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Effect of organic materials extract from *Bunium incrassatum* (Talghouda) roots on hematological and histological parameters of the adrenal glands in the pregnant rabbits, *Oryctolagus cuniculus*

N. Hammoudi[a, c]*, H. Hafid[b], Y. Moumen[a], S. Chentouh[a], H. Djebaili[a,c] and S. Boulahbel[a]

[a]*Laboratory of Bio-molecules and Plant Breeding, Faculty of Exact Sciences and Sciences of Nature and Life, Larbi Ben Mhidi University, Oum EL Bouaghi, 04000, Algeria*
[b]*Laboratory of Natural Resources and Management of Sensitive Environments, Faculty of Exact Sciences and Sciences of Nature and Life, Larbi ben M'Hidi University, Oum El Bouaghi, 04000, Algeria*
[c]*National Center for Biotechnology Research, Ali Mendjeli Constantine, 25016, Algeria*

*Corresponding author, email: n.hammoudi@outlook.fr

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Abstract

This study aims to evaluate the effect of the treatment by organic materials extract of Bunium incrassatum roots on hematological and histological parameters of the adrenal gland in the pregnant rabbits during the last third of pregnancy. Twenty-four pregnant rabbits were divided into four groups. The group C received distillated water, served as control. Groups G1, G2, G3 received treatment of 50, 100, 200 mg/kg/day respectively. After treatments, the animals were sacrificed. The rabbit blood was collected in heparin tubes and the adrenal glands were removed, fixed in 10 % formalin and stained with hematoxylin-eosin. The treatment induces a significant increase in cholesterol levels in G1 and G2, in G3 being very highly significant. It also induces a significant increase in cortisol serum level in all treated groups. In addition, our results showed a progressive decrease in glycemia, which had significant in G2 and high significant in G3. The histological measurement of cortex layer thickness showed a very highly significant increase in fasciculate layer in G2 and G3. This suggests that Bunium incrassatum had a hypoglycemic effect and is safe for use up to 100 mg/kg/day for both nutritional and medicinal purposes which open new perspectives for this plant in the bioactive materials technology.

Keywords: Adrenal gland; Bunium incrassatum; Cholesterol, Cortisol; Pregnant rabbits.

1. Introduction

The use of plants for their medicinal properties is a very old practice. It finds its origins in the oldest civilizations and still well preserved for many centuries all over the world [1]. In Algeria, phytotherapy is an integral part of the local culture; population has an important indigenous knowledge acquired empirically through the generations. Its geographical location and climatic diversity have allowed the development of a very rich and highly diversified flora; which was used since time immemorial to treat several diseases[2-4].

The genus *Bunium* belonging to *Apiaceae* family consists of seven species in Algerian flora, four of which are endemic [5]. This genus is close to *Carum*. *Bunium* and *Carum* are two of the most important aromatic and medicinal plants, whose seeds and essential oils have been used in food and medicine all over the world for so long [6]. *Bunium incrassatum* species, locally known as "Talghouda" are extensively used in eastern Algeria; it is an economically important medicinal plant. Since ancient times, *Bunium incrassatum* has been traditionally used as galactagogues to improve breast milk production in the farm animals. The roots of this plant are quite nutritious and usually eaten as potato. In the indigenous system of medicines, dried and powdered tubers are regarded as found to be useful against inflammatory hemorrhoids and, in addition, for bronchitis and cough. Phytochemical analysis of the roots of this plant reveals the presence of coumarins, *Beta*-sitosterol, sucrose and oleic acid [7]. Previous phytochemical studies of this species have shown that this plant accumulates more than 45 compounds that fit into the chemical composition of essential oil [8]. However, to the best of our knowledge, we have no research on the impact of this plant's extracts in vivo. The present study was intended to continue the work undertaken in our laboratory Chentouh *et al* [9]; on the impact of administration of organic extract of *Bunium incrassatum* in pregnant rabbits on adrenal gland and serum lipids, as well as on body weight of pregnant rabbit and fetal weight and also to correlate the histological adaptive changes in adrenal gland in response to different doses.

2. Materials and Methods

2.1. Collection and identification of plant material

The *B. incrassatum* was collected in September 2015. It has been identified by herbalist. Plants were washed thoroughly with water; roots were removed and dried at room temperature, in the shade, for a few days and ground into fine powder.

2.2. Organic extract

Plant extraction was carried out according to the protocol of Bousetla *et al* [7], 100g of plant powder were macerated into 200 ml of ethanol/chloroform solvent (1:1, v/v) , during 24h in the shade. The homogenate obtained is filtered through Wattman paper (3mm). Solvents were totally recovered from the filtrate by rotary evaporator (BUCHI®) followed by oven-dried at 60°C and air-dried. 3 ml of the crude organic extract was obtained without any traces of solvent. Doses were prepared from this extract by dilution with distilled water.

2.3. Animals

Animal ethics approval was obtained from Animal Care Committee of the National Biotechnology Research Center in Algeria and was in accordance with guidelines of the International Bioethics Committee, European Institute of Bioethics and World Organization of Animal Health.

This study was conducted in autumn 2016, rabbits (*Oryctolagus cuniculus*) were purchased from the Technical Institute of Breeding, Hamma Bouziane, Constantine; Algeria. They were allowed ad libitum access to water and food (scraping food supplied by the National Office for Animal Feed (O.N.A.B.)) under natural conditions (temperature, photoperiod and humidity).

A total of 24 multiparous rabbits (3-4 parturition; 2.66-3.66 kg) were mated to fertile males. The day of mating was considered to be "zero day" of pregnancy which was confirmed by abdominal palpation on the 12th day.

2.4. Treatment methods

The pregnant rabbits were divided into 4 equals groups (n=6)and treated by gavage for 10 days from the 17th to the 27th day of gestation, organogenesis ends in 18th or 19th day of gestation and fetal growth is very rapid during this last third [10]. Groups G1, G2 and G3 received 50, 100, 200 mg/kg/day doses of *B. incrassatum* roots organic extract respectively, while the control group (C) received distilled water to maintain the same experimental conditions for all animals.

2.5. Sacrifice

In the 28th day and between 9:30 am and 10:00 am, the rabbits were sacrificed under non-stress conditions by rapid cervical decapitation. The time of sacrifice and blood sampling are very important for the blood cortisol determination, in order to limit any variations due to the nycthemeral cycle [11].

2.6. Blood sampling

Animals were sacrificed by decapitation; blood samples were collected in polyethylene tubes containing heparin and were taken immediately for the analysis of various hormonal (cortisol) and biochemical (cholesterol, triglyceride and glycaemia) parameters.

Analysis were carried out in the Ibn Sina laboratory Constantine; Algeria. The serum concentration of cortisol is measured by the kit Mini Vidas, Biomerieux Diagnostic, Automated Immunoassay Analyzer. Biochemical parameters were estimated by the kit BIOBASE BK200 Fully Automatic chemistry analyzer.

2.7. Histological studies

Adrenals glands were dissected out, cleaned from adherent fat and weighed (absolute weight). The relative gland weight was determined by the following equation:

Relative organ weight = Absolute organ weight (g) × 100/ Body weight of animal on sacrifice day (g).

After that, glands were fixed in Formalin (10%), dehydrated in ethyl alcohol, cleared in xylene and then embedded in paraffin. section of 5 µm thickness were stained with hématoxyline and eosin. Slides were observed and photographed under a light microscope (B-150 OPTIKA)® connected to a digital camera.

Morphometric analysis was performed using ImageG software. Tissue thickness was measured under a ×10 objective from the 24 adrenal sections. Based on the study done by Aknoun-Sail *et al* [12], Eight measurements were taken around each adrenal section firstly for whole cortical thickness and then for each zonae thickness. Measurements were averaged for each animal.

2.8. Statistical study

Data Analysis was done using XLSTAT software, version 2014. Results were expressed as mean ± standard deviation (mean ± SD). Means were analyzed using a one-way ANOVA, followed by student's test to compare the difference between the control and the treated values and $P<0.05$ was considered significant [13].

3. Results

Table 1 showed that the oral administration for 10 days of the organic extract of *B. incrassatum* roots to pregnant rabbits has non-significant changes of the rabbit's weight progress, relative weight of adrenal gland and fetal weight.

Table1: Effect of organic extract of *B. incrassatum* roots on the weight of the pregnant rabbits, theirs adrenal glands and fetuses, in the last third of pregnancy period.

	Parameters	Groups				P value
		C	G1	G2	G3	
Weight progress	Initial weight (Kg)	3,35±0.389	3,39±0.410	3,00±0.332	2,95±0.273	
	Final weight (Kg)	3,55±0.421	3,56±0.343	3,14±0.355	3,20±0.398	
	Gained weight (Kg)	0,2±0.120	0,17±0.067 ^{NS}	0,14±0.060 ^{NS}	0,25±0.134 ^{NS}	
	Augmentation rate	5,97%	5,01%	4.66%	8.74%	0.64
Fetal weight(g)		36.00±2.79	38.66±1.12^{NS}	35.89±4.14^{NS}	33.32±1.82^{NS}	0.64
Relative adrenal gland weight	Relative adrenal gland weight (g)	0.0061±0.0015	0.0066±0.001^{NS}	0.0049±0.0005^{NS}	0.0047±0.001^{NS}	0.62
	Relative right AG weight (g)	0.0061±0.0015	0.0071±0.001^{NS}	0.0049±0.005^{NS}	0.0043±0.001^{NS}	0.47
	Relative left AG weight (g)	0.006±0.002	0.006±0.001^{NS}	0.005±0.0005^{NS}	0.0051±0.001^{NS}	0.75

(NS: non-significant)

In our experiment, four parameters were examined, triglyceride, cholesterol, glycemia and cortisol (table 2). We found a very significant increase in cortisol levels (p = 0.009), supplemented by a very highly significant increase in cholesterol levels (p = 0.0001), where they reached a top level with the treatment of 200 mg/kg/day, with a cholesterol and cortisol levels of 0.301g/l, 21.50µg/dl, comparing to the control group with 0.082g/l, 6.23 µg/dl respectively. In the other hand, the triglyceride levels had a non-significant decrease, which extended to 0.542g/l, 0.539g/l, 0.526g/l in G1, G2 and G3 respectively, comparing to the control group with 0.643g/l. The glycemia levels had a significant decrease that reached 0.998g/l in the second group (G2) and very significant decrease where it reached its lowest level with 0.862g/l in the third group (G3) compared to the control group with 1.172g/l.

Table2: Effect of organic extract of *B. incrassatum* roots on the blood biochemical and hormonal parameters in the last third of pregnancy period.

Parameters	C	G1	G2	G3	P value
Triglycerides level (g/l)	0.64±0.12	0.55±0.15 ^{NS}	0.54±0.09 ^{NS}	0.53±0.07 ^{NS}	0.79
Cholesterol level (g/l)	0.08±0.03	0.11±0.02 ^{NS}	0.15±0.03 ^{NS}	0.3±0.04***	0.0001
Cortisol level (µg/dl)	6.24±3.69	14.83±2.43*	13.51±4.24*	21.50±2.94**	0.009
Glycemia level (g/l)	1.17±0.09	1.04±0.18^{NS}	1.00±0.31*	0.86±0.07**	0.001

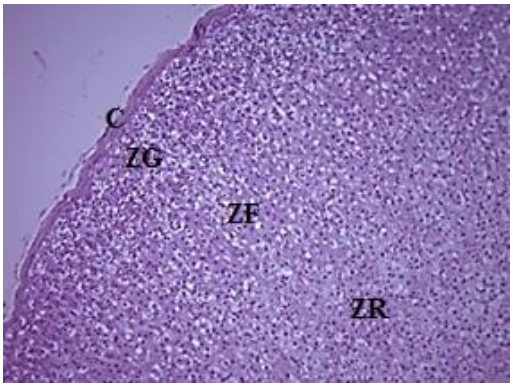
(NS = non-significant, *significant, **highly significant, ***very highly significant)

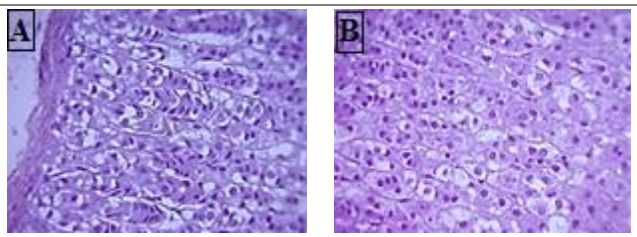
Figure 1 showed the three concentric cortex zones of the adrenal gland, which are glomerulosa, fasciculata and reticularis. The histological measurements of these layers (table 3) showed that the general thickness of the cortical zone was not significantly increased throughout the different doses. While a very highly significant increase (p = 0.0001) in the thickness of fasciculate layer is marked in parallel with a significant decrease (p = 0.04) in the thickness of reticular layer in both G1 and G2 compared to the control group. However, the thickness of Glomerulosa layer was not significantly increased.

Table3. Effect of organic extract of *B. incrassatum* roots on the cortical thickness layers, for rabbit in the last third of pregnancy period.

Groups	C	G1	G2	G3	P value
Cortex (µm)	0.81±0.09	0.82±0.03^{NS}	0.87±0.03^{NS}	0.88±0.05^{NS}	0.1
Glomerulosa (µm)	0.17±0.03	0.16±0.04^{NS}	0.18±0.01^{NS}	0.18±0.04^{NS}	0.57
Fasciculata (µm)	0.33±0.05	0.38±0.03*	0.49±0.06***	0.50±0.03***	0.0001
Reticularis(µm)	0.30± 0.09	0.28±0.05^{NS}	0.19±0.08*	0.19±0.06*	0.04

(NS = non-significant, *significant, **highly significant, ***very highly significant)

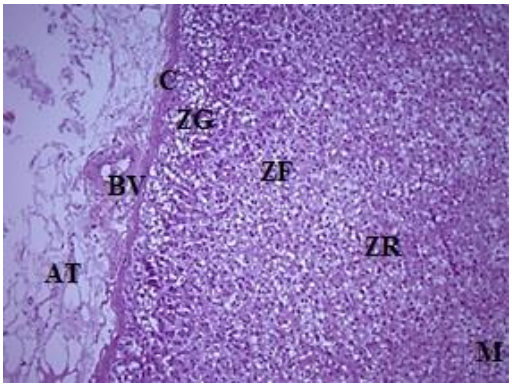


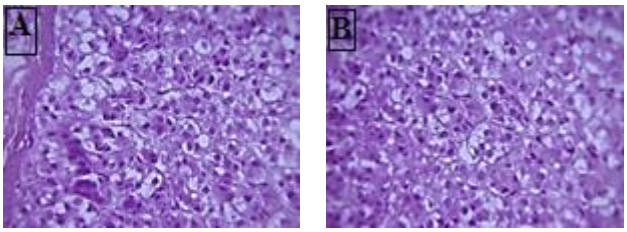


A: Zona Glomerumoza (×40), **B**: Zona Fasiculata (×40).

← (×10), **C**: capsule, **ZG**: zona glomerulosa, **ZF**: zona Fasciculata, ZR: zona reticularis.

Fig.1-1. Control

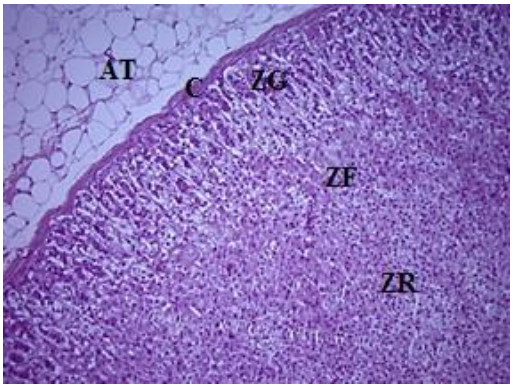


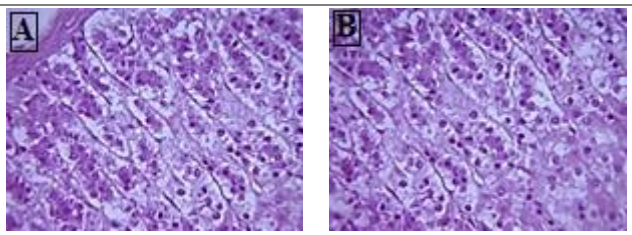


A: Zona Glomerumoza (×40), **B**: Zona Fasiculata (×40).

← (×10), **AT**: adipause tissue, **BV**: blood vessel, **C**: capsule, **ZG**: zona glomerulosa, **ZF**: zona Fasciculata, ZR: zona reticularis, **M**: medulla.

Fig.1-2. Treated with 50 mg/kg/day

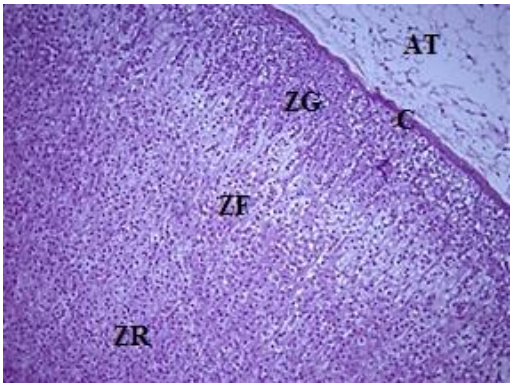


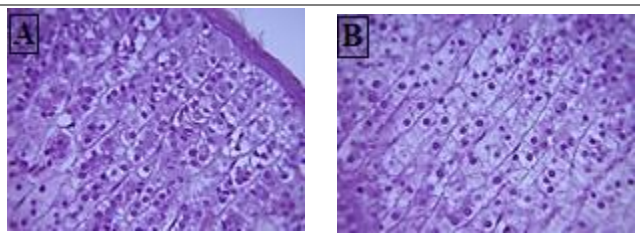


A: Zona Glomerumoza (×40), **B**: Zona Fasiculata (×40).

← (×10), **AT**: adipause tissue, **C**: capsule, **ZG**: zona glomerulosa, **ZF**: zona Fasciculata, ZR: zona reticularis

Fig.1-3. Treated with 100 mg/kg/day





A: Zona Glomerumoza (×40), **B**: Zona Fasiculata (×40).

← (×10), **AT**: adipause tissue, **C**: capsule, **ZG**: zona glomerulosa, **ZF**: zona Fasciculata, ZR: zona reticularis

Fig.1-4. Treated with 200 mg/kg/day

Figure 1. Histological section of adrenal gland in the last third of gestation for rabbits treated with *Bunium incrassatum* organic extract.

Regarding animals behavior it was normal either in the control or in treated groups. In effect, No treatment-related deaths and clinical signs of toxicity were observed throughout the study period for all experimental groups whatever the doses.

4. Discussion

The objective of this work is to assess the effect of the treatment of pregnant rabbits by organic extract of *B. incrassatum.*

Our results show that the increase in the final body weight of pregnant rabbits compared to their initial weight was not statistically significant in G1 and G2 but for G3 the increase was more appreciated. Analogous results were found by Dhandapani *et al* [14], in which they reported weight gain after using *Bunium persicum* which may due to the fact that both plants belong to the same genus of the *Apiaceae* family. We also observed no statistically significant decrease ($p > 0.05$) in relative weight of adrenal in treated animals in comparison with the control. The cholesterol is essential for the synthesis of progesterone and 17 beta-estradiol, hormones that actively participate to sustain gestation. Othmani & Benazzoug [15] found that gestation in rabbit causes a progressive decrease in the concentration of the cholesterol until reaching levels not detectable at term. The current study reveals that oral administration of the organic extract of *B. incrassatum* for last period of gestation causes a very highly significant increase ($p < 0.001$) in cholesterol in group G3 treated with 200 mg/kg/day and significant increase in G1 and G2 treated with 50 and 100 mg/kg/day respectively, compared to the control. Also, we have observed a no significant decrease in triglyceride in all treated groups compared to the control. The same results were found by Montoudis *et al* [16] who reported that treatment of pregnant rabbit by dietary enriched cholesterol (DEC) increased levels of cholesterol and free fatty acids in maternal plasma compared to untreated rabbits. HMG-CoA reductase is a rate-limiting enzyme for mevalonate and cholesterol synthesis. Montoudis *et al* [17] reported that during gestation in rabbits, the hepatic activity of HMG-CoA-reductase and that of cholesterol-7a-hydroxylase is reduced by DEC, whereas ACAT activity is not affected, but reduced by gestation. Our results can be interpreted by the presence of β-sitosterol whose structure is similar to that of cholesterol. Othmani & Benazzoug [15] reported that in the first week of rabbit gestation, a decrease in blood glucose was recorded, then becomes significant at J30 ($P \leq 0.01$). nevertheless our results showed that after treatment with 50, 100 and 200 mg/kg/day using organic extract of *B. incrassatum* during the last period of pregnancy, no significant decrease in blood glucose in G1 (1.04±0.18), significant decrease in G2 (1.00±0.31) and high significant decrease in G3 (0.86±0.07) compared to the control (1.17±0.09). These drops can be explained by the presence of two compounds in the extract namely β-caryophyllene (BCP) and β- sitosterol. Rafeek Hidhay *et al* [18] noticed that oral administration of BCP increased insulin with concomitant decrease in glucose levels in diabetic rats. An enhancement in plasma insulin levels in BCP treated rats implies its insulin-tropic property. β-sitosterol was shown to have antidiabetic and antioxidant effects in animal models. It is well established that the adrenal gland is known to be one of the main organs that accumulate phytosterol [19]. The metabolite of β-sitosterol in a cultured swine adrenal slice is cortisol [20]. These studies suggest that β-sitosterol is metabolised in the same way as cholesterol, leading to the sterol skeleton entering the cholesterol biotransformation pathways .Many studies have demonstrated that circulating cortisol levels increase clearly to about three fold levels by the third trimester of pregnancy [21]. This rise in cortisol is partly due to estrogen stimulation of corticosteroid-binding globulin with a rise in free (or bioavailable) cortisol levels [22-23]. Our results showed that the cortisol values in control animals were significantly lower than the values in those treated by *B. incrasstum* extract and the increase in cortisol secretion is important in all treated groups. Cortisol is well known for its lipogenic effects and several studies have shown an association between cortisol concentrations and cholesterol concentrations [24-27]. Among the hormones that increase during pregnancy, cortisol probably has the greatest effect on lipids and on lipoproteins even though lipids are known to increase during acute and chronic stress [24-25-28]. Glucose plays a key role in the intrauterine development of the fetus [29-30]. Indeed, it represents for the fetus a source of energy and a growth factor and covers at the end of pregnancy 80% of the foeto-placental oxidative requirements [31]. Maternal basal plasma glucose concentration tends to decrease with the progression of pregnancy even in the presence of a twofold increase of insulin level [32, 33]. We can explain this by the presence of β-sitosterol in the extact which inhibits glucosidase enzyme present in the epithelium of the small intestine, which works to facilitate the absorption of glucose by the small intestine. Histological study of the gland showed that the general thickness of the cortical zone was not significantly increased throughout the different doses. While the fasciculate layer, responsible of cortisol secretion, had a very highly significant progression, with all diverse doses respectively, and that lead rationally to an increasing of cortisol levels. This increase was joined by a significant reticular layer decrease. This is link between cortisol levels and histological modification in the different layer of adrenal gland. A probable simultaneous transformative change of reticular cells into fascicular cells in response of the stimulation by the extract to deal with the enhanced demand of glucocorticoide might have been responsible for the increase in the thickness of Zona Fasciculata. In the present study, we have recorded that the reduction of offspring weight in G2 and G3 could be related to high increase maternal cortisol level and reduction of glucose metabolism and several studies demonstrate that birth weight is the best indicator of fetal growth and newborn's health. [34]Conclusion

Our preliminary study, performed to evaluate the effect of the treatment by organic materials extract from *Bunium incrassatum* (Talghouda) roots on hematological and histological parameters of the adrenal glands during the last third of pregnancy period in rabbits, is realized for the first time. The results indicate that the components of this

medicinal plant can, at specific doses, have a positive effect on some reproductive parameters. When administered at higher doses to pregnant rabbit caused highly significant decrease in blood glucose and highly significant increase in cortisol such as moderate reduction in fetal weight .This suggests that *B. incrassatum* has a hypoglycemic effect and is safe for use up to 100 mg/kg/day for both nutritional and medicinal purposes which opens new perspectives for this plant in the bioactive materials technology.

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