ISSN 1413-2087

Reprodução & Climatério

Volume 27 • Número 1
Janeiro/Abril 2012

Sociedade Brasileira de Reprodução Humana
Sociedade Brasileira do Climatério
Sociedade Brasileira de Ginecologia Endócrina

Reprodução & Climatério

A revista REPRODUÇÃO & CLIMATÉRIO, anteriormente denominada REPRODUÇÃO, é órgão oficial de divulgação da SBRH, SOBRAGE e SOBRAC. Está registrada sob nº ISSN 1413-2087, e indexada no Index Medicus Latino Americano. Sua distribuição se faz a todos os sócios das sociedades participantes e aos principais serviços universitários da América Latina.

Diagramação, revisão e projeto gráfico

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Original Article

An experimental investigation on effect of *Laurus nobilis* L. on gestation

Investigação experimental sobre o efeito de *Laurus nobilis* L. na gestação

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Abstract

Laurus nobilis L. is an aromatic plant commonly used for culinary and medicinal purposes. However, berry or leaf decoction is taken to induce menstruation and abortion. In this sense, this study was conducted to evaluate its abortive and teratogenic potential. Aqueous and hydroalcoholic extracts of *L. nobilis* leaves were orally administered to mice from the first to the third day of pregnancy (preimplantation period), from the fourth to the sixth day of pregnancy (implantation period) or from the seventh to the ninth day of pregnancy (early organogenesis). On the 18th day of pregnancy, the number of *corpora lutea*, implantation sites, embryonic resorptions, and fetuses was recorded. The fetuses were examined for malformations and skeletal anomalies. Embryos were collected on the fourth gestational day for morphological analysis. Estrogenic activity was verified by uterine assay on sexually immature females. The presence of degenerated embryos and dead fetuses resulted in a significant amount in the group treated with hydroalcoholic extract from the first to the third gestational day. In this group, placenta and fetus' weight were significantly decreased, and many fetuses presented a delayed bone development. When embryos collected on the fourth gestational day were analyzed, a higher frequency of oocyte or zygote to ten cell-embryo and a lesser frequency of morula or blastocyst were found in *L. nobilis*-treated groups. No uterotrophic effect was observed. The findings obtained in this experimental investigation suggest embryotoxicity by *L. nobilis*.

Uniterms: *Laurus nobilis*; Medicinal plant; Abortive agent; Embryotoxicity; Pregnancy.

Resumo

Laurus nobilis L. é uma planta aromática comumente usada para propósitos culinários e medicinais. Entretanto a decocção dos frutos ou folhas é tomada para induzir a menstruação e o aborto. Nesse sentido, este estudo foi conduzido para avaliar seu potencial abortivo e teratogênico. O extrato aquoso e o extrato hidroalcoólico das folhas de *L. nobilis* foram administrados oralmente a camundongos do primeiro ao terceiro dia de gestação (período pré-implantação), do quarto ao sexto dia de gestação (período em que ocorre a implantação) ou do sétimo ao nono dia de gestação (início da organogênese). No 18º dia de gestação, foi registrado o número de corpos lúteos, sítios de implantação, reabsorções embrionárias e fetos. Os fetos foram examinados para a presença de malformações e anomalias esqueléticas. Embriões foram coletados no quarto dia de gestação para análise morfológica. Atividade estrogênica foi verificada pelo ensaio uterino em fêmeas sexualmente imaturas. Um número significativo de embriões degenerados e fetos mortos foi observado no grupo tratado com o extrato hidroalcoólico do primeiro ao terceiro dia de gestação. Nesse grupo, o peso das placentas e dos fetos diminuiu significativamente, e muitos fetos apresentaram um desenvolvimento ósseo retardado. Na análise dos embriões coletados no quarto dia gestacional, uma frequência maior de oócito ou zigoto a embrião de dez células e uma frequência menor de mórula ou blastocisto foram encontradas nos grupos tratados com *L. nobilis*. Efeito uterotrófico não foi observado. Os resultados obtidos nesta investigação experimental sugerem embriotoxicidade por *L. nobilis*.

Unitermos: *Laurus nobilis*; Planta medicinal; Agente abortivo; Embriotoxicidade; Gestação.

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Fonte de financiamento: Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) e Pró-reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (UFRGS).
Conflito de interesse: nada a declarar.

Introduction

Laurus nobilis L. (Lauraceae family) is commonly used to medicinal and culinary purposes. However, berry or leaf decoction is taken to correct menstrual delays and to induce abortion[1,2]. This study was conducted to verify its abortive potential and mechanism of action. The same methodology employed to evaluate other plants was used to achieve this aim[3-6]. The experimental model was the pregnant mouse, and the reproductive parameters were evaluated after the administration on the preimplantation, implantation or early organogenesis period. The fetuses were examined for anomalies and malformations, because if pregnancy is not interrupted, the herb might be teratogenic. Embryotoxicity activity was assessed by morphological analysis of the preimplantation embryos, and estrogenic activity was investigated by uterine bioassay.

Material and Methods

Plant material

L. nobilis leaves were collected from a garden at Caxias do Sul, in Rio Grande do Sul state, and they were dried in a ventilated place at room temperature for 15 days.

The aqueous extract was prepared by decoction, boiling for 15 minutes 5 g of dried leaves with 300 mL of distilled water. After lyophilization, 10 mL of the aqueous extract had 4.7 mg of dry matter. Therefore, the yield was 2.82% in relation to the initial material.

The hydroalcoholic extract was made with 100.389 g of ground leaves using pestle and mortar and 1,000 mL of 70% ethanol. After maceration by 72 hours, the extract was filtered, concentrated in a rotary evaporator at 40ºC and it was lyophilized, resulting 17.022 g. The yield was 16.96% in relation to the dried plant material.

The aqueous and lyophilized hydroalcoholic extracts were stored frozen from where they were taken, when required.

Animals

This study was carried out in the Laboratory of Reproduction Biology from the Department of Morphological Sciences at *Universidade Federal do Rio Grande do Sul* (UFRGS), and the procedures were approved by the UFRGS Animal Care and Ethics Committee. CF1 mice were maintained under temperatures from 22 to 26ºC and 12-hour light/dark cycle, with pellet diet (Nuvilab Cr 1, Nuvital, Colombo, PR, Brazil) and water *ad libitum*.

Abortive activity evaluation

Nulliparous female mice (two to three-month-old) were mated with male mice in a 2:1 ratio and examined for a vaginal plug in the following morning. The day when the vaginal plug was observed was considered as first gestational day (GD).

The females received 1,000 mg/kg/day of hydroalcoholic extract (suspended in distilled water in a proportion of 1,000 mg to 4 mL) or 4 mL/kg/day of aqueous extract (or distilled water as Control Group). The dose of 1,000 mg/kg was selected because it corresponds to that of the limit test in the guideline for reproduction and teratogenicity tests[7].

The administration was oral, using a curved feeding needle and a 1 mL disposable syringe, restricted to the first half of the gestation, segmented in the periods preimplantation (first to third GD), implantation (fourth to sixth GD), and post-implantation (seventh to ninth GD). According to recommendations for reproductive toxicity using rodents, each group contained 20 animals[7-9]. The animals were weighed on the first GD, on the first day of administration, on the day after the last dose, and on the 18th GD, when they were killed by cervical dislocation. The ovaries were collected, weighed and placed in a Petri dish with saline for counting their *corpora lutea* (or albicans) under a stereomicroscope. The uterus was opened for counting of live and dead fetuses, degenerated embryos and late resorptions, and it was incubated in 10% ammonium sulfide for ten minutes to count implantation sites and early resorptions[10]. The placentae and the live fetuses were weighed.

The fetuses were examined for external malformations and fixed either in Bouin's fluid for posterior analysis of internal malformations[11] or in 95% ethanol for staining with alizarin red S[12] and identification of skeletal anomalies, observation of the skull plates and counting of metacarpals, metatarsals, sternebrae and xiphisternum, ribs, lumbar vertebrae, and sacral and caudal vertebrae.

Preimplantation embryos analysis

Aiming at elucidating an embryotoxic effect, females received 1,000 mg/kg/day of hydroalcoholic extract or 4 mL/kg/day of aqueous extract (or distilled water) from the first to the third GD. Each group contained 20 animals. They were killed on the fourth GD between 2 and 5 p.m., and the embryos were collected in an embryo dish by flushing the uterine horns with 1 mL/horn of saline. The embryos were counted under stereomicroscope. After they were transferred to a microscope slide, using a mouth pipette, observed and photographed under light microscope in order to identify the development phase and morphological abnormalities.

Estrogenic activity evaluation

Sexually immature females (23 to 25-day-old) received 1,000 mg/kg/day of hydroalcoholic extract or 4 mL/kg/day of aqueous extract or distilled water orally for 3 days. Each group contained ten animals. If the vagina was opened, smears were taken and stained by Shorr's technique[13] to evaluate the extent of vaginal cornification. The females were weighed on the first day of administration and on the day after the last dose, when they were killed, and uteri were collected and weighed[14].

Statistical analysis

The weight of the body, ovary, placenta, fetus and uterus and the number of *corpora lutea*, implantation sites and live fetuses were expressed as mean ± standard deviation and analyzed by one-way ANOVA, post hoc multiple comparisons Dunnett *t* test. The initial and final weight difference was analyzed by the Student's *t*-test.

The number of resorptions, degenerated embryos and dead fetuses, the reproductive indices, the skeletal and teratological data and the results obtained from embryos analysis were expressed as median and quartiles range, and they were analyzed by the Kruskal-Wallis test when all groups were compared or the Mann-Whitney's U-test when a treatment group was compared to the Control Group[9,15,16].

A probability level of less than 5% was considered as significant.

Results

Abortive activity evaluation

There was significant corporal gain in all groups, even during the administration period. There was no significant difference in body weight between the treated and control groups.

The effect of *L. nobilis* on the reproductive parameters and rates is shown in Tables 1 and 2. The number of *corpora lutea*, implantation sites, resorptions, and live fetuses was not altered by extracts. However, the sum of degenerated embryos and dead fetuses was significant in the hydroalcoholic extract-treated group from the first to the third GD (Table 1). Out of the 20 treated females, 9 had 13 degenerated embryos and dead fetuses in total.

Table 1 – Effect of *Laurus nobilis* on reproductive parameters

Group	*Corpora lutea*	Implantation sites	Embryonic resorptions	Degenerated embryos and dead fetuses	Live fetuses
First to third GD					
Control	15.85±1.84	9.95±6.49	1 [0–2]	0 [0–0]	8.15±5.99
Aqueous extract	15.8±1.58	12.05±4.67	2 [1–3]	0 [0–0]	9.45±4.36
Hydroalcoholic extract	15.85±2.0	11.9±4.93	1.5 [0–3.5]	0 [0–1]*	8.95±4.55
Fourth to sixth GD					
Control	17.25±2.02	13.1±4.53	1 [0–3]	0 [0–0]	11.25±4.36
Aqueous extract	15.8±2.21	10.95±5.23	1 [0–4]	0 [0–0]	8.55±4.68
Hydroalcoholic extract	16.15±1.93	13.4±5.28	2 [1–2]	0 [0–0]	11.15±4.33
Seventh to ninth GD					
Control	16.2±2.44	11.1±5.92	1 [0–2]	0 [0–0.5]	9.65±5.16
Aqueous extract	15.35±1.95	10.75±6.22	1 [0–4]	0 [0–0]	8.35±5.51
Hydroalcoholic extract	16.83±2.57	11.55±5.84	2 [0–3]	0 [0–0]	9.6±5.07

GD: gestational day; *significant difference: p=0.01, Kruskal-Wallis; p=0.004, Mann-Whitney's U-test.

Table 2 – Effect of *Laurus nobilis* on reproductive rates (%)

Group	Implantation rate[a]	Resorption rate[b]	Death rate[c]	Birth rate[d]
First to third GD				
Control	85.4 [22.5–97.2]	4.6 [0–29.2]	0 [0–0]	86.2 [53.5–100]
Aqueous extract	87.1 [67.4–96.7]	14.3 [7.4–31.3]	0 [0–0]	82.3 [62.0–87.9]
Hydroalcoholic extract	82.8 [61.5–94.3]	13.3 [0–27.8]	0 [0–7.2]*	77.4 [56.4–89.9]
Fourth to sixth GD				
Control	83.3 [65.0–92.3]	6.1 [0–20.1]	0 [0–0]	89.3 [75.7–97.2]
Aqueous extract	81.6 [48.5–97.4]	15 [0–28.1]	0 [0–0]	76.8 [47.7–91.4]
Hydroalcoholic extract	93.3 [80.6–97.2]	11.2 [6.5–16.0]	0 [0–0]	85.2 [75.7–91.3]
Seventh to ninth GD				
Control	81.6 [39.8–100]	7.9 [0–12.9]	0 [0–3.1]	85.7 [76–93.5]
Aqueous extract	87.5 [47.2–100]	8.4 [0–26.7]	0 [0–0]	73.3 [28.6–91.6]
Hydroalcoholic extract	76.1 [58.7–92.8]	14.8 [0–21.8]	0 [0–0]	80.5 [72.7–87.5]

GD: gestational day; [a]implantation rate = (number of implantation sites/number of corpora lutea) x 100; [b]resorption rate = (number of resorptions/number of implantation sites) x 100; [c]death rate = (number of degenerated embryos and dead fetuses/number of implantation sites) x 100; [d]birth rate = (number of live fetuses/number of implantation sites) x 100; *significant difference: p=0.011, Kruskal-Wallis; p=0.003, Mann-Whitney's U-test.

In this period of administration, a control female had one, and four females treated with aqueous extract had five degenerated embryos and dead fetuses. Due to the high incidence of degenerated embryos and dead fetuses in the hydroalcoholic extract-treated group, the death rate was significantly different between the groups (Table 2).

In the experiment with administration from the first to the third GD, the weights of the placentae and fetus were significantly different between the groups, due to the lesser weight in the hydroalcoholic extract-treated group (Table 3). The weight of the fetus was also significantly lesser in the group treated with this extract from the seventh to the ninth GD.

Statistical analysis did not identify significant differences to distribution of external and internal malformations and skeletal anomalies between the groups, but a significant inferior number of metacarpals, metatarsals, sternebrae, sacral and caudal vertebrae was observed in the group treated with the hydroalcoholic extract from the first to the third GD and groups treated with the extracts from the seventh to ninth GD.

Preimplantation embryos analysis

When the extracts were administered from the first to the third GD for morphological analysis of the preimplantation embryos, many females had weight loss, resulting in a significant loss in the aqueous extract-treated group and in an absence of significant gain in the hydroalcoholic extract-treated group. By observation of the embryos collected on the fourth GD, a higher frequency of oocyte or zygote to ten cell-embryo was found in the groups treated with aqueous and hydroalcoholic extracts, decreasing the frequency of morula and blastocyst, respectively (Table 4, Figures 1 to 3).

Table 3 – Effect of *Laurus nobilis* in the weight of ovary, placenta, and fetus (mg)

Group	Ovary	Placenta	Fetus
First to third GD			
Control	12.15±1.81	111.51±26.48	980.09±118.03
Aqueous extract	11.93±2.75	101.04±16.91	921.69±93.11
Hydroalcoholic extract	12.45±2.18	90.88±7.37*	876.70±61.13*
Fourth to sixth GD			
Control	12.6±1.52	98.65±11.90	913.66±90.13
Aqueous extract	11.65±2.07	104.7±12.57	920.68±81.56
Hydroalcoholic extract	12.73±1.75	92.64±11.52	873.27±75.12
Seventh to ninth GD			
Control	12.65±2.21	98.30±10.13	944.60±71.93
Aqueous extract	12.73±1.85	103.88±16.64	917.82±93.03
Hydroalcoholic extract	13.88±2.41	97.75±11.82	877.01±84.86**

GD: gestational day; *significant difference: p=0.004, ANOVA post-hoc Dunnett's *t*-test; **significant difference: p=0.041, ANOVA post-hoc Dunnett's *t*-test.

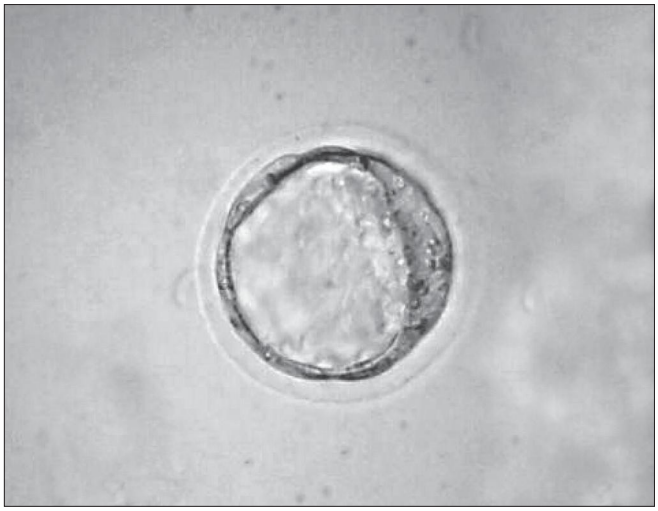

Figure 1 – Normal blastocyst obtained from Control female on the fourth gestational day.

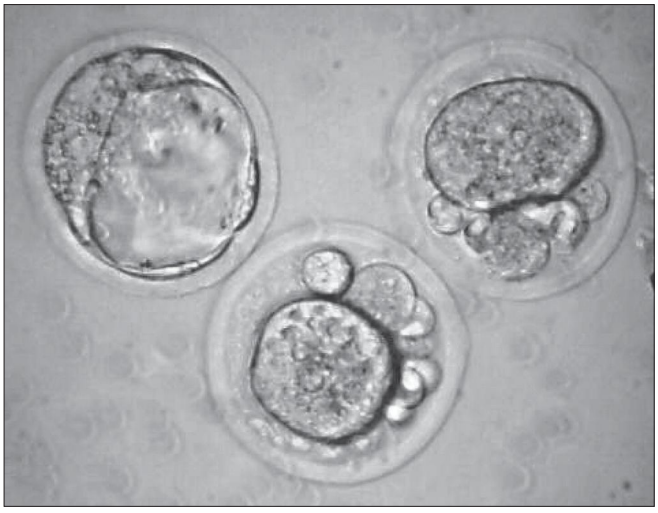

Figure 2 – Normal blastocyst and two fragmented oocytes/zygotes obtained from a female treated with *Laurus nobilis* aqueous extract.

Table 4. Effect of *Laurus nobilis* administered during the preimplantation period on the development phase and morphological aspect (%) of embryos on the fourth gestational day

Group	Oocyte or zygote to 10 cell-embryo	Morula	Blastocyst	Normal morphology
Control	0 [0–10.1]	10 [0–22.7]	86.1 [61.3–96.5]	96.2 [79.5–100]
Aqueous extract	9.1 [0–34.5]	0 [0–3.6]*#	84.5 [43.6–95.9]	88.2 [46.6–100]
Hydroalcoholic extract	22.7 [0–60.7]**	10.1 [0–16.1]	61.9 [11.1–88.9]	77.4 [36.6–100]

Difference between the groups: *p=0.021, Kruskal-Wallis; difference to the Control Group: #p=0.007; **p=0.056, Mann-Whitney's U-test.

Estrogenic activity evaluation

The sexually immature females had neither vaginal opening and cornification nor uterotrophic effect with the administration (Table 5). Therefore, *L. nobilis* decoction and extract demonstrated no estrogenic activity by this bioassay.

Discussion

L. nobilis (laurel) is commonly used in culinary and herbal Medicine, but berry or leaf decoction is taken to induce menstruation and abortion. Aiming at evaluating their effect on gestation, this study was delineated.

Dried leaf aqueous and hydroalcoholic extracts were prepared, resulting in a yield of 2.82 and 16.96%, respectively. A minimum dose and a maximum dose were administered: 4 mL (1.88 mg)/kg/day of aqueous extract and 1,000 mg/kg/day of hydroalcoholic extract.

In the abortive activity assessment, female mice received orally the extracts on the preimplantation (first to third GD), implantation (fourth to sixth GD) or early organogenesis (seventh to ninth GD) period, and the reproductive parameters were evaluated on 18th GD. The presence of degenerated embryos and dead fetuses was significant in the group treated with the leaf hydroalcoholic extract from the first to the third GD. In this group, the weights of the placenta and the fetus were significantly lesser, and the fetuses exhibited reduced ossification, supporting the findings of lesser fetuses.

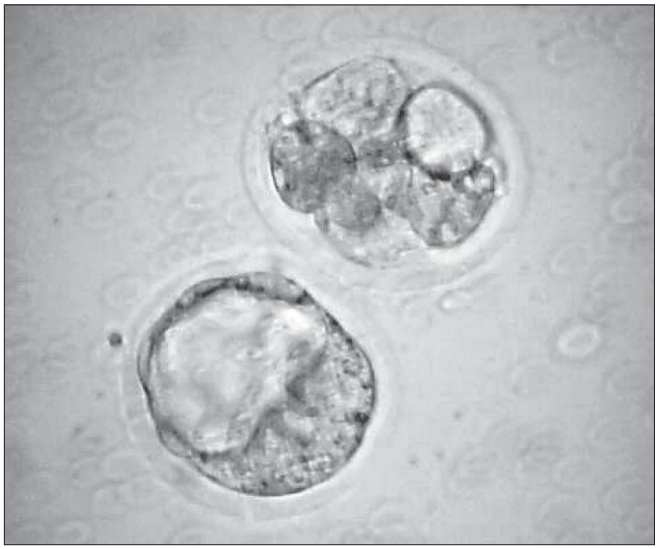

Figure 3 – Blastocyst and an abnormal six cell-embryo obtained from a female treated with *Laurus nobilis* hydroalcoholic extract.

Table 5 – Effect of *Laurus nobilis* on body weight (g) and relative uterine weight (mg/100 g) of immature females

Group	Initial weight	Final weight	Relative uterine weight
Control	10.68±1.47	13.59±1.72	85.92±15.45
Aqueous extract	10.83±1.22	13.66±1.92	84.01±16.58
Hydroalcoholic extract	10.73±0.60	13.88±0.93	92.48±12.0

These results suggested embryotoxicity by *L. nobilis*, and experiment for evaluating the development phase and the morphological aspect of the preimplantation embryos was executed. The aqueous and hydroalcoholic extracts were administered from the first to the third GD, and the dams were killed on fourth GD. A higher frequency of oocyte or zygote to ten cell-embryo and a lesser frequency of morula or blastocyst were found in the treated groups.

The essential oil of *Cinnamomum zeylanicum*, other species from Lauraceae family, significantly decreased the number of nuclei of embryos collected on the fourth GD[17].

Using immature sexually females, the extracts did not promote an uterotrophic effect. Therefore, estrogenic activity was not exhibited by *L. nobilis*.

The results suggest that *L. nobilis* has an abortive potential, and its mechanism of action is non-hormonal, possibly related to cytotoxicity. *L. nobilis* is also used popularly to treat cancer and skin diseases, and investigations *in vitro* found an anti-tumoral and cytotoxic effect, via inhibition of the NF$^{\kappa}$B pathway[18].

Conclusion

Significant presence of degenerated embryos and dead fetuses was observed in the *L. nobilis* hydroalcoholic ex- tract-treated group on the preimplantation period. In this group, the weights of placenta and fetus were significantly decreased, and many fetuses presented a delayed bone de- velopment. When embryos collected on the fourth GD were analyzed, a higher frequency of oocyte or zygote to ten cell- embryo and a lesser frequency of morula or blastocyst were found in *L. nobilis*-treated groups. These findings suggest em- bryotoxicity by *L. nobilis*.

Acknowledgments

The authors thank Professor Grace Gosmann from the Faculty of Pharmacy for using evaporator and lyophilizer, and Professor Matilde Elena Achaval from the Department of Morphological Sciences for using photographic microscope.

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Recebido em: 15/11/2011
Aprovado para publicação em: 20/12/2011