neuronal circuitries induced by *B. frutescens* L.

Introduction

Humans have used traditional medicine (TM) ancestrally. Some people use it as the mainstay of healthcare delivery and others as complementary medicine (CM).[1] Nearly 80% of the world's population relies on TM and plant-derived drug compounds to improve health, as opposed to licensed conventional medicines (40%).[2] Herbal medicine practices are usually not supported by studies on their effectiveness or safety, and there are concerns about the potential risks involved in their use.[3] For this reason, the WHO Traditional Medicine Strategy 2014–2023 was created to set the course for TM and CM.[1] In Mexico, we have around 4,500 medicinal plant species, and many of them are recognized and used by ~ 90% of the general population. Approximately 85% of healthcare professionals know about herbal medicines, and ~ 75% recommend their use.[4]

Pregnant women make use of TM for several reasons: lack of fetal turning, overdue delivery, breech pregnancy and false labor, morning sickness, abdominal pain, constipation, heartburn, dirty or air-filled uterus, sexually transmitted diseases, and high blood pressure.[5,6] Holst *et al.*[7] found that more than 50% of the pregnant women they studied used herbal remedies.

Bocconia frutescens L. is a plant native to tropical America, where it is commonly known as gordolobo, guauchilli, palo amarillo de México, llora sangre, mano de león, palo santo[8], or boconnia, parrotweed, plume poppy, sea oxeye daisy, tree poppy, and tree celandine. The infusion of its leaves has been traditionally used in extensive parts of the American continent for the treatment of gastrointestinal disorders, skin ulcers, dermatitis, some respiratory tract infections, and tuberculosis.[9,10] Additionally, in rats, *B. frutescens* L. exhibited potent antidiarrheal activity[9] and reduced peristalsis activity.[11]

Phytochemical analyses of *B. frutescens* L. have detected high levels of saponins and coumarins, as well as flavonoids, sesquiterpene lactones, alkaloids,[10] and cardiotonic in chloroformic, ketonic, and ethanolic extracts, whereas tannins and sterols were not detected. The effects of consuming some metabolites such as alkaloids during pregnancy must be evaluated since exposure to these compounds may cause defects in developing embryos and fetuses.[12-16] In fact, in a previous study, we showed that orogastric consumption of *B. frutescens* L. at concentrations equivalent to those used for human consumption[11] during the

organogenesis period produces teratogenic effects, including external (umbilical hernia and growth retardation) and internal alterations (central rib ossification).[17]

In addition, during gestation, external factors such as inadequate maternal diet and exposure to pollutants and toxins significantly affect the development of the fetal nervous system and may therefore increase the risk of neurodevelopmental/psychiatric disorders.[18,19] It is unknown whether consumption of *B. frutescens* L. during pregnancy alters the neurodevelopment of the progeny. Given that maturation of neurological reflexes and motor coordination are hallmarks of nervous system development,[20] in the present study, we used a battery of noninvasive tests to quantify the sensory and motor function of rat pups; these tests included measurements of physical development, reflex testing, and locomotor maturation.[21] The aim of this study was to investigate the possible association between the gestational consumption of *B. frutescens* L. at a dose equivalent to that consumed by humans and the neurological development of rat progeny.

Materials and method

Plant material

Leaves of *B. frutescens* L. (Papaveraceae family) were collected in Ixhuatlancillo, Veracruz-México (18° 53′ 32″ N, 97° 8′ 51″ W), in June 2017. Plant material was collected by S. Delgado Rodríguez no. 510, and the exsiccate was deposited at the herbarium of the Biological Research Institute, Universidad Veracruzana, under no. 25585UV. In this study, we used a wild plant collected from the ecotone between tropical and temperate zones, and our work posed no risk toward this species. During the collection, we carefully collected a small amount of leaves so as not to cause any damage to the plants.

Preparation of extract

Plant material was dried in an oven at 35°C–40°C for a period of 72 h and ground in a conventional mechanical mill until a fine powder was obtained. The powdered plant material (50 g) was then macerated for 72 h with 300 ml of reagent-grade hexane at room temperature and protected from light for a week. The solvent was removed at reduced pressure using a rotary evaporator (Heidolph Laborota 4001) at 35°C–40°C to obtain a green oily residue. This residue was dried in a vacuum oven (Thermo Scientific) to constant weight (0.4 g) with a yield of 0.8%, and the recovered plant material was macerated in 96% methanol (300 ml) for 72 h and protected from light at room temperature. The methanol was removed in a rotary evaporator (Heidolph Laborota 4001) at reduced pressure to obtain a green oily residue which was dried in a vacuum oven (Thermo Scientific) to constant weight (1.3 g) with a yield of 2.6%. Subsequently, the product was lyophilized (lyophilizer Labconco, USA) at −50°C, pressure 0.002 bar, to obtain the plant extract. For administration to the animals, the extract was diluted in canola oil (Capullo®) heated at 50°C as a vehicle.

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy

The ^{1}H-NMR data was recorded on an Agilent Technologies Model DD2 nuclear magnetic resonance spectrometer at 25.0°C, operating at a proton NMR frequency of 500 MHz. For this process, 30 mg of the extract was dissolved in 0.6 ml methanol-*d4* (99.8% D, Sigma-Aldrich Co., St Louis, MO, USA). The parameters consisted of 64 scans and relaxation time = 2 s. A pre-saturation sequence was used to suppress the residual H_2O signal with low selective irradiation power at the H_2O frequency during the recycling delay. Prior to the Fourier transform, a line broadening factor of 0.3 Hz was applied to the free induction decay data. Tetramethylsilane 0.00 parts per million was used as a reference, and NMR data were processed using MestReNova v12.0.4 software (MestreLab Research SL). The presence of alkaloids and flavonoids was detected by comparison with previously reported studies.

Animals and experimental design

The experiments were carried out with Wistar rats from the Animal Facility in the Health Sciences Unit, Universidad Veracruzana. All procedures described here fully complied with the National Guidelines for Production, Care, and Use of Laboratory Animals (Norma Oficial Mexicana NOM-062-ZOO-1999) and followed the ethical principles of animal care based on the international standards.[22]

Virgin female Wistar rats, weighing 200 ± 10 g, were housed in polypropylene cages (32 × 47 × 20 cm), with a stainless-steel wire cover. The cages containing a bed of pine shaving and a maximum of five animals per cage were kept in a temperature-controlled room (22°C ± 2°C) with a 12/12-h light/dark cycle. The rats had free access to food (Rodent Lab Chow 5001®, Purina) and water.

After a two-week period of habituation to laboratory conditions, the rats were mated with sexually experienced males. We considered gestational day 0 the day that sperm cells appeared in a vaginal smear. Pregnant animals were housed individually and randomly assigned to the control group (Ctrl, $n = 7$) or the *B. frutescens* L. group (Bocf, $n = 7$). Bocf females were administered lyophilized extract of *B. frutescens* L. in a single orogastric dose of 300 mg/kg/day, using an adapted intravenous catheter of 16G × 47 mm. Given that no toxicity studies for rats have been reported with this extract, we decided on a dose of 300 mg/kg/day, which is closely equivalent to one cup of *B. frutescens* L. used by Mexican and Central American people in infusions or decoctions taken three times/day for the treatment of gastrointestinal disorders such as diarrhea.[11] This dose also corresponds to that used in studies with animal models.[9,11]

Ctrl rats were administered only canola oil orogastrically. We administered *B. frutescens* L. extract or vehicle from day 7 to day 13 of gestation, which constitutes the period of organogenesis. The body weight of each mother was recorded on gestational days 0 and 21.

Assessment of pup development

Litter features and physical development

The weight of each litter was recorded at birth, as well as the number of pups per litter. The days each pup opened both eyes and their two incisors erupted were also recorded.

Sensorimotor maturation and reflex testing

One day after parturition, entire litters were removed from their does and one male and one female pup from each litter were randomly selected, marked, and individually tested in a different temperature-controlled room (32°C ± 2°C), with a very low level of ambient noise. At the end of the daily tests (3–5 min), the two selected pups per litter were returned to the litter and then simultaneously returned to the respective doe cage. Tests included auditory startle, grasp reflex, negative geotaxis, inclined board, and righting reflex;[21] all tests were conducted blindly by the same researcher.

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a) Auditory startle. A clicker was used at a distance of 30 cm above each pup, and we observed if the animal displayed a startle response immediately after the click. The day of appearance of this reflex was recorded.

b) Grasp reflex. Each pup was held in the air, and the forelimbs were gently stroked with a thin rod. The reflex was considered present when the animal closed the stimulated paw around the rod, and the first day of grasping was recorded.

c) Negative geotaxis. Each pup was placed on an inclined board with its head facing downward. The time taken for the pup to turn around 180° to put its head upward was recorded. If the pup took longer than 60 s, the test was stopped and recorded as 60 s. From postnatal day (PD) 1 to 10, the board had an inclination of 20° and from PD 11 to 17, an inclination of 50°.

d) Inclined board test. For this test, we used a wooden board with a previously graduated inclination scale. In the beginning, pups were placed on the inclined board with their head facing upward at an angle of 20°. Every 5 s, the board inclination was elevated by 5°, and the maximum angle the pup maintained its position on the board was recorded. The test was carried out daily from PD 10 to 21.

e) Righting reflex. Each pup was placed in the supine position, with the dorsum of its head and trunk in contact with the surface. The time taken for the pup to turn around its longitudinal axis and to put its four paws in contact with the surface was recorded. If the pup took longer than 120 s, the test was stopped and recorded as 120 s. This test was carried out daily from PD 1 to 10.

Statistical analysis

The body weight of each mother (difference between gestational day 0 and day 21) and the number of pups per dam were analyzed using Student's *t*-tests. Temporal comparisons of pup weight, righting reflex, negative geotaxis, and inclined board between groups were analyzed using a generalized linear model with a design of a repeated measures factor (day) across testing sessions and comparison between treatment and sex. Since there was no difference in pup weight between females and males ($F_{1,24} = 0.67$, $p = 0.4$), data of pups from the same litter were pooled, and the factor sex was excluded for statistical parsimony of the repeated measures models. Two-way Analysis of Variance (ANOVA) model analyses were applied for eye opening, incisor eruption, auditory startle, and grasp reflex, variables that only had a single record in the study subjects. In addition, we estimated Cohen's d for the variables number of pups, eye opening, incisor eruption, auditory startle, and grasp reflex to measure the effect size. The data were tested previously for homogeneity and normality of variances and rank-transformed when necessary,[23] and the results were expressed as mean ± standard error (SE). Differences between groups were considered statistically significant at $\alpha = 0.05$. All statistical analyses were performed using JMP Pro 14.0.0 (SAS Institute, Inc. Cary, NC, USA, 1989–2014).

Results

Body weight gain during gestation of Ctrl and Bocf females was similar (Ctrl: 33.77 ± 5.31 SE, Bocf: 24.46 ± 5.31 SE, *t*-test = −1.24, $p = 0.87$). Also, days of gestation did not differ between Ctrl and Bocf groups (Ctrl: 22.71 ± 0.12 SE, Bocf: 22.71 ± 0.12 SE, *t*-test = 0.1, $p = 0.5$). However, Ctrl dams had more pups per litter than Bocf mothers (*t*-test = 3.63, $p = 0.003$; Fig. 1). Even so, Ctrl pups were heavier at birth than Bocf pups, and the difference increased during lactation (Table 1 and Fig. 2a). Differences in the physical development of the pups were also evident in eye opening and incisor eruption. In both variables, the offspring of the Bocf group showed a delay of more than 2 d with respect to the control group (Table 2 and Fig. 3a, b). In both cases, we found a very large Cohen's *d* between the Ctrl and Bocf groups (Fig. 3).

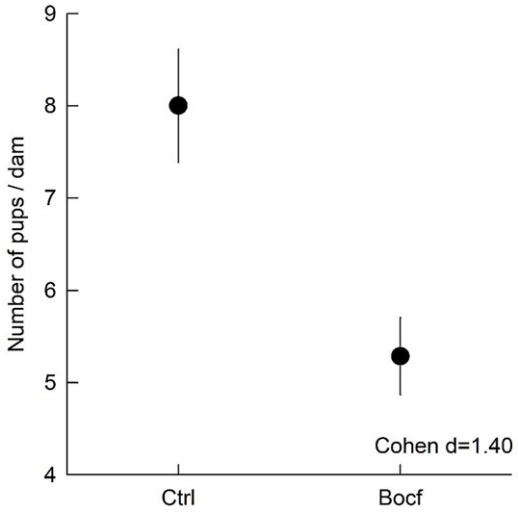

Figure 1. Number of pups per dam. Independent student *t*-test was performed. Values represent the mean ± SE, and size of the effect is indicated with Cohen distance.

We tested pups for negative geotaxis with a board inclined 20° from PD 1 to 10 and from PD 11 to 17 with an inclination of 50°. We found significant differences between groups; the offspring of the Bocf group took longer to turn 180° (Table 1 and Fig. 2b).

In the inclined board test, there were significant differences between groups, showing that Bocf pups had less strength to maintain their position on the inclined board from PD 11 to 14 (Table 1 and Fig. 2c).

The righting reflex test showed a better ability of Ctrl pups to turn around their longitudinal axis since birth. During the first 3 PD, Bocf pups took more time to complete the test, and it was not until the fourth PD that both groups recorded similar latencies (Fig. 2d). The statistical analysis revealed a significant group effect (Table 1).

We also recorded the day of appearance of the auditory startle and grasp reflex. Like our findings with eye opening and incisor eruption, Bocf pups exhibited the appearance of the auditory and grasp reflexes 2 d later than Ctrl pups (Table 2 and Fig. 3c, d). For both reflexes, we found a large effect size between Ctrl *vs* Bocf animals when estimated by Cohen's d.

Finally, ^{1}H-NMR analysis showed signals for both flavonoids and alkaloids in the *B. frutescens* L. extract (Fig. 4).

Discussion

Previously, we reported the teratogenic effects of *B. frutescens* L. in rats.[17] Now, our results demonstrate that the orogastric consumption of its extract at concentrations equivalent to those of human consumption during pregnancy may compromise the neural development of the progeny.

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Table 1. Variation source of repeated measures ANOVA for pup weight and reflex tests

		Pup weight	Negative geotaxis	Inclined board	Righting reflex
Variation source	Df	*F*			
Intercept	1	341.65***	596.48***	362.37***	356.03***
Treatment	1	71.48***	13.28**	27.47**	4.63*
Sex	1	1.34	0.001	0.17	0.04
Treatment × sex	1	0.001	0.34	0.14	0.35
Error	24				
Days	1	0.01	0.01	0.004	0.01
Days × treatment	1	1.22	1.84*	6.53***	2.34*
Days × sex	3	0.89	0.79	0.53	1.53
Treatment × days × sex	3	0.59	0.80	0.17	0.88
Error	480				

DF, Degrees of freedom; F, Fisher's test in ANOVA. Values with asterisks represent *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

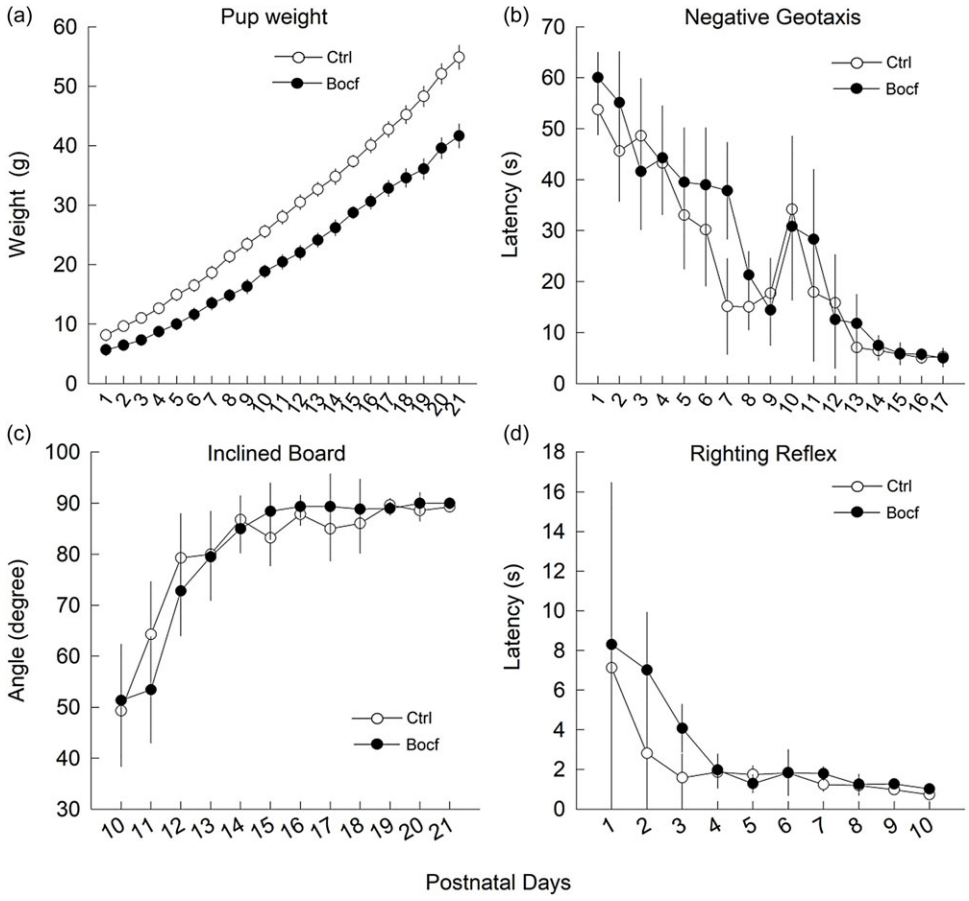

Figure 2. Pup weight, negative geotaxis, inclined board, and righting reflex. Pup weight between control (Ctrl) and *B. frutescens* L. (Bocf) groups (a). Effect of the Bocf treatment on daily performance in negative geotaxis 20° at PD 1 to 10 and 50° at PD 11 to 17. Latency refers to the time taken by the pup to turn around 180° to put its head upward (b). Effect of the Bocf treatment on daily pup performance in the inclined board test. The maximum angle of the position the pup maintained on the board is presented (c). Effect of the Bocf treatment on daily pup performance in the righting test. Latency refers to the time taken by the pup to turn around its longitudinal axis from a supine to a prone position (d). PD: postnatal day. The values represent the mean ± SE.

B. frutescens L. administration decreased the number of pups per litter and the weight of the pups. The causes of these effects are not clear; however, there are other medicinal plants whose consumption during pregnancy causes similar effects; for example, the administration of St John's wort (*Hypericum perforatum* L.) in experimental animals is associated with low birth weight.[24]

Eye opening and incisor eruption occurred 2 d later in Bocf pups. Accordingly, delayed eye opening and incisor eruption have been described in neurotoxicity studies. For example, in Sprague-Dawley rats exposed to inorganic arsenic.[25] Moreover, there are also reports of developmental delays in rat offspring when exposed to caffeine[26] or alcohol[27] or fed a high-fat diet.[28] Furthermore, there are a few studies showing developmental delays associated with medicinal plants, for example, *Mauritia flexuosa* L. f., a palm tree native to the South American Amazon rainforest commercialized as medicine; Medeiros *et al.*[29] reported negative impacts on the

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Table 2. Variation source of two-way ANOVA for eye opening, incisor eruption, and reflex tests

		Eye opening	Incisors	Auditory startle	Grasp
Variation source	Df	*F*			
Intercept	1	316.61***	126.09***	308.99***	136.43***
Treatment	1	72.99***	14.58***	71.67***	18.47***
Sex	1	0.87	0.13	0.23	0.01
Treatment × sex	1	0.1	0.73	0.002	0.05
		$R^2 = 0.73$	$R^2 = 0.30$	$R^2 = 0.72$	$R^2 = 0.37$
Error	24				

DF, Degrees of freedom; F, Fisher's test in ANOVA. Values with asterisks represent ***$p < 0.001$.

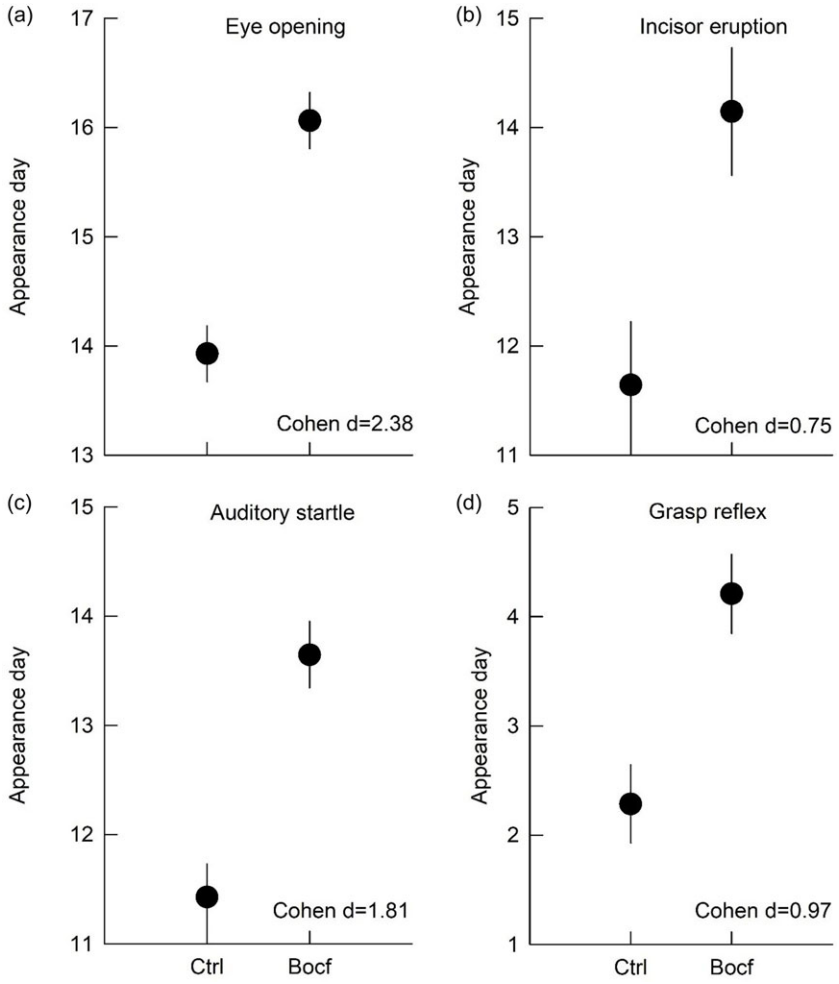

Figure 3. Appearance day of physical and sensory-motor development indexes of the control (Ctrl) and *B. frutescens* L. (Bocf) groups. Eye opening (a). Incisor eruption (b). Auditory startle (c). Grasp reflex (d). Values represent the mean ± SE, and the size of the effect is indicated with Cohen distance.

growth and maturation of neonatal rats after its consumption and attributed it to essential fatty acids and vitamins present in the plant.

Phytochemical analyses of *B. frutescens* L. have detected the presence of alkaloids, and the exposure of a developing embryo or fetus to these alkaloids from plants, plant products, or plant-derived extracts has the potential to cause developmental alterations.[13] For example, swainsonine, an indolizidine alkaloid, is the principal toxic component of leguminous plants of the *Astragalus* genus. It stimulates neurologic symptoms inducing neuron apoptosis through a death receptor pathway and endoplasmic reticulum stress.[30] Ibogaine, a psychoactive indole alkaloid characteristic of the shrub *Tabernanthe iboga*, may cause neurodegeneration in rats, probably mediated by stimulation of the inferior olive,[31] which coordinates spinal cord signals to regulate motor coordination.

An ^{1}H-NMR comparison of different samples of Mexican gordolobo showed characteristic signals for flavones gnaphaliin A,

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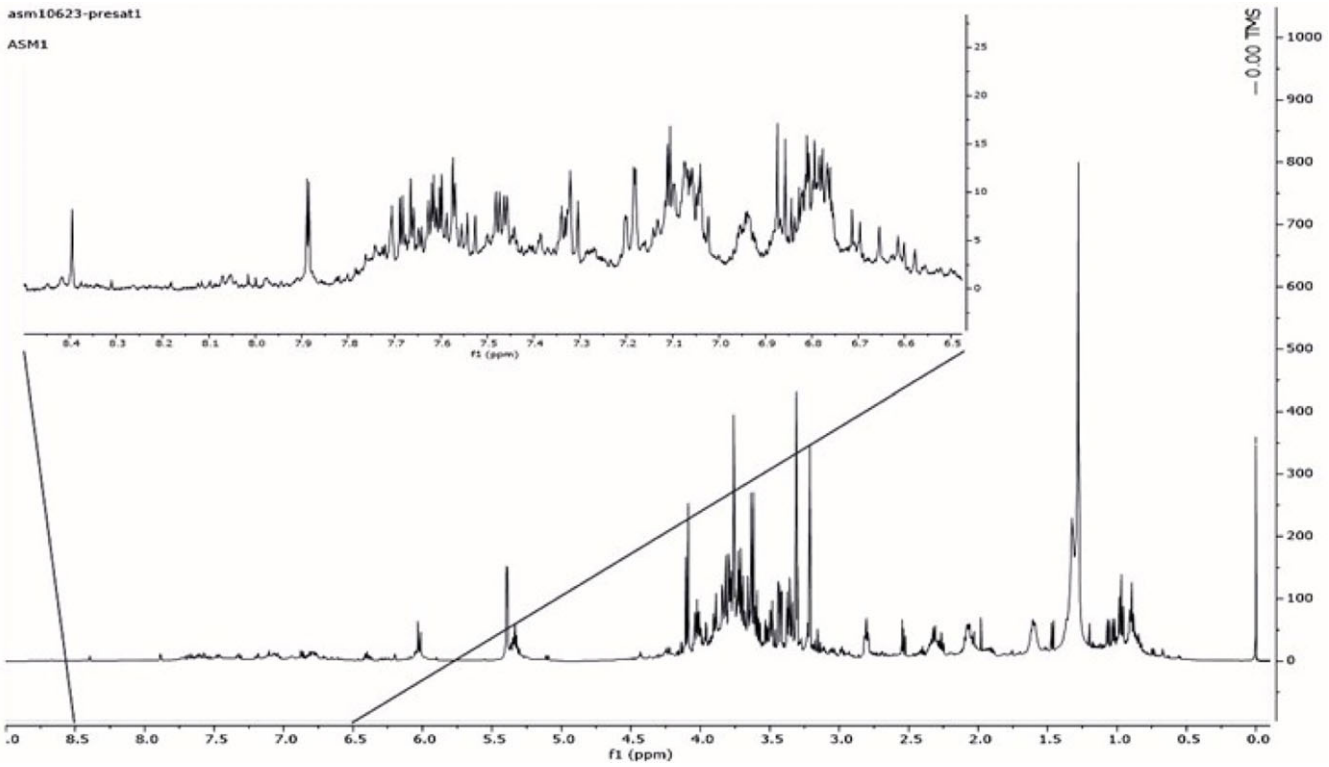

Figure 4. ^{1}H-NMR spectrum (500 MHz) of *Bocconia frutescens* L. extract δ 0.00–8.5 ppm. The amplified region corresponds to the aromatic region (δ 6.5–8.5 ppm).

gnaphaliin B, araneol, and 3,5,7-Tri-O-methylgalangin.[32] Additionally, the extract produced signals related to those reported for flavonoids and phenolic compounds in the aromatic region (δ 6.5–8.5 ppm). It is important to note that the presence of benzophenanthridine alkaloids such as chelerythrine, sanguilutine, sanguirubine, chelirubine, chelilutine, sanguinarine, nitidine, and fagaronine have also been reported in the aromatic region.[33] In the present study, we observed signals for both flavonoids and alkaloids in the *B. frutescens* L. extract by ^{1}H-NMR analysis. Benzophenanthridine alkaloids have been reported to possess cytotoxic effects by affecting DNA replication and cell cycle arrest. Specifically, sanguinarine, a quaternary benzophenanthridine alkaloid, can induce fragmentation of DNA,[16,34] and there is evidence *in vivo* and *in vitro* that it may cause negative effects on mouse embryonic development, both pre- and postimplantation, via dietary intake.[35]

As we mentioned previously, *B. frutescens* L. also contains coumarins, which induce developmental delay in the offspring of female mice who received a toxic isocoumarin, ochratoxin A, which is derived from *Aspergillus ochraceus* and several *Penicillium* species.[36]

The potential danger of environmental factors during intrauterine development is of particular concern because of its irreversible nature. Although herbs are natural, not all are safe to be ingested during pregnancy. It is necessary to determine the chemical components and mechanism of action of traditional plants before recommendation.

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Competing interests. None.

Ethical standard. The authors assert that all animal procedures contributing to this work were strictly in accordance with the ethical standards of the relevant national guidelines on the care and use of laboratory animals (Norma Oficial Mexicana NOM-062-ZOO-1999) and has been approved by the Animal Use Ethics Institutional Committee (Comité Interno para el Cuidado y Uso de Animales de Laboratorio del Instituto de Ciencias de la Salud CICUAL-ICS), under the number 2020-003. Furthermore, this study was conducted according to ARRIVE guidelines[37] and international standards.[22]

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