HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2017.01.010

Effects of the hydro-ethanolic extract of *Marrubium vulgare* in female rats

Rim Aouni[1,2], Mossadok Ben Attia[1,3], Mohamed Habib Jaafoura[4], Amina Bibi-Derbel[5], Mustapha Haouari[2,6]✉

[1]*Faculty of Sciences of Bizerte, Department of Life Sciences, 7021 Jarzouna, University of Carthage, Tunisia*

[2]*INNTA, SURVEN Research Laboratory (Monitoring and Nutritional Epidemiology in Tunisia), 1006 Tunis, Tunisia*

[3]*Laboratoire Biomonitoring of the Environment, Faculty of Sciences of Bizerte, 7021 Jarzouna, University of Carthage, Tunisia*

[4]*Orthopedic InstituteMohamed Kassâb, Laboratory of Cyto-Morphology and Anatomy Pathologic, Av Habib Bourguiba 2010 Ksar said, Tunis, Tunisia*

[5]*National Institute of Nutrition and Food Technology, Clinical Laboratory of Biochemistry, Bab Saâdoun 1007, Tunis, Tunisia*

[6]*Ecole Superior of Science and Technology of Health of Tunis, University of Tunis El Manar, Street 4021, Tunis, Tunisia*

ARTICLE INFO

Article history:
Received 20 Oct 2016
Received in revised form 11 Nov 2016
Accepted 10 Dec 2016
Available online 21 Jan 2017

Keywords:
Vegetal extract
Abortificiant effect
Hematological parameters
Fetuses
Uterus
Histology

ABSTRACT

Objective: To evaluate the effects of ethanol–water (80:20) extract of *Marrubium vulgare* (*M. vulgare*) on the hematological parameters, macroscopic and histological aspects of the uterus and fetus in non-pregnant and pregnant rats.

Methods: Female rats were divided into 4 equal groups ($n = 9$), group N (normal rats) and group G (pregnant rats) considered as control groups, group NE (normal rats treated with the ethanol–water (80:20, v/v) extract of *M. vulgare*) and group GE (pregnant rats treated with the extract). The ethanol–water (80:20) plant extract was administered in a single daily dose 1 g/kg at the morning, during 19 d. On the 19 day of the experiment, animals were sacrificed, the uterus and fetuses were removed for the morphological and histological studies and the blood was collected in EDTA tubes for the measurement of hematological parameters with the use of an automate 'HORIBA ABX Micros 60 Hematology Analyzer'.

Results: Our results showed, in group NE and GE, a significant decrease on hematological parameters: red blood cells (NE: 18.6%; GE: 38.4%), hematocrit (NE: 13.8%; GE: 20.4%), hemoglobin (NE: 12.1%; GE: 8.3%) and mean corpuscular volume (NE: 6.4%; GE: 2%) with P more less a 0.05. Indeed, the extract of *M. vulgare* caused a significant decrease on the mean implantations of fetuses (82.5%, $P < 0.001$) and their size (47.2%, $P < 0.01$). As for the macroscopic and histological appearance of uterus, our data showed no change in normal treated rats. In contrast, the treated pregnant rats showed a severe histological change characterized by the existence of location of stopped gestation. Furthermore, it was also found in the uterus of these rat lyses placental and embryo tissue.

Conclusions: All these results support the hypothesis of an abortifacient effect of *M. vulgare*.

1. Introduction

Marrubium vulgare L. (*M. vulgare*, Lamiaceae), commonly known as 'White Horehound', is a robust perennial herb, densely cottony stems with white flower. Several studies have reported that treatment with low doses of *M. vulgare*, has many beneficial effects such as: anti-bacterial effect [1] and antioxidant action [2–4]. It was also proved that this plant lowers blood pressure in rats when used in traditional Moroccan medicines (hypotensive activity) [5]. Other effects are hypoglycemic and hypolipidemic effects that have been proved by the administration of *M. vulgare* in diabetic rats with 500 mg/kg/d [6,7]. Scientific research about *M. vulgare* has shown that the treatment with different doses, in adult rats, induces a slight increase in body weight over time [8]. Also, the treatment improves liver function in rats [9,10]. These actions were demonstrated both in humans and animals. Four groups of

First author: Rim Aouni, Faculty of Sciences of Bizerte, Department of Life Sciences, 7021 Jarzouna, University of Carthage, Tunisia.
E-mail: rimouni1@yahoo.fr
✉Corresponding author: Mustapha Haouari, Ecole Superior of Science and Technology of Health of Tunis, University of Tunis El Manar, Street 4021, Tunis, Tunisia.
E-mail: haouari.mustapha@yahoo.com

Peer review under responsibility of Hainan Medical University.
Foundation project: This work, supported by grants from the Ministry of Higher Education and Research, was conducted in laboratory of research: Monitoring and nutritional epidemiology in Tunisia, LR 12SP05.

female rats received by gavage a single daily dose of 1 g/kg of body weight during nineteen days. The present study was conducted to investigate the hypothesis of a toxic effect of ethanol–water (80:20, v/v) of *M. vulgare* in high doses causing, in pregnant rats, an abortion and/or growth retardation of some embryos. In this context, we follow the perturbations on hematological parameters, on one hand, and the probable changes on the macroscopic and histological appearances of the uterus and fetuses, on the other.

2. Material and methods

2.1. Plants and extraction

The leaves of *M. vulgare* were collected in the month of October, in the morning at a temperature of 9 °C of the region of Haidra located in the center-west of Kasserine, Tunisia. Then it was dried in the shade converted into a fine powder. Regarding the extraction, as the method of Ghnimi *et al.*, 2014 [11], the dried leaves (1 kg) were homogenized with 5 L ethanol–water (80:20, v/v) for 24 h. After filtration with Whatman paper the extract was evaporated to dryness *in vacuum* using a rotary evaporator at 37 °C. The percentage yield was calculated as 65.5 g per kg of powdered material and was stored at room temperature in opaque bottles. According to analysis by LC–MS and GC–MS, it was found that this plant was rich with saponins, tannins, alkaloids, terpenes, polyphenols, flavonoids and fatty acids.

2.2. Animals and experimental protocol

2.2.1. Animals and extract administration

Adult female albino rats Wistar weighed between 180 and 220 g were purchased from SIPHAT (Tunisia). The animals were housed in room temperature (22 ± 2) °C with 12/12 h (light/dark cycle). All the rats were provided with commercially available rat normal pellet diet (Almes, Mateur, Tunisia) and water *ad libitum*. The animals were kept for 2 weeks under standard conditions. All the experiments were conducted according to the Tunisian Ethical Committee for animal's laboratory (approval number: FST/LNFP/Pro 152012). Animals were divided into four groups with 9 in each group: group N (normal rats) and group G (pregnant rats) considered as control groups, group NE (normal rats treated with the ethanol–water (80:20, v/v) extract of *M. vulgare*) and group GE (pregnant rats treated with the extract). The plant extract was administered in a single daily dose 1 g/kg B.W., for 19 $_{d.}$ On the 19th day of the experiment, animals were sacrificed by decapitation; the blood was collected in EDTA tubes.

2.2.2. Vaginal smear

Vaginal smear was collected to identify the different phases of the estrous cycle. At the end of the pro-estrus, the adult virgin female rats were subjected to males to be fertilized. The presence of spermatozoa in the vaginal smear indicated successful mating and was considered as day one of gestation according to Sakraoui, 2008 and Keshri *et al.*, 2003 [12,13].

2.2.3. Hematological analysis

All measurements of the biological values were performed in a clinical laboratory of national institute of nutrition and food technology of Tunis. For measurement of hematologic parameters, an automate HORIBA ABX Micros 60 Hematology Analyzer was used to determine the values of red blood cells number (RBC), hematocrit parameters (HCT), hemoglobin rate (HGB) and mean corpuscular volume (MCV).

2.2.4. Morphological and histological analysis

The uteri of normal and pregnant rats were removed. Then the fetuses were harvested and weighed. The number of fetuses was determined and the mean implantations were calculated as total implantations sites divided by the number of pregnant rats, untreated and treated groups. The anti-implantation activity was calculated and compared to control groups.

$$\text{Antiimplantation activity } \% = \left(\frac{\text{No. of implants in control group–No. of implants in treated group}}{\text{No. of implants in control group}}\right) \times 100$$

The samples were fixed in 10% buffered formalin solution, dehydrated in graded ethanol series, embedded in paraffin for posterior cross sections of 5 µm. The slides were stained in hematoxylin eosin, mounted in balm for observation in an optic microscope [14].

2.3. Data analysis

Significant differences between the treated groups mean and its control group was performed by Student's *t* test. Differences were considered to be significant if $P < 0.05$. Data were analyzed with Excel and expressed as mean ± SEM.

3. Results

3.1. Hematological analysis

Treated normal rats (NE), demonstrated that the ethanol–water (80:20, v/v) extract of *M. vulgare* administrated by gavage during 19 d induced a significant decrease in the different hematological parameters (Table 1). The

Table 1
Effect of daily treatment with the water–ethanol (80:20, v/v) extract of *M. vulgare* on values of RBC (million/mm^3), HGB (g/L), HCT (%) and MCV (µ^3) in group N, NE, G and GE (n = 9).

Group	RBC	HGB	HCT	MCV
N	5.3 ± 0.8	15.8 ± 0.9	25.4 ± 1.4	53.2 ± 1.0
NE	4.3 ± 0.4**	13.9 ± 2.3**	21.9 ± 3.5**	49.8 ± 3.3*
G	3.9 ± 0.6	13.3 ± 0.9	23.6 ± 1.8	50.4 ± 1.5
GE	5.4 ± 1.3***	12.2 ± 0.9*	18.8 ± 2.9**	49.4 ± 0.9*

The measurements were performed on the 19th day of treatment. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$, compared to respective control group.

percentages of the treatment induced falls were (18.6%, $P < 0.01$), (12.1%, $P < 0.01$), (13.8%, $P < 0.01$) and (6.5%, $P < 0.05$) for respectively RBC, HGB, HCT and MCV. Similarly, the same tendency of variations in the treated pregnant rats GE was detected. Indeed, a significant decrease of the studied hematological parameters was detected in treated pregnant rats, compared to its respective control group G, with a lower values of both, HGB (8.27%, $P < 0.05$), HCT (20.33%, $P < 0.01$) and MCV (2.21%, $P < 0.05$). However, it showed a significant increase on the RBC (31.22%, $P < 0.001$).

3.2. Anti-implantations activity

The macroscopic appearances of the uterus, in pregnant treated rats during 19 d, showed very serious perturbations compared to her respective control group. In fact, the oral administration of the ethanol–water (80:20, v/v) extract of *M. vulgare* induced a significant decrease on the mean implantations of fetuses and their corporal size (the length between the head and the tail bone). The rates of these effects were 82.5% and 47.2% respectively. The implantation number was significantly declined in the pregnant treated group (0.5 ± 0.2) compared to pregnant control group (2.6 ± 0.2) ($P < 0.001$). The average size of fetuses in the pregnant treated group (1.5 ± 0.5) was also declined significantly compared to its control group (2.8 ± 0.6) ($P < 0.01$). The anti-implantation activity of the pregnant treated group and its control group were 82.5% and 0% respectively.

3.3. Morphological and histological analysis

The comparative study between the macroscopic morphology of the uteri in the normal treated rats (Figure 1B) and her respective control group (Figure 1A) showed no change between the two uteri. Further, the histological examination of the uterus in normal rats (Figure 1C) showed a presence of glandular epithelium that separates light of the uterus and endometrium, which contained several secretor glands regularly arranged. The myometrium was formed by a muscle bilayer with different dispositions, circular internal and longitudinal external. Such architecture of uterus morphology did not show any changes after administration of water ethanol–water (80:20, v/v) extract of *M. vulgare* in normal rats (Figure 1D).

In pregnant group of rats, our data demonstrated that the number of fetal rat for up to 12, with an average of eight as shown in the selected picture. In addition, it was observed that fetus's distribution between the two horns of untreated pregnant rats uterus was generally balanced, giving the body a beaded appearance (Figure 2A). In the contrary, the treatment by the ethanol–water extract during pregnancy caused uneven distribution fetuses between the two horns (Figure 2B and C).

The administration of ethanol–water (80:20, v/v) extract of *M. vulgare* by gavage, in pregnant rats, caused significant fetal growth retardation at certain stages (Figure 2D and 2E). Moreover, the histological examination in the uterus of treated pregnant rats showed the presence of remnants of placental and embryonic tissue lyses in light of their uterus (Figure 3).

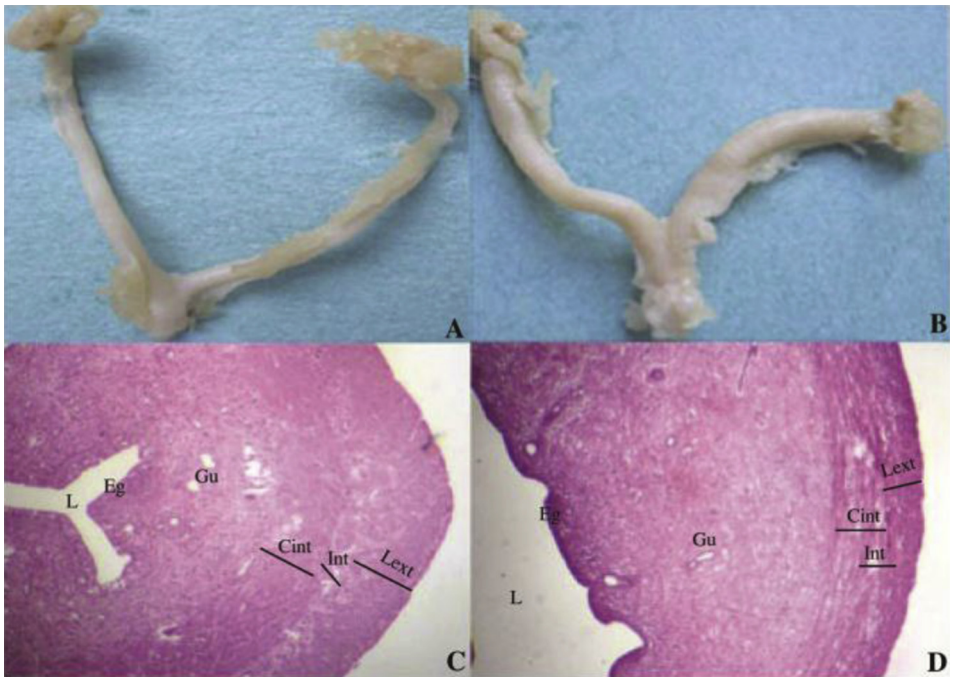

Figure 1. Effect of daily treatment with the ethanol–water (80:20, v/v) extract of *M. vulgare* on macroscopic and histological appearance of uteri in female normal rats.

(A) Macroscopic appearance of uterus in normal rats. (B) Macroscopic appearance of uterus in normal treated rats. (C) Histological appearance of uterus in normal rats (100×). (D) Histological appearance of uterus in normal treated rats (60×). L: light of the uterus; Eg: epithelium glandular; Gu: glands uterine; Cint: circular internal; Int: intermediate; Lext: longitudinal external. The observation was made on the 19th day of treatment.

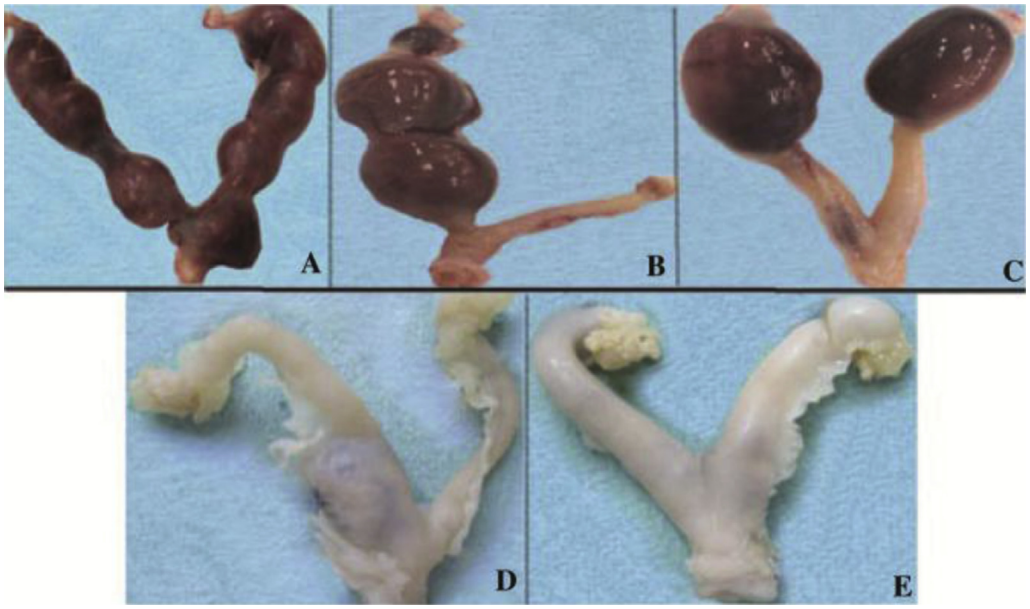

Figure 2. Effect of daily treatment with water–ethanol (80:20, v/v) extract of *M. vulgare* on the macroscopic appearance of uterine cavity in full-term pregnancy in rats.
(A) Macroscopic appearance of uterine cavity in full-term pregnancy in untreated pregnant rats. (B–E) Macroscopic appearance of uterine cavity in full-term pregnancy in treated pregnant rats. The figure demonstrated a significant delay in fetal growth at different stages of gestation.

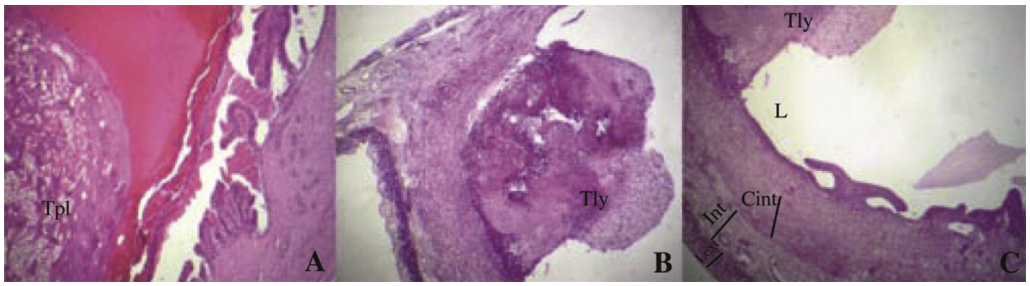

Figure 3. Effect of daily treatment with water–ethanol (80:20, v/v) extract of *M. vulgare* on histological appearances of uterus in pregnant rats.
The histological observations of uterus (40×) were made on the 19th day of treatment. L: light of the uterus; Cint: circular internal; Int: Intermediate; Lext: longitudinal external; Tly: lysed tissue; Tpl: placental tissue.

4. Discussion

Decreased values of RBC, HCT percentage, HGB and MCV in rats treated with ethanol–water (80:20, v/v)extract of *M. vulgare* (1 g/kg) during the three weeks of pregnancy supported the hypothesis suggesting that this extract in pregnant rats cause the appearance of a microcytic anemia. Taking into account the fact that pregnancy and lactation are physiologically prepared by a series of metabolic changes attributed to fetal growth, particularly affecting hematological values, we have, therefore, tried to detect the impact of treatment with the extract of *M. vulgare* on these parameters in pregnant rats. This disturbance may be related, at least in part, to inadequate nutritional bioavailability induced by treatment in pregnant rats whose needs are physiologically increased especially towards the end of pregnancy. These perturbations during pregnancy could be partially overcome by the hypertrophy of the intestinal mucosa, attributed to estrogen [15], to ensure better physiologically nutritional bioavailability, as our previous results have demonstrated [16], in the particular case of iron [17] demonstrated both in humans and in animals [18]. Furthermore, an increase in the value of transferring [19] with iron storage elevation is noticed towards the end of gestation, confirmed by an increase on the values of ferritin [20]. Considering these results, which show that treatment with a dose of 1 g/kg/d of ethanol–water (80:20, v/v) extract of *M. vulgare* during pregnancy in rats causes microcytic anemia. Therefore, the possibility of disruption of pregnancy following treatment with a high dose of ethanol–water extract of *M. vulgare*, would be entirely plausible. In agreement with this hypothesis, Léon *et al.*, 2014 and Sifakis *et al.*, 2000 have shown that anemia in pregnant women causes serious disturbances of pregnancy, including fetal growth retardation or even the risk of abortion [21,22].

To answer the question: 'How does pregnancy was affected in treated rats with the dose of 1 g/kg/d of extract of *M. vulgare* during 19 days?', our observations have revealed a significant reduction on the mean implantations and the size of the fetuses, compared to those from a normal gestation. According to these macroscopic observations of the uterus, we could detect a significant decrease on the number of fetuses per rat, with the appearance of cases of fetal growth being stopped. In addition, our histological observations showed that *M. vulgare* extract treatment in pregnant rats induced: (1) The presence of lysed tissue corresponding to embryonic and placental tissue; (2) A decrease in the thickness of the glandular epithelium and a

distension of the endometrium of the uterine wall; (3) A narrowing of the uterine myometrium with the outbreak of lysed pregnancy. Therefore, the assumption of a disturbance, relating to an action of the ethanol–water extract of *M. vulgare*, in the intestinal mucosa hypertrophy similar to that detected in the uterine tissue could explain, at least in part, the appearance of microcytic anemia. To better elucidate the mechanisms of the abortion action of *M. vulgare* extract, further studies will need to explore profoundly the eventual actions disrupting physiological levels of hormones ensuring good course of gestation.

In conclusion, the treatment by gavage of the ethanol–water extract of *M. vulgare* causes, in pregnant rats, a significant decrease on the hematological parameters. As for pregnancy perturbations, our data showed a decrease on the mean implantations of fetuses and their size. Furthermore, histological appearances of pregnant rat uterus revealed the presence of lysed tissue corresponding to embryonic and placental tissue. In conclusion, our study might be considered as the first report on the abortive properties of *M. vulgare* used at higher doses.

Funding

This work, supported by grants from the Ministry of Higher Education and Research, was conducted in laboratory of research: Monitoring and nutritional epidemiology in Tunisia, LR 12SP05. This research received no specific grant from any other funding agency in the public, commercial, or not-for-profit sectors.

Authors' contributions

Aouni R, Haouari M., Jâafoura M. H. and Bibi, A. participated in the collection and verification of data. Ben Attia M. performed the statistical analysis. Aouni R. and Haouari M. wrote the manuscript. Aouni R. and Haouari M. participated in the design of this study and edited the manuscript. All authors have read and approved the final manuscript.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors thank to the technical staffs of the laboratories of histopathology of the Institute of Orthopedic Mohamed Kassab-Tunis and of clinical biology of the National Institute of Nutrition and Food Technology of Tunis.

References

[1] Mubashir MH, Bahar A, Iqbal ZM, Saroor KA, Shamshir K, Singh P. Antibacterial activity of whole plant extract of *Marrubium vulgare*. *Afr J Biotech* 2008; **7**(2): 086-087.

[2] Kadri A, Zarai Z, Békir A, Gharsallah N, Damak M, Gdoura R. Chemical composition and antioxidant activity of *Marrubium vulgare* L. essential oil from Tunisia. *Afr J Bio* 2011; **10**(19): 3908-3914.

[3] Pukalskas A, Venskutonis PR, Salido S, De Waard P, Van Beek T. Isolation, identification and activity of natural antioxidants from horehound (*Marrubium vulgare* L.) cultivated in Lithuania. *Food Chem* 2012; **130**(3): 695-701.

[4] Orhan IE, Belhattab R, Senol FS, Gülpinar AR, Hosbas S, Kartal M. Profiling of cholinesterase inhibitory and antioxidant activities of *Artemisia absinthium, A. herba-alba, A. fragrans, Marrubium vulgare, M. astranicum, Origanum vulgare* subsp. glandulossum and essential oil analysis of two *Artemisia* species. *Ind Crop Prod* 2010; **32**(3): 566-571.

[5] El-Bardai S, Lyoussi B, Wibo M, Morel N. Comparative study of the antihypertensive activity of *Marrubium vulgare* and of the dihydropyridine calcium antagonist amlodipine in spontaneously hypertensive rat. *Clin Exp Hypertens* 2004; **26**(6): 465-474.

[6] Elberry A, Fathalla HM, Salah GA, Nagy A, Abdelsattar E. Methanolic extract of *Marrubium vulgare* ameliorates hyperglycemia and dyslipidemia in streptozotocin-induced diabetic rats. *Int J Diabetes Mellit* 2015; **3**(1): 37-44.

[7] Sfaxi A, Haouari-Oukerro F, Haouari M, Nagati K. Les effets du Marrube blanc '*Marubium vulgare.* L' sur le gain de poids, la glycémie, la triglycéridémie et la cholestérolémie à jeun, chez le rat. *Revue Méd Pharm Afr* 1994; **8**(2): 141-143.

[8] Haouari M, Sfaxi A, Nagati K, Kallal Z. Le Marrube blanc: *Marrubium vulgare* L. (Lamiaceae) plante hypoglycémiante. *Rev Méd Pharm Afr* 1991; **5**(2): 11-16.

[9] Elberry AA, Harraz FM, Ghareib SA, Nagy AA, Gabr SA, Suliaman MI, et al. Anti-hepatotoxic effect of *Marrubium vulgare* and *Withania sommifera* extracts on carbon tetrachloride-induced hepatotoxicity in rats. *J Basic Clin Pharm* 2010; **1**(4): 247-254.

[10] Ahmed B, Masoodi MH, Siddique AH, Khan S. A new monoterpene acid from *Marrubium vulgare* with potential anti-hepatotoxic activity. *Natprodres* 2010; **24**(18): 1671-1680.

[11] Wafa G, Amadou D, Larbi KM, Hela EFO. Larvicidal activity, phytochemical composition, and antioxidant properties of different parts of five populations of *Ricinus communis* L. *Ind Crops Prod* 2014; **56**: 43-51.

[12] Sakraoui F. *Etude de l'effet d'un traitement subaigu au chrome hexavalant (potassium dichromate) sur la fonction de reproduction et de développement embryonnaire chez le rat*. PhD Thesis. Tunisia: Université of Carthage; 2008.

[13] Keshri G, Lakshmi V, Singh MM. Pregnancy interceptive activity of *Melia azedarach* Linn in adult female Sprague–Dawley rats. *Contraception* 2003; **68**(4): 303-306.

[14] Sakraoui F, Bouzidi N, Bouakkaz A, Maurel D. Impact nociceptif du chlorobenzene chez la ratte gestante Wistar: aspects toxicologiques, histopathologiques et teratogenes. *Eur J Scien Res* 2008; **23**: 392-399.

[15] Haouari M, Jâafoura H, Sfaxi A, Kammoun A, Zouaghi H. Effets de l'oestradiol sur les protéines sériques et l'histo-morphologie duodénal, chez la ratte castrée. *Eurobiologiste (Paris)* 1998; **233**: 25.

[16] Suhail M, Patil S, Khan S, Siddiqui S. Antioxidant vitamins and lipoperoxidation in non-pregnant, pregnant, and gestational diabetic women: erythrocytes osmotic fragility profiles. *J Clin Med Res* 2010; **2**(6): 266-273.

[17] Haouari M, Haouari-Oukerro F, Alguemi C, Nagati K, Zouaghi H, Kamoun A. Effects of oestradiol-17 beta on small intestine iron absorption and iron uptake in to blood and liver. *Horm Metab Res* 1994; **26**(1): 53-54.

[18] Cao J, Chavez ER. Comparative trace mineral nutritional balance of first-litter gilts under tow dietary levels of copper intake. *J Trace Elem Med Biol* 1995; **9**: 102-111.

[19] Baynes RD, Lamarelli RDV, Beeawoda WRD, Dajee D, Van der Walt LA. Plasma lacto ferrin content in pregnancy. *South Afr J* 1989; **76**(10): 531-534.

[20] Abbas A, Sniijders RJM, Sadullah S, Nicolaides KH. Fetal blood ferritin and cobalamin in normal pregnancy. *Fetal Diagn Ther* 1994; **9**(1): 14-18.

[21] Savadogo LGB, Salimata O, Tamini C, Kinda M, Donnenet P. Characteristics of severely anemic pregnant women and perinatal outcomes in Banfora Regional Hospital, Burkina Faso: an epidemiological study. *Open J Obst Gynecol* 2014; **4**: 34-38.

[22] Sifakis S, Pharmakides G. Anemia in pregnancy. *Ann N Y Acad Sci* 2000; **900**: 125-136.