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Effect of *Morus nigra* aqueous extract treatment on the maternal–fetal outcome, oxidative stress status and lipid profile of streptozotocin-induced diabetic rats

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ABSTRACT

Ethnopharmacological relevance: Morus nigra, commonly known as black mulberry, is widely used in Brazilian folk medicine for the diabetes treatment.
Aim of this study: To evaluate the effect of *Morus nigra* aqueous extract treatment on maternal lipid and oxidative stress profile, reproductive outcomes, and also fetal anomaly incidence from diabetic and non-diabetic rats.
Materials and methods: Diabetes was induced by streptozotocin (40 mg/kg) in virgin female Wistar rats. *Morus nigra* leaf aqueous extract (400 mg/kg) was administered from day 0 to 20 of pregnancy. At day 21 of pregnancy, all rats were anesthetized and killed to obtain blood samples and maternal–fetal data.
Results and conclusion: After treatment with *Morus nigra* extract, non-diabetic and diabetic rats presented no glycemic changes. Fetuses from diabetic dams, regardless of *Morus nigra* treatment, were small for pregnancy age. In diabetic dams, plant treatment caused reduced MDA, cholesterol, triglycerides and VLDL levels, and decreased placental index and weight as compared to diabetic group. The fetuses from diabetic rats treated with *Morus nigra* extract had lower frequency of skeletal and visceral anomalies as compared to diabetic group. Thus, *Morus nigra* leaf aqueous extract failed to control hyperglycemia in diabetic rats. However, *Morus nigra* treatment had antioxidant effect, contributing to reduce incidence of internal anomalies in offspring from diabetic dams.

1. Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and/or action, or both. Several pathogenic processes are involved in diabetes development (Reece et al., 2004; American Diabetes Association, 2011). In pregnancies complicated by diabetes, hyperglycemia and lipid metabolism alterations are associated with both maternal and fetal complications (Merzouk et al., 2000; HAPO Study Cooperative Research Group, 2008). Excessive oxidative stress has been implicated in the pathology and complications of diabetic pregnancy (Zhao and Reece, 2005). During pregnancy, diabetes leads to reproductive abnormalities that enhance spontaneous abortion, congenital anomalies, and neonatal morbidity and mortality (Van Assche et al., 1998; Eriksson et al., 2003). The rates of spontaneous abortion are three times more common in diabetes and pregnancy association. Despite advances in obstetric care and diabetes management, the risks for morbidity and mortality are increased for the human with diabetes and their offspring (Diabetes and Pregnancy Group, 2003; Dunne et al., 2003).

Several drugs are used to control diabetes, however perfect glycemic control is rarely achieved (Cooppan, 2005). From ancient times, plants have been used for treatment of *Diabetes mellitus* (Volpato et al., 2002; Damasceno and Volpato, 2008). The use of medicinal plants as alternative therapy is widely spread in the populations of underdeveloped countries, which have limited access to medical assistance. Diabetic women often use aqueous extracts of plants during pregnancy without any concern as to their possible outcomes. The effects of many of these plants have already been proven experimentally in animals and humans while others require further investigations (Prince et al., 1998).

The use of medicinal plants such as *Morus nigra* L. (family: Moraceae), known as black mulberry, and other members of its genus can be found in many countries. However, almost all the parts of the tree are used for pharmacological actions all over the world (Singab et al., 2005; Pawlowska et al., 2008). Its berries, bark and leaves are used medicinally, the berries for inflammation and to stop bleeding, the bark for toothache, and the leaves for snakebites and as an antidote to action poisoning. In Europe, black mulberry leaves have been used to stimulate insulin production for diabetes

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treatment (Ody Mnimh, 2000; Naderi et al., 2004). The hypoglycemic effect of the black mulberry leaves is similar to the antidiabetic activity of glibenclamide in patients with type 2 diabetes (Andallu et al., 2001). Experimental studies have also demonstrated the hypoglycemic effect of black mulberry extract (Hosseinzadeh and Sadeghi, 1999; Michinori et al., 2001; Petlevski et al., 2001). Previous study in our laboratory demonstrated that *Morus nigra* leaf extract did not present toxicological and hypoglycemic effects on diabetic and non-diabetic pregnant rats (Volpato et al., 2005).

Oxidative reaction has also been implicated by low density lipoprotein (LDL) oxidation in atherosclerosis (Rakesh et al., 1999). There are many reports concerning the structural changes of biological membranes induced by active oxygen species (Naderi et al., 2004). Black mulberry contains soluble plant chemicals known as bioflavonoids. These powerful antioxidants may be responsible for their medicinal properties (Ursell, 2000; González et al., 2010). Therefore, it is important to study *Morus nigra* effect in oxidative stress and lipid profile of experimental diabetic pregnancy.

Several diabetic pregnant women that arrived for medical investigation in the Botucatu Medical School, São Paulo, Brazil, report the use of medicinal plants, such as black mulberry leaves, to treat diabetes because of the high cost of the antidiabetic drug. Thus, the objective of the present study was to investigate the oral treatment effects with *Morus nigra* leaf aqueous extract on maternal reproductive outcomes, lipid and oxidative stress profile, and also fetal anomaly incidence from the streptozotocin-induced diabetic and non-diabetic rats.

2. Materials and methods

2.1. Extraction of plant materials

The leaves of *Morus nigra* were collected from Botucatu, São Paulo State, Brazil, between January and May 2007, in the morning. The plant was identified and authenticated by experts from Botanical Department (Unesp – Botucatu, São Paulo State, Brazil), where a voucher specimen (BOTU 25412) has been deposited. The leaves of the plant were dried at 50 °C for a period of 24 h in an aerated stove, ground and a powder was prepared, similarly to the folk-medicine preparation method. *Morus nigra* aqueous extract was prepared by boiling 50 g of the powder of the leaves of the plant in a flask containing 1 L of water for 5 min. The extract was agitated and covered until it reached room temperature. The residue was removed by filtration and the extract was then suitably concentrated in a rotary evaporator (final concentration: 50 mg/mL). A sample was separated for determination of the solid concentration, and the extract was divided into aliquots stored at −20 °C until further use.

2.2. Experimental animals

Female and male Wistar rats (190–210 g) were obtained from Unesp – Botucatu Breeding Center, and were maintained under standard laboratory conditions (22 ± 3 °C, 12-h light/dark cycle), with pelleted food (Purina rat chow, Purina®, São Paulo, SP, Brazil) and tap water *ad libitum*. The animals were cared for in accordance with the principles of the Guide for Care and Use of Experimental Animals. The local Committee of Ethics in Animal Experimentation approved all experimental procedures of this study (Protocol number 545/2004).

After two weeks of acclimatization, diabetes was induced in female rats with streptozotocin (STZ, Sigma Chemical Company, St. Louis, Millstone) (Froede and Medeiros, 2008). STZ was administered (i.v.) in a dose of 40 mg/kg dissolved in citrate buffer (0.1 M, pH 6.5). Control rats received (i.v.) citrate buffer. Blood glucose concentrations were measured by One Touch Ultra Johnson & Johnson® glucometer 7 days after induction of diabetes, and concentrations exceeded 200 mg/dL confirmed the diabetic state (Volpato et al., 2009).

2.3. Experimental groups

After diabetic state was confirmed, virgin female Wistar rats were mated overnight with non-diabetic male Wistar rats. The morning on which sperm were found in the vaginal smear was designated gestational day 0 (Francia-Farje et al., 2010). This study was composed of four experimental groups (n minimum = 13 animals/group): non-diabetic; non-diabetic treated with *Morus nigra* extract; diabetic and diabetic treated with *Morus nigra* extract. The treatment dose of 400 mg/kg/day of the *Morus nigra* extract was orally given, by gavage, from day 0 until the day 20 of pregnancy.

2.4. Course of pregnancy

Glycemia was measured about every seven days up to the end of pregnancy, at approximately 9 a.m. At day 21 of pregnancy, the rats were anesthetized by sodium pentobarbital and blood samples were collected by decapitation for biochemical parameters. The gravid uterus was weighed and dissected to count dead and live fetuses, resorption, implantation, and corpora lutea numbers. The number of implantation sites was determined by the Salewski method (1964). The fetuses and placentas were weighed to calculate the placental index as: placental weight/fetal weight. The male/female percentage ratio was calculated. The mean birth weight of the control pups (G1) was 5.4 ± 0.4 g. Newborns in the experimental groups whose birth weights did not diverge more than ±1.0 standard deviation (SD) from the G1 mean (i.e., those that were within the 5.0–5.8-g range) were classified as adequate for pregnancy age (APA). Those whose weights were at least 1.0 SD greater than the G1 mean birth weight were classified as large for pregnancy age (LPA). Those whose birth weights were at least 1.0 SD lower than the G1 mean birth weight were classified as small for pregnancy age (SPA) (Volpato et al., 2008), and evaluated in a microscope with respect to incidence of external anomaly. After external analysis, half the fetuses were fixed in Bouin's fluid and serial sections were prepared as described by Wilson (1965) for visceral examination. The remaining fetuses were prepared for examination of the skeletons by the staining procedure of Staples and Schnell (1964).

2.5. Biochemical profile analysis

One part of blood samples was collected from each rat and so put into anticoagulant-free test tubes, maintained at ice for 30 min and then centrifuged at 1300 × g during 10 min at 4 °C. The supernatant was collected as serum and stored at −80 °C for further determination of biochemical parameters.

Serum concentrations of total cholesterol (CHO), triglycerides (TG) and high-density lipoprotein (HDL) were determined by using the enzymatic method; and total protein (TP) concentrations were estimated by colorimetric method (Young, 2000), all of them by Sigma assay kits. The values were expressed in milligrams (mg) per deciliter (dL). Very-low-density lipoprotein (VLDL) serum level estimated value was calculated through the triglyceride concentrations (Friedwald et al., 1972).

For oxidative stress analysis, another blood portion was put into anticoagulant tubes, and then centrifuged at 90 × g for 10 min at 25 °C. Plasma content was discarded and so erythrocyte contents were washed using phosphate-saline buffer followed by centrifugation at 1300 × g during 1 min at 4 °C. This procedure was done for three times for assay of oxidative stress biomarkers, as described by de Souza et al. (2010). Oxidative stress biomarkers measured were

Table 1
Biochemical profile of non-diabetic and diabetic rats treated or not with a *Morus nigra* aqueous extract during pregnancy.

	Groups			
	Non-diabetic	Non-diabetic treated	Diabetic	Diabetic treated
TP (g/dL)	7.02 ± 2.14	6.88 ± 3.54	7.06 ± 2.87	3.56 ± 1.76
CHO (mg/dL)	119.15 ± 18.70	112.88 ± 29.06	349.23 ± 286.96*	137.99 ± 40.91#
TG (mg/dL)	352.46 ± 112.90	503.00 ± 261.91	1186.98 ± 652.55*	545.44 ± 349.87#
HDL (mg/dL)	33.51 ± 20.43	53.45 ± 21.38	41.26 ± 17.76	45.14 ± 15.38
VLDL (mg/dL)	67.55 ± 24.35	143.27 ± 114.54	237.40 ± 130.51*	109.09 ± 69.97#
MDA (nM/g Hb)	49.85 ± 10.24	108.82 ± 70.73	685.84 ± 789.36*	46.74 ± 29.68#
SOD (UI/mg Hb)	1.77 ± 0.71	13.33 ± 7.15*	11.64 ± 11.19	7.62 ± 2.42

Data shown as mean ± standard deviation (SD).
* $p < 0.05$ compared to non-diabetic group (Bonferroni test).
# $p < 0.05$ compared to diabetic group (Bonferroni test).

superoxide dismutase activity (SOD) and malonaldehyde (MDA) as lipid peroxidation index.

2.6. Statistical evaluation

The Kruskal–Wallis test, followed by Dunn's test, was used for comparison between experimental groups as regards the number of implantations, live fetuses, resorptions and fetal weight. For glycemia, biochemical parameters, number of corpora lutea, gravid uterine weight, and maternal weight gain was applied Analysis of Variance (ANOVA), followed by Bonferroni test. The percentual values were calculated by the Fisher Exact Test (Zar, 2009). Differences were considered statistically significant when $p < 0.05$.

3. Results

As shown in Fig. 1, non-diabetic group presented a glycemic level under 100 mg/dL. Blood glucose levels were higher than 300 mg/dL in the diabetic pregnant rats. The treatment with *Morus nigra* aqueous extract did not interfere with glycemias from non-diabetic or diabetic groups compared to respective untreated groups (Fig. 1).

Table 1 shows the profile evaluation of biochemical parameters of all experimental groups studied. There were not any significant differences for total protein and HDL-lipoprotein levels and GSH-Px activity among different groups. The non-diabetic rats treated with the plant extract presented increased SOD activity. Diabetic status was observed in this study by increased CHO, TG and VLDL-lipoprotein levels as compared to non-diabetic group. The diabetic rats treated with *Morus nigra* had significantly reduced MDA, CHO, TG and VLDL serum concentrations.

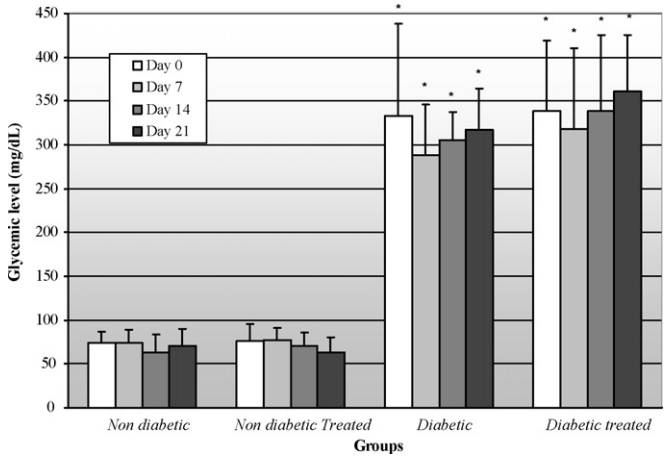

Fig. 1. Glycemia on days 0, 7, 14 and 21 of non-diabetic and diabetic rats treated or not with a *Morus nigra* aqueous extract during the pregnancy. Data shown as mean ± standard deviation (SD). *$p < 0.05$ compared to non-diabetic group (Bonferroni test).

There were no changes in the parameters of the reproductive outcome in the non-diabetic group treated with plant extract compared to non-diabetic group. The diabetic group, regardless of *Morus nigra* treatment, presented increase in placental index, placental weight, percentage of resorption per implantation sites, and SPA fetuses; reduced percentage of live fetuses per implantation sites, maternal weight gain, maternal weight gain free of gravid uterus, fetal body weight, and percentage of APA and LPA fetuses as compared to non-diabetic group. Corpora lutea and live fetus numbers, and gravid uterus weight in the diabetic dams were significantly lower than those of non-diabetic group. *Morus nigra* treatment in diabetic group reduced placental index and weight compared to diabetic group (Table 2).

There were no significant differences among the four experimental groups with respect to frequency of external anomaly. Similarly, there were no significant differences in frequencies of skeletal and visceral anomalies in non-diabetic group treated with the plant extract as compared to non-diabetic group. The diabetic groups (untreated and treated) presented significant decrease in ossification centrum and increase in number and percentage of fetus per litter with skeletal anomalies (dumbbell ossification of vertebral centrum, supernumerary rib, sternebra agenesis, incomplete ossification of sternebra, and abnormally shaped sternebra) and total number of fetuses with visceral alteration as compared to non-diabetic group. The diabetic group also presented high percentage of fetus with visceral anomalies per litter (microphthalmia, hydroureter, and hydronephrosis) as compared to non-diabetic rats. The fetuses from diabetic dams exposed to *Morus nigra* extract had smaller incidence of skeletal anomalies, such as cleft palate, bipartite ossification of vertebral centrum, supernumerary rib, sternebra agenesis, incomplete ossification of sternebra, and abnormally shaped sternebra compared to diabetic group. The treatment with the plant extract in diabetic rats also decreased the number and percentage of fetus per litter with visceral anomalies, and incidence of fetal microphthalmia and hydroureter compared to diabetic group (Table 3).

4. Discussion and conclusions

The importance of use of medicinal plants is being widely experimentally studied at about 40 years all over the world, and the information used about the plant effects is collected and very knowledged by the folk reports. Besides, *Morus nigra* is also included, its leaves have been shown to possess diuretic, hypoglycemic, and hypotensive activities, whereas the root bark of mulberry trees has long been used for antiinflammatory, antitussive, and antipyretic properties (Pawlowska et al., 2008).

Diabetic patients have used *Morus nigra* extract for the treatment of diabetes, and diabetic women often use aqueous extracts

Table 2
Reproductive outcome from non-diabetic and diabetic rats treated or not with a *Morus nigra* aqueous extract during the pregnancy.

	Groups			
	Non-diabetic	Non-diabetic treated	Diabetic	Diabetic treated
Pregnant females (*N*)	17	20	18	14
Pregnant at term (*N*)	17	20	15	12
With total resorptions (*N*)	0	0	3	2
Corpora lutea				
Total (*N*)	239	267	215	167
Mean ± SD	14.06 ± 1.68	13.35 ± 1.53	11.94 ± 1.98*	12.50 ± 1.91
Implantation[a]				
Total (*N*)	210	251	201	161
Mean ± SD	12.35 ± 2.37	12.55 ± 2.42	11.17 ± 1.58	11.50 ± 2.31
Live fetuses[a]				
Total (*N*)	199	242	145	131
Mean ± SD	11.71 ± 2.34	12.10 ± 2.40	9.06 ± 3.34*	10.92 ± 2.68
Per implantation sites (%)	94.76	96.00	72.14*	81.37*
Dead fetuses[a]				
Total (*N*)	2	0	2	1
Mean ± SD	0.12 ± 0.33	0.10 ± 0.31	0.13 ± 0.34	0.08 ± 0.29
Resorptions[a]				
Total (*N*)	9	7	54	29
Mean ± SD	5.16 ± 5.75	3.26 ± 6.23	22.63 ± 57.13	6.81 ± 11.76
Per implantation sites (%)	4.29	2.78	26.87*	18.01*
Sex ratio (M/F)[b]	80/95	120/122	58/75	72/59
Maternal weight gain (g)	134.00 ± 22.17	138.35 ± 21.22	64.67 ± 44.96*	77.21 ± 45.57*
Gravid uterus weight (g)	82.62 ± 15.39	84.21 ± 16.44	65.96 ± 17.65*	70.37 ± 17.67
Maternal weight gain minus gravid uterus weight (g)	49.08 ± 17.27	54.14 ± 14.69	15.31 ± 15.74*	19.38 ± 23.88*
Fetal body weight (g)[a]				
Mean ± SD	5.40 ± 0.38	5.38 ± 0.42	4.44 ± 0.71*	4.73 ± 0.62*
SPA Fetuses (%)[b]	23.5	27.4	77.8*	79.4*
APA fetuses (%)[b]	47.3	53.5	17.7*	15.6*
LPA fetuses (%)[b]	29.2	20.1	4.5*	5.0*
Placental weight (g)				
Mean ± SD	0.47 ± 0.05	0.46 ± 0.05	0.66 ± 0.28*	0.52 ± 0.05*,#
Placental index (g)				
Mean ± SD	0.09 ± 0.01	0.09 ± 0.01	0.16 ± 0.07*	0.11 ± 0.02*,#

N, number. Data shown as mean ± standard deviation (SD) and proportions (%).
* $p < 0.05$ compared to non-diabetic group (Bonferroni; [a]Kruskal–Wallis test; [b]Fisher Exact Test).
# $p < 0.05$ compared to diabetic group (Bonferroni; [a]Kruskal–Wallis test; [b]Fisher Exact Test).

of plants during pregnancy, including *Morus* species, without any concern as to their possible repercussions. In the present study, *Morus nigra* treatment led no antihyperglycemic effect in diabetic dams and caused no hypoglycemic action in non-diabetic pregnant rats. These data did not support folk reports, but they confirmed previous results found in our laboratory (Volpato et al., 2005). This might be justified due to administered dose, short treatment period, presence of uncontrolled diabetes and the difference of sensitivity among the plants used and the animals tested, which would prevent the effective action by the plant extract. Contradictorily, there was evidence that *Morus nigra* extract (Hosseinzadeh and Sadeghi, 1999; Mudra et al., 2007) and other species of *Morus* (Andallu et al., 2001; Singab et al., 2005; Naowaboot et al., 2009) present hypoglycemic effects.

Our data showed that *Morus nigra* extract caused reduction in cholesterol, triglycerides and VLDL levels in diabetic dams, decreasing to similar concentrations as compared to non-diabetic rats. Costa et al. (2007) also verified that *Croton cajucara* presented hypolipidemic effect in rats due to presence of terpenoids. *Morus nigra* has significant flavonoid concentrations (Pawlowska et al., 2008) and, similar to terpenoids, flavonoids possess also hypolipidemic action (Jung et al., 2006).

In this study, the diabetic dams presented exacerbated lipoperoxidation as confirmed by increased MDA level. Many reports have been published that support the use of antioxidant supplementation at reducing oxidative stress and at slowing or preventing the development of diabetic complication in animals (Kaul et al., 1995; Keegan et al., 1995). The non-diabetic rats treated with plant extract in our study presented increased level of enzymatic antioxidant activity (SOD), showing *Morus nigra* presented antioxidant effect. In diabetic state, the aqueous extract treatment decreased levels of MDA, suggesting increased SOD consumption led to normal MDA rates. Naderi et al. (2004) also showed antioxidant effect of *Morus nigra* treatment *in vitro*. There are several vegetal compounds that may influence the up-regulation about antioxidant profile, like betulinic acid (Zheng et al., 2011). This substance was already identified by HPLC in leaf extracts of *Morus nigra*, showed in Padilha et al.'s (2010) study. Besides betulic acid, these authors also identified germanicol and β-sitosterol, members of the class of triterpenes and steroids.

In diabetic group, there was decrease in corpora lutea and live fetuses, and consequently, a decreased gravid uterus weight. The treatment with *Morus nigra* prevented these complications. In women with uncontrolled diabetes, miscarriages are frequent (Eriksson et al., 2003). The diabetic state caused higher frequency of embryonic deaths leading to increased number of resorptions and decreased percentage of live fetuses per implantation sites. *Morus nigra* treatment did not prevent the development of these complications in the diabetic rats and did not interfere with these parameters in non-diabetic rats. There were higher rates of small fetuses for pregnancy age, corroborating with experimental studies performed in our laboratory, which showed that rats with severe diabetes also presented a fetuses with intrauterine growth restriction (Rudge et al., 2007; Volpato et al., 2008; Damasceno et al., 2011). Maternal hyperglycemia causes fetal hyperglycemia, which contributes for fetal hypoinsulinemia. The decreased fetal insulinemia has also been suggested in the etiology of fetal growth restriction. If insulin is decreased in the fetuses from diabetic mother, hypoinsulinemia causes fetal growth restriction seen in diabetic pregnancy (Holemans et al., 2003). In our study, the

Table 3
Frequency of fetal anomalies from non-diabetic and diabetic rats treated or not with a *Morus nigra* aqueous extract during the pregnancy.

Variables	Groups			
	Non-diabetic	Non-diabetic treated	Diabetic	Diabetic treated
External anomalies				
Number fetuses examined (litter)	201 (17)	243 (20)	117 (12)	132 (12)
Total number of fetuses (%) with alteration	0 (0.0%)	0 (0.0%)	1 (0.9%)	0 (0.0%)
Mean % fetuses with alteration per litter (mean ± SD)	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.6	0.0 ± 0.0
Gastrosquises	0 (0.0%)	0 (0.0%)	1 (0.9%)	0 (0.0%)
Exencephaly	0 (0.0%)	0 (0.0%)	1 (0.9%)	0 (0.0%)
Skeletal anomalies				
Number fetuses examined (litter)	105 (17)	71 (12)	60 (12)	67 (12)
Mean of total number of ossification centrum (mean ± SD)	38.0 ± 3.3	39.3 ± 4.8	28.9 ± 6.0[a]	32.2 ± 3.2[a]
Total number of fetuses (%) with alteration	38 (36.2%)	25 (35.2%)	47 (78.3%)*	46 (68.7%)*
Mean % fetuses with alteration per litter (mean ± SD)	35.5 ± 22.7	37.4 ± 28.3	82.7 ± 33.3[a]	70.7 ± 25.8[a]
Incomplete ossification of cranius	0 (0.0%)	0 (0.0%)	2 (3.4%)	1 (1.5%)
Cleft palate	1 (0.9%)	0 (0.0%)	17 (28.3%)*	0 (0.0%)#
Dumbbell ossif. of vertebral centrum	2 (1.9%)	0 (0.0%)	15 (25.0%)*	11 (16.4%)*
Bipartite ossif. of vertebral centrum	2 (1.9%)	2 (2.8%)	13 (28.3%)*	1 (1.5%)#
Supranumerary rib	17 (16.2%)	13 (18.3%)	38 (63.3%)*	30 (44.8%)*,#
Wavy rib	0 (0.0%)	0 (0.0%)	1 (1.7%)	0 (0.0%)
Sternebra agenesis	2 (1.9%)	1 (1.4%)	17 (28.3%)*	7 (10.4%)*,#
Unossified sternebra	3 (2.8%)	1 (1.4%)	3 (5.0%)	1 (1.5%)
Incomplete ossification of sternebra	13 (12.4%)	10 (14.0%)	28 (46.7%)*	18 (26.9%)*,#
Extra ossification site of sternebra	0 (0.0%)	0 (0.0%)	1 (1.7%)	0 (0.0%)
Abnormally shaped sternebra	7 (6.7%)	10 (14.0%)	48 (80.0%)*	32 (47.8%)*,#
Visceral anomalies				
Number fetuses examined (litter)	93 (17)	112 (19)	57 (12)	65 (12)
Total number of fetuses (%) with alteration	16 (17.2%)	13 (11.6%)	31 (54.4%)*	23 (35.4%)*,#
Mean % fetuses with alteration per litter (mean ± SD)	17.1 ± 21.1	11.3 ± 16.7	57.4 ± 26.0[a]	36.7 ± 21.3
Microphthalmia	1 (1.0%)	0 (0.0%)	6 (10.5%)*	2 (3.1%)#
Ectopic kidney	2 (2.1%)	6 (5.4%)	2 (3.5%)	10 (15.4%)*,#
Dilated renal pelvis	7 (7.2%)	5 (4.5%)	6 (10.5%)	11 (16.9%)
Hydroureter	5 (5.2%)	2 (1.8%)	10 (17.5%)*	1 (1.5%)#
Hydronephrosis	1 (1.0%)	2 (1.8%)	11 (19.3%)*	4 (6.2%)

* $p < 0.05$ compared to non-diabetic group (Fisher Exact Test).
\# $p < 0.05$ compared to diabetic group (Fisher Exact Test).
[a] $p < 0.05$ compared to non-diabetic group (Kruskal–Wallis test).

placental weight and index were greater in the diabetic rats. However, this increased placental weight was insufficient for fetal nourishment. As a result, there was a higher proportion of small for pregnancy age fetuses in the diabetic groups, thus confirming the existence of placental dysfunction in maternal–placental–fetal exchanges (Calderon et al., 1992). *Morus nigra* extract was not able to increase the rates of fetuses classified as APA and LPA in the diabetic group.

Hyperglycemia during embryogenesis has been associated with birth defects in pregnancies complicated by diabetes (Damasceno et al., 2002). The anomalies most often affect the central nervous system, heart and large vessels, kidneys, and axial skeleton (Schaefer-Graf et al., 2000). In our investigation, *Morus nigra* treatment in non-diabetic group did not alter the incidence of external, skeletal, and visceral anomalies compared to non-diabetic group. In the same way, the treatment of *Morus nigra* did not alter the incidence of external anomaly in diabetic group. The diabetes caused increase in skeletal and visceral anomalies. The most common skeletal anomalies in fetuses were cleaf palate, dumbbell and bipartite ossification of vertebral centrum, supernumerary rib, and in sternebra (agenesis, incomplete ossification, and abnormally shaped). About the visceral anomalies, the diabetic group presented high incidence of microphthalmia, hydroureter, and hydronephrosis. The treatment of *Morus nigra* presented significant decrease in rate of these anomalies in diabetic group. There was also decreased total number of fetuses with visceral alteration and percentage of fetuses with alteration per litter. Nevertheless, maternal glicemic control was not the mechanism by which *Morus nigra* treatment reduced the rates of these anomalies. This fact could be related to the presence of substances present in this extract, which might act in response to an increased oxidative stress caused by diabetes. Support for this hypothesis comes mainly from evidence that antioxidant enzymes provide protection against free radical-induced malformations (Reece and Eriksson, 2004). It was shown in our laboratory that *Bauhinia forfocata* extract, containing exogenous antioxidant, significantly reduced the anomaly rate in offspring from diabetic rats (Volpato et al., 2008).

The assessment of the ossification sites of the fetuses, proposed by Aliverti et al. (1979), is important in determining the fetal stage of maturity at birth. Diabetes resulted in delayed skeletal development as evidenced by the smaller number of ossification centrum in fetuses compared to fetuses from non-diabetic groups. Chahoud and Paumgartten (2005) verified strongest correlation between body weight and degree of ossification. These retarded ossification and the lower fetal body weight indicated that diabetes caused prenatal growth retardation and impaired fetal somatic development, and the *Morus nigra* treatment did not alter these findings.

In conclusion, *Morus nigra* leaf aqueous extract was not toxic at the dose level used in this study, but it failed to control the maternal hyperglycemia, pregnancy rate, and placental–fetal development in diabetic rats. However, *Morus nigra* treatment had antioxidant effect contributing to reduce incidence of skeletal and visceral anomalies in offspring from diabetic dams. The effects of medicinal plants and other co-adjuvant resources, which may affect antioxidant status during the course of experimental diabetes in pregnancy, as an approach to devising inexpensive therapeutic measures for the management of the many and varied disturbances of embryofetal development of clinical diabetes.

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