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Experimental Research

Pharmacology Section

Antioxidant Activity and Teratogenicity Evaluation of *Lawsonia Inermis* in BALB/c Mice

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ABSTRACT

Background and Aim: *Lawsonia inermis* is a medicinal plant with abortive properties. There has been no scientific study to evaluate the teratogenicity of this plant. This study was performed to determine the antioxidant activity and the possible side effect of *L. inermis* hydroalcoholic extract on development of congenital abnormalities in BALB/c mice.

Materials and Methods: In this experimental study, 120 female mature BALB/c mice were assigned to four groups and after mating and confirming the vaginal plug, the animals in the first group (G1) were kept with no intervention, and the second (G2), third (G3) and fourth (G4) groups were intraperitoneally (ip) injected with respectively saline (0.3 ml), and 10 and 100 mg/kg of *L. inermis* extract (for 7 days). On the 19th day, caesarean section was performed on the mice and embryos were examined for abnormalities. Their height and weight were measured. Data were analysed by ANOVA and post-hoc least significant difference tests.

Results: There were significant differences between G3 and G4, and G1 ($p<0.001$); no significant difference was seen between G3 and G4. At 100 mg/kg dose of *L. inermis*, the parietal bones were absent in 90% of embryos and more extra ribs were observed in both G3 and G4 ($p = 0.01$).

Conclusion: *L. inermis* may have teratogenicity and should be used cautiously during pregnancy.

Keywords: Herbal medicines, Medicinal plant, Pregnancy, Safety, Toxicity

INTRODUCTION

Due to the potent adverse effects of synthetic drugs and increasing contraindications to their usage, there is an increasing interest in the use of medicinal plants [1,2]. Recent studies on medicinal plants have also shown promising results in treatment or prevention of hard curable conditions such as diabetes [3,4], atherosclerosis [5,6], hypertension [5,7], and cancer [8,9]. Medicinal plants have also the capacities to diminish drug induced adverse effects [10,11] and heavy metal toxicities [12]. Most women believe that herbal medicines are safe and therefore pregnant women may use these herbs or their combinations during pregnancy [13].

Although medicinal plants are considered to be safe however, some of them have been shown to be teratogen. *Carthamus tinctorius* causes complications such as eyelid defects or brain, renal, and hepatic toxicity [14]. Hydroalcoholic extract of *Stachys lavandulifolia* may cause significant decrease in height and weight as well as hepatotoxicity [15,16]. Therefore, investigation of herbal plants toxicity, especially during pregnancy and lactation, is necessary. *Lawsonia inermis* L. is a perennial shrub from the family of Lythraceae which is distributed in tropical zones of Africa and Asia [17]. It is used for a wide range of diseases such as skin fungal diseases and the relief of rheumatic pains. It has antidiarrheal, anti-inflammatory, analgesic, and antipyretic effects, antibacterial effect especially in gram positive bacteria, antifungal properties against trichophyton, sporotrichum, and Cryptococcus [18]. Moreover, it is used as a colouring agent especially for skin and hair [19].

The leaves of *L. inermis* include a pigment named lawsone or hydroxynaphthoquinone (3.1-22.0%), various glycoside phenols, coumarin, xanthone, quinoids, glycoside, β-Sitosterol, flavonoids like luteolin , 6% fat, 2-3% resin, 7-8% tannins, and 1.2% essence [20]. Other components of the leaves include 5-7% glycoside derivatives, gallic acid, 1&4 naphthoquinone, 1&2 laxatone, and some alkaloids [21]. The leaves of *L. inermis* cause hemolysis because it contains a substance with oxidant property named lawsone with the chemical name of 2-Hydroxy-1,4-naphthoquinone. In high doses, it causes headache and poisoning. The available lawsone in *L. inermis* causes bradycardia [22]. Although some pharmacologic and toxic effects of *L. inermis* have been investigated, however, the teratogenic effects of this plant on the embryo at pregnancy have not been scientifically clarified. Therefore, in this study the teratogenic effects of *L. inermis* were studied in BALB/c mice.

MATERIALS AND METHODS

Materials

Female BALB/c mice between 8-12 wk were purchased from Tehran Pasteur Institute, Iran. Normal saline was purchased from Darupakhsh Pharmaceutical Company and other chemicals from Merck Company (Germany). *L. inermis* was collected in Yazd (Iran).

Experiments

In this experimental study, 120 female BALB/c mice between 8-12 week weighing 25-28 g were prepared and kept in proper health and light conditions (12/12 h light/darkness) without any food access limitation. After ten-day habituation to the environment, one male mouse and two female mice were placed in a cage for two days [23,24]. The vaginal plug was considered as the sign of zero day of pregnancy. Since this method was not certain, an eosin smear 3% was prepared from the vaginal discharges of the female mice and the existence of sperm was regarded as fertility [15]. Another sign of fertility was female mice's weight gain after fertility. Since the mice weight increases by 3-10% daily since the fourth day of pregnancy, the weight gain up to this level or more was regarded as the sign of fertility. Then, the mice were assigned to four groups. The animals in the first group (G1) were kept with no intervention, and the second (G2), third (G3) and fourth (G4) groups were intraperitoneally (ip) injected with respectively saline (0.3 ml), and 10 and 100 mg/kg of *L. inermis* extract (for 7 days) [25].

The two doses of *L. inermis* and saline were injected daily since the zero day of pregnancy till the seventh day. On the 19th day of

pregnancy, caesarean operation was done under anaesthesia with chloroform. The caesarean cut was in shape of inverse Y. After the caesarean section, fallopian tubes were opened and embryos were removed and placed in normal saline. Embryos were weighed by a digital sensitive scale and their heights were measured by a caliper from crown to rump. The embryos were assessed in terms of observable abnormalities. Then, after using Alizarin dye and Alcian Blue, the skeletal abnormalities were examined [15].

Extraction method

The aerial organs of *L. inermis* were collected from Yazd suburbs and after being authenticated by a botanist in the Research Center of Jahad-e-Keshavarzi, a herbarium sample was prepared and deposited in the Herbarium Unit of Medical Plants Research Center of Shahrekord University of Medical Sciences, Iran (Code number: 234). The collected plants were dried at normal temperature and the extraction was done by maceration method. To 500 gm of the plant powder in an appropriate container, 500 ml of ethanol 80% was added, and after 48 hour it was filtered. The solvent was removed at 35°C using a rotary evaporator machine. The extract was incubated for two days at 40°C to dry. Then, it was kept in refrigerator until the usage time [22].

Standardization of the extract

To standardize, the level of flavonoids and phenolic compounds as well as antioxidant capacity of the extract were measured as follows:

Measurement of flavonoid compounds level

Aluminum chloride colorimetric and rutin method were run to assay the total flavonoids [26]. First, standard solutions (rutin in methanol 60%) at concentrations of 25, 50, 100, 250, and 500 ppm were prepared. Then, one ml of these solutions was transferred into test tubes and mixed with one ml of chloride aluminum 2%. Then, 6 ml of potassium acetate 5% was introduced and the optical density was read after 40 minutes at 415 nm wavelength. The concentration levels of the standard solutions were assayed in three intervals. To measure the overall level of flavonoid in the extracts, 0.01-0.02 g of the extracts was dissolved with methanol 60%, reaching 10 ml. Then, the total level of flavonoid was measured by chloride aluminum colorimetry. However, instead of the standard solution, one ml of the extract was added. The total flavonoid level was calculated in mg/gr extract, equivalent to rutin.

Measurement of total phenolic compounds

Total phenolic compounds were assayed equivalent to gallic acid by Folin-Ciocalteu colorimetry [27]. The standard solutions were prepared at concentrations of 12.5, 25, 50, 62.5, 100, and 125 ppm of gallic acid in methanol 60%. Then, 0.1 ml of each sample was transferred into a test tube and 0.5 ml Folin-Ciocalteu 10% was introduced as reactive agent. The solutions were left for 8 minutes at room temperature and 0.4 ml of sodium carbonate 7.5% was added. The tubes were maintained for 30 minutes at the laboratory temperature and then assayed in three intervals by a spectrophotometer (Unico UV-2010, Japan) at 765 nm wavelength. To measure the overall phenol in the extracts, 0.01-0.02 g of the extracts was solved with 60% methanol, reaching 10 ml and the overall level of phenol was measured by Folin-Ciocalteu method. However, instead of the standard solution, 0.1 ml of extract solution was added. Finally, the overall phenol level was derived from the read optical density in mg/gr extract in gallic acid equivalent.

Measurement of antioxidant activity

To measure the antioxidant activity of the extract β-carotene model was used [27]. In a suitable container 500 µL chloroform, 0.2 ml Tween 40, 5 ml β-carotene (0.2 mg) and 20 ml linoleic acid (20 mg) were mixed, and incubated at 50°C for 10 minutes to remove the solvent. The solution was diluted using distilled water and mixed with 4 ml of aliquots in the following manner. The control solution, consisting of 0.2 ml ethanol and 0.2 ml of the extract sample with 0.15 ml ethanol and 0.05 ml turmeric extract, was prepared. The optical density in the G1 was recorded at t=0 and t=90 at 470 nm wavelength, similar to the standard group. The specimens were incubated in a bain-marie at 50°C. The antioxidant activity of the samples was measured based on the samples ability to prevent washing of β-carotene. The antioxidant activity was measured by the formula below [28]:

(1) $AA = 100 \{1 - (Ao - At) / (Aoo - Aot)\}$

Where,

Ao: optical density at t = 0

At: optical density of the sample at t = 90

Aoo and Aot: optical density values in the control samples at t = 0 and t = 90, respectively.

STATISTICAL ANALYSIS

Data were presented as frequency, relative frequency, mean, standard deviation and were analysed using ANOVA, post-hoc least significance difference (LSD) test and Fisher's exact test using SPSS 11.5 software.

RESULTS

Each 100 gr of *L. inermis* powder yielded 11.7 gr hydroalcoholic dried extract. The calculated levels for flavonoid and phenolic compounds were 94.19 mg/gr rutin equivalent and 126.38 mg/gr gallic acid equivalent, respectively. The extract antioxidant activity was 23.66%. The indices of embryos' height and weight were measured [Table/Fig-1]. There were embryos in all selected mice; however, post-hoc LSD test showed that both doses of the extract caused a significant decrease in embryos' height and weight in comparison with the G1 ($p<0.001$); however, no significant difference was observed in G3 and G4 regarding these parameters ($p>0.05$).

Variable Group	Extract 10 mg/kg	Extract 100 mg/kg	Distilled Water	Control
Height (mm)	16.2 ± 0.19*	16.08 ± 0.24*	19.9 ± 0.06	20.07 ± 0.4
Weight (gr)	0.49 ± 0.02*	0.46 ± 0.02 *	1.15 ± 0.16	1.20 ± 0.11

[Table/Fig-1]: The comparison of the mice embryos' height and weight mean (± standard deviation) in the groups under study
* p<0.001 in comparison with control and distilled water groups

Group abnormalities	Extra Rib		Anencephaly		Exencephaly		Parietal Bone	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Extract 10 mg/kg	3	10	0	0	0	0	7	23.3
Extract 100 mg/kg	6	20	1	3.2	1	3.3	27	90
* Total 10 and 100 mg/kg	9**	30	1**	3.2	1**	3.3	34**	113.3
Distilled Water	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0

[Table/Fig-2]: The frequency of observed abnormalities in mice's embryos*
*N = 30 in each group
** Due to small sample size, it was not possible to compare the abnormalities' frequency in the four groups based on chi-Square test. Therefore, through merging the results of the two groups receiving the extract and control and distilled water groups, the abnormalities in control and distilled water groups were compared, which showed a significant difference (P<0.01)

Skeletal abnormalities including rib and parietal bone abnormalities, anencephaly and exencephaly of embryos in G3 and G4 are summarized in [Table/Fig-2]. The rate of extra rib, anencephaly, exencephaly, and parietal bone abnormality of the mice embryos was zero in G1 and G2. These rates were 10, 0, 0, and 23.3 for respectively extra rib, anencephaly, No significant difference in anencephaly and exencephaly was observed between the G3 and G4, and G1 ($p = 1$). exencephaly, and parietal bone abnormality of the mice embryos in the G3. Moreover, these rates were 20, 3.2, 3.3 and 90%, for respectively extra rib, anencephaly, exencephaly, and parietal bone abnormality of the mice embryos in the G4. Since, it was not possible to compare the frequency of abnormalities in the four groups based on chi-square test, through merging the results of G3 and G4, and G1 and G2, those of G1 and G2 were compared and the difference was obtained significant ($p<0.01$).

Based on Fisher's exact test, the abnormalities of the extra rib and parietal bone were significantly higher in G3 and G4 than G1 ($p<0.01$). In addition, no case of extra rib or parietal bone abnormality was observed in G1 and G2 [Table/Fig-2]. At 100 mg/kg of *L. inermis*, there were no parietal bones in 90% of the embryos. Higher frequency of extra rib was observed in G3 and G4 ($p<0.01$). The rate of abnormalities was higher in the higher dose of the extract in comparison with the lower dose.

DISCUSSION

This study tried to examine the teratogenicity of *L. inermis* plant in BALB/c mice. Although *L. inermis* has been used as a medical herb in traditional medicine for therapeutic purposes, there has been no scientific study on its teratogenicity during pregnancy.

In the present study, the extract of *L. inermis* caused skeletal abnormalities and height and weight loss in embryos. The comparison between the G3 and G4, and G1 and G2 showed that there was a significant difference in external and skeletal abnormalities and the extract of *L. inermis* caused anencephaly, exencephaly, extra rib abnormalities, and lack of parietal bone in mice embryos ($p<0.05$). In this study, abnormalities of extra rib and lack of parietal bone were observed in G3 and G4; however, their incidence was higher with dose of 100 mg/kg. Moreover, anencephaly and exencephaly abnormalities were only observed in the G3. Therefore, it can be said that the incidence of these abnormalities depends on the dose. What substance with which mechanism (s) causes abnormalities in mice is not clear and requires examining of the fundamental components of the extract.

Previous studies have shown that *Hypericum perforatum* has teratogenic effects and these effects are due to the existence of two combinations of apigenin and alpha pinene [1]. The flowers that have high amounts of apigenin have been used to treat insomnia, convulsion, and asthma and to relieve nervous pains. Apigenin is an estrogen flavonoid available in aromatic plants. Apigenin has a slow metabolism and the adsorption and desorption phases happen slowly and therefore accumulation of this flavonoid in the body is probable [29]. Because it has been already shown that *L. inermis*, especially its hydroalcoholic extract, has high amounts of apigenin [30], the presence of this component in *L. inermis* hydroalcoholic extract could be one of the reasons for external and skeletal abnormalities.

Among the abnormalities developed in aborted embryos in this study are skeletal abnormalities (extra rib, lack of parietal bone, anencephaly, and exencephaly). Previous studies have shown a negative correlation between the lack of absorption of folic acid and skeletal abnormalities. The teratogenicity of lamotrigine in animals has also been attributed to reduced absorption of folate [1]. On the other hand, cineol is one of the probable combinations available in *L. inermis* hydroalcoholic extract and studies have shown that this component causes skeletal abnormalities (extra rib and lack of parietal bone) by affecting the liver storage of folic acid [31]. Regarding the mentioned components and their teratogenic effects on the skeletal system, the teratogenic effects of *L. inermis* could be related to cineol and loss of folate storage in pregnant mice.

The results of the study showed that *L. inermis* extract caused a significant reduction in mice embryos' height and weight. This result should, at least in part, be related to the available component of 2-Hydroxy-1,4-naphthoquinone in *L. inermis* extract. This component has oxidant properties and causes hemolysis. It is metabolized in liver and turns into toxic metabolites that cause a significant reduction in height and weight in critical stages of organogenesis in mice embryos [32].

CONCLUSION

In view of the obtained results about external and skeletal abnormalities including extra rib and lack of parietal bone, it seems that *L. inermis* should be taken cautiously during pregnancy. This study showed that *L. inermis* plant was able to create abnormalities in mice, and its teratogenic effects were dose-dependent. On the other hand, this plant was shown to reduce embryos' height and weight. Of course, the teratogenic effects of this plant should be studied more precisely.

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