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Histo-biochemical evaluation of spearmint dried leaves extract on selected organs of pregnant albino rats and their neonates: A long-term study

Uzma Ali[1], Zaima Umar[2], Amber Salman[2], Momna Riaz[2], Muhammad Usman[3], Humaira[1], Muhammad Ahsan Naeem[4], Talha Umar[5] and Saba Zulfiqar[6]

[1]Department of Anatomy, Faisalabad Medical University, Faisalabad, Pakistan
[2]Department of Anatomy, The University of Faisalabad, Health Sciences Wing, Faisalabad, Pakistan
[3]Department of Basic Sciences (Histology), KBCMA College of Veterinary and Animal Sciences, Narowal, Pakistan
[4]Department of Basic Sciences (Pharmacology), KBCMA College of Veterinary and Animal Sciences, Narowal, Pakistan
[5]Department of Clinical Medicine Huazhong Agriculture University, Huazhong, China
[6]Department of Biochemistry, The University of Faisalabad, Health Sciences Wing, Faisalabad, Pakistan

Abstract: The primary focus of this study is to elucidate the adverse effect of dried leaf extract of spearmint (SE) on histomorphometry of ovaries and uterus of pregnant albino rats along with weight and crown-rump length (CRL) of their fetuses. Albino rats (n=30) were split into three groups, viz; Group A (Placebo), Group B (0.3g/kg SE) and Group C (0.6g/kg SE). The SE was administered for 30 days through gastric intubation. On 31st day of experiment, a male was introduced for 5-days in each group to make the female rats pregnant. SE was administered till the parturition of the neonates. At parturition, the maternal uteri and ovaries and neonates' renal and hepatic tissues were histological analyzed along with the blood for progesterone level. Results means were compared by Mann-Whitney test. The body-weights of experimental groups decreased significantly while counter-part of ovaries followed reverse trend. The ovaries showed atrophied antral, graafian-follicle and corpora lutea. The layer thickness of uteri was also decreased. The degenerative changes in the renal and hepatic tissues were observed with decreased weight and crown-rump-length of neonates. In the light of these results, further research work should be carried out to evaluate its other toxic effects on different organs of the body.

Keywords: Spearmint extract, pregnancy, fetal growth, histology.

INTRODUCTION

Herbs are plants valued for their medicinal and aromatic characteristics and are often grown for these unique properties (Mimica-Dukic *et al.*, 2003; Brahmi *et al.*, 2017; Naseer *et al.*, 2022). For thousands of years, the knowledge of herbs has been passed down from one generation to another (Sulieman *et al.*, 2011; Salsabil *et al.*, 2022; Velázquez-Antunez *et al.,* 2023). Many often used culinary herbs, such as basil, mint, rosemary, sage, savoury, marjoram, oregano, thyme, lavender and perilla, are present in the plants, which are frequently scented throughout. Spearmint is also a member of the mint family (Naghibi *et al.*, 2005). It is a member of the order Mentheae, family Lamiaceae, subfamily Nepetoideae, kingdom Plantae, order Lamiales, genus *Mentha* and specie *Mentha* Spicata (Brahmi *et al.*, 2017). It is indigenous to Egypt and North Africa, where it was originally identified in the 18th century. It is invasive in the Great Lakes region where it was first sighted in 1843 (Rahman *et al.*, 2008). In all fairly temperate areas, spearmint thrives. Its aggressive, spreading rhizomes force gardeners to grow it frequently in pots or planters (Abbaszadeh *et al.*, 2021). In spearmint, you can find carvone (29-70%), volatile oil (21-2.1%), limonene (4-24%) and cierole (3-8%). Menthone makes up 2% of its oil as well. Its main constituent, carvone, has a moisture content of 76%, an ash content of 3.4% and significant nutritional elements including protein, fat and fibre. It also has considerable amounts of sodium, calcium, potassium, iron and carbohydrate. Additionally, it has bioactive flavonoids including epicatechin and catechin (de Sousa Barros *et al.*, 2015; Ouakouak *et al.*, 2019; Giménez-Santamarina *et al.*, 2022). Carvone was rapidly eliminated from blood as the calculated half-life was 2.5 hrs. It is metabolized mainly in the liver into its metabolites like carvonic acid, dihydrocarvonic acid, uroterpenolone and carveol. Early physicians recommended spearmint tea to treat colds, headaches and stomach issues like indigestion, gas and vomiting. Physicians often treated infant colic with a bit of spearmint tea It is a common herb for the treatment of chest problems like cough, bronchitis, common cold and fever (Tayarani-Najaran *et al.*, 2013; Brahmi *et al.*, 2017; Ouakouak *et al.*, 2019; Sener *et al.*, 2019). By inhibiting the inflammatory response to lipopolysaccharides, such as the suppression of interleukin-1 and prostaglandin E2, it functions as an anti-inflammatory drug. Additionally it has antifungal properties. It works as an antispasmodic medication in the treatment of irritable bowel syndrome

**Corresponding author:* e-mail: usman.asif@uvas.edu.pk

because it is a competitive calcium channel antagonist in smooth muscle (Bulat *et al.*, 1999; Goncalves *et al.*, 2008, 2010). Its essential oil is also used as larvicidal against mosquito larvae. Rosmarinic acid, an antioxidant found in spearmint, is beneficial in preventing autoimmune rejection in human skin transplants and treating some autoimmune illnesses, such as autoimmune arthritis (Snoussi *et al.*, 2015; Kapp *et al.*, 2020). Rosmarinic acid is a well-known compound for "'HIV-1 reverse transcriptase inhibitor"' which is why it is used as an alternative therapy for acquired immunodeficiency syndrome (AIDS) (Ayoola *et al.*, 2008; Govindarajan *et al.*, 2012). Despite its beneficial potentials, some studies addressed its toxic influence reproductive system, renal and hepatic tissue. There is no mention of the precise spearmint dosage that has been shown to be detrimental to humans. The females who had 4 cups of spearmint tea each day experienced its negative consequences, including menstruation issues and a lowered libido. Sever histopathological changes has been reported in uterine, testicular, renal and liver tissues caused by long term use of spearmint (Akdogan *et al.*, 2004; Kumar *et al.*, 2008). There is paucity of literature describing the possible toxic effect of long term use of spearmint in pregnancy and its possible adverse effect on offspring's. This study was designed to evaluate the long-term effects of the dried leaf extract of spearmint on histological parameters of the ovaries and uterus of pregnant albino rats along with weight and crown-rump length (CRL) of their fetuses. Then renal and hepatic tissues of their fetuses were evaluated histologically to check the impact of spearmint leaves extract.

MATERIALS AND METHODS

This experimental investigation was carried out at the Shaikh Zayed Postgraduate Medical Institute's Department of Anatomy in Lahore, Pakistan. This experiment was conducted with the ethical approval of the Board of Research Ethic of Shaikh Zayed Medical Institute vide No TUF/Addl Reg/P-5/156.

Collection of animals

Adult female albino rats (n=30), weighing between 200-250 g were purchased from Veterinary Research Institute, Lahore. The animals are kept in the experimental room for acclimatization for two weeks before the start of the experiment.

Preparation of spearmint leaves extract

Fresh spearmint leaves were acquired from the market and taxonomic identification was done by a qualified taxonomist of Institute of Horticultural Sciences, University of Agriculture Faisalabad. An extract was made at the PCSIR Laboratories Complex in Lahore. After drying in the shade, the leaves were ground into powder. Refluxing distilled ethanol through it allowed for the extraction of the ethanol. The extract was vacuum dried using a speed vacuum device. 20% of the extract was produced as powder after being air dried. The doses were represented as dry-weight extract after the extract was dissolved in distilled water. Quantification of the active ingredient of spearmint tea was done by gas chromatography-Mass Spectrometry (GC-MS) from Chemistry Department, Forman Christian College, Lahore, Pakistan.

Experimental design

Food and water were available to the animals without restriction. Rats were given a commercial brand of chick feed No. 1. The experimental animals are alienated into three groups (n=10); Group A (Control): received distilled water 15ml/Kg body weight through gastric intubation. Group B (Low dose): received 20g/L (0.3g/Kg body weight) spearmint leaves extract for 30 days through gastric intubation. Group C (High dose): received 40 g/L (0.6g/Kg body weight) spearmint leaves extract for 30 days through gastric intubation. This treatment was given to the rats for one month. On the 31^{st} day of the experiment, an intact male was introduced for five days in each group to make the female rats pregnant. The pregnancy was confirmed through the formation of a vaginal plug. The spearmint leaves extract treatment was given to the rats up to the parturition of the neonates. On the day of parturition, the dam and the three neonates from each mother were humanely euthanized under gaseous anesthesia for the collection of samples. The weight of the live rats was also recorded on weekly basis. The crown-rump length (CRL) of neonates was also recorded from the vertex to the point where the tail started.

Collection of blood

Blood samples from the dam were collected at the end of the experiment for assay of serum progesterone for all three groups. At the end of the study, these rats were weighed properly.

Collection of histological samples

The rats were stretched out on the dissection tray in the supine position. The abdomen was opened through a midline incision running from the pubic region to the lower end of the sternum. Then ovaries and uterus were identified, removed and weighed followed by fixed in 10% Natural Buffered Formaldehyde solution for histological evaluations. Similarly, neonates' kidneys and liver were collected and preserved for any kind of pathological changes.

Histological analysis

To study the histological parameters, fixed tissues were cut into small pieces and tissue blocks were made through the paraffin embedding technique which includes dehydration, clearing, infiltration and paraffin embedding. The tissue blocks were sectioned at 5um thickness with a rotary microtome and stained with Hematoxylin & Eosin (H & E) (Savarna *et al*., 2019). For micrometry of these sections was done through the image analysis software ImagJ®.

Screening parameters

The ovaries of the dam were evaluated for histomorphometric parameters like the thickness of the capsule, diameter of corpus leutum and follicles. While the uteri sections were analyzed for endo- and myometrium thickness along with any kind of congestion and cellular damage. The histological sections of neonates' kidney and liver were also examined for any kind of pathological changes in the renal tubules and hepatic cells.

STATISTICAL ANALYSIS

Software SPSS 20.0 was used to enter and analyze data. The quantitative data for body weight, ovarian weight, follicle diameter, uterine layer thickness, serum progesterone level and CBC were reported as mean S.D., with comparisons between groups performed using ANOVA (one way). The Chi-square test was used to compare groups. The value of P is 0.05 regarded as significant.

RESULTS

Dam's parameters

Weekly weight of live rats

The pattern of weekly weight gain is represented in fig. 1. The maternal weight of rats in the control and the treated group was recorded on weekly basis during the experimental trial. The spearmint-treated groups showed decreasing trend as compared to the control group. However, a more decrease was observed in the high-dose treated group. The pregnancy was confirmed in the all-female rats of the control group but in Group B (GB) and Group C (GC), out of ten females, seven and five females got pregnancy.

Morphological parameters of ovary and progesterone pattern

At the end of trial ovarian weight (grams) was determined and recorded significantly ($P<0.05$) high in GB (0.36±0.09) and GC (0.49±0.05) in comparison to that of the control group (0.19±0.02). Albeit the increase in the ovarian weight, the serum progesterone level followed the reverse trend and found significantly low (35.87±4.97, 23.64±4.96) than that of control group (53.75±10.01) in the animals treated with low and high dose of MT, respectively (table 1) Carpus luteus followed the same trend as that of the ovarian weight.

Histological parameters

Different histological sections of the ovaries of each group were examined for histological features. The ovaries were observed for peripheral broad zone, the cortex and the inner area (medulla). The cortex contained different developmental stages of ovarian follicles, corpus hemorrhagic and atretic follicles. While the medulla contained coiled blood vessels (fig. 2). The control group indicated well-developed ovarian follicles, corpus luteum, capsule and cellularity. Although, histological parameters about the cellularity and corpus luteum were seen significantly affected by the MT treatment in the form of a decreasing pattern the capsular thickness remained unaffected by the MT treatment. The most evident histopathological changes in the ovaries of females of GB & GC are congestion of medullary blood vessels and vacuolation in luteal cells along with the infiltration of the macrophages. Vacuolation of interstitial cells and granulosa cell layers is activated.

The maternal uteri were also analyzed and different parameters like endometrium thickness, uterine glands and myometrium thickness were also measured. The uteri from the normal group animals represented the intact uterine epithelium with evenly distributed uterine glands in the endometrium. The myometrium layers are composed of two muscle layers namely the inner circular and outer longitudinal smooth muscle layer and stratum vasculum, blood vessels between these layers, with the normal lumen. The MT treatment led to a significant reduction in the thickness of endometrium and myometrium of the uteri collected from GB and GC. Mild dilated blood vessels in the stratum vasculum were found in the GB while this effect became more severe in high dose treated group (GC) in the form of congested blood vessels engorged with blood. Moreover, the low and high dose treatment of Mt also cause eruptions on the epithelium of the uteri which led to decreased epithelial thickness (table 3 & fig. 2).

Neonates' parameters

The typical course of pregnancy (21 days) was completed by all animals in the MT and control groups. In the MT and control groups, there were no abnormalities or pathologies in the sucking/rooting reflex, motor movements, colour, anal and urethral openings, or eye and ear openings. The crown-rump length was measured of 90 rats, 30 from each group.

Morphological parameters

The weight of the fetuses delivered from the dams of different groups was significantly affected by the treatment (table 2). The most significant ($P<0.05$) weight reduction (4.2±0.6g) was observed in the fetuses of Group C than that of the normal group's dam.

The effect of MT treatment was significant ($P<0.05$) on the fetuses delivered from the dams treated with high dose (GC) in the form of least CRL value (33.20±10.87mm) whereas the CRL estimated from the fetuses (40.76±9.39 mm) of the dams of GB (Low dose) of the low dose treated group (GB) remained unaffected by the treatment (table 2).

Histological parameters

The histological examination of the renal sections of different groups of fetuses revealed significant changes in comparison to the normal structure. The renal corpuscle was observed significantly ($P<0.05$) shrunk in the fetuses delivered from MT-treated groups as compared to the normal group. Besides, fetuses of GB and GC showed mild and severe tubular degeneration, respectively, in the cortical region with the infiltration of inflammatory cells. The MT administration during pregnancy showed perturbative effects on the thickness of cortical and medullary regions of the kidneys of fetuses which is more marked in the fetuses delivered from dams of group C (table 2 & fig. 2). Similarly, liver fetuses were also evaluated microscopically and detail is given in table 4. The liver section of fetuses of the normal group' dam had a normal cellular architecture like intact hepatocytes which were in the form of a regular radiating pattern around the central vein with no hemorrhages. In group B fetuses, although mild to moderate hepatocyte degeneration with congested central vein and hemorrhages were seen but hepatocyte pattern was severely affected and disrupted as compared to the normal hepatic tissue. High-dose treatment of MT during pregnancy caused a significant alteration in the hepatic tissue of fetuses. There were degenerated hepatocytes with hydropic or fatty alteration with an increased degree of necrosis. Moreover, the congested or dilated central vein engorged with blood along with severe petechial hemorrhages were present and they were associated with mononuclear cells of variable intensity. A distinct disruption of the hepatic cells and the sinusoids with conspicuous nucleus and swelling were also observed in the group C dam, fetuses.

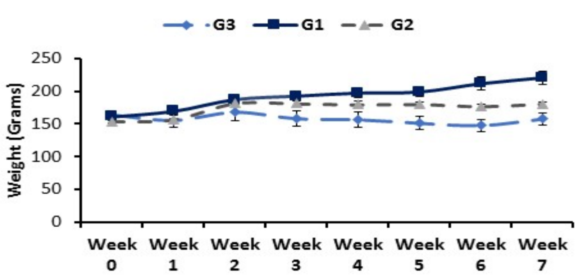

Fig. 1: Weekly representation of the live weight of rats during the experimental period.

DISCUSSION

There is a plenty of material available that describes how nutrition affects the foetus' intrauterine growth during the gestation period. It is claimed that herbal teas, foods and beverages taken during pregnancy have an impact on the postnatal morphometric development of the foetus. (ŞENER *et al.*, 2019) However, there are no studies in the literature that addressed adverse effect of gestational consumption of spearmint on postnatal morphometric growth. Therefore, this study was designed primarily to evaluate the long-term effect of MT leave extract on pregnancy and fetuses in dose dependent manner. Maternal weight during pregnancy depends upon many factors and has direct influence on the development of offspring. Weight gain during pregnancy has been demonstrated to have an effect on foetal growth, birth weight and gestational length. Low weight gain early in pregnancy is linked to preterm birth. (Sekiya *et al.*, 2007). Decreased fetus weight in spearmint treated group (B&C) are in consistence with the findings of (ŞENER *et al.*, 2019) There is no explained mechanism underline this phenomenon, however, this may be attributed to the degenerative histological changes in the uterine tissue under the spearmint treatment. In our study decrease weight gain during gestation observed in rats in the MT treated group may also be attributed to the toxic effects of spearmint. Spearmint caused reduction in body weight as its constituents suppress release of growth hormone from anterior pituitary gland that leads to malabsorption and indigestion. Another probable explanation is that it increases serotonin levels in the blood, which is responsible for activating the brain's satiety centres (Malihezaman *et al.*, 2012). Increased lipid per oxidation brought on by spearmint also results in a reduction in body weight (Zulfiqar *et al.*, 2022). However, other factors may be held accountable for this finding. Although the foetal CRL of the spearmint-treated group was significantly impaired, which may be connected to lower weight gain during pregnancy, all newborn pups in the MT and control groups had normal sucking/rooting reflexes, movements, colour, anal and urethral openings, eye and ear openings. There are contemporaries studies available to compare these results that address the adverse influence of the spearmint in gestation. M. spicata, herbal plant, recommended and used for many digestive and reproductive disorders like polycystic ovaries. Ovaries showed sever effect of MT treatment in the form of congestion of medullary vessels, infiltration of inflammatory cells and vacuolation and shrinkage of luteal cells. The spearmint treatment caused increase in the ovarian weight of the rats. The similar result was reported by (Thakur *et al.*, 2009) who observed the effects of carum carvi (member of lamiacae having same constituent like spearmint) on adult ovaries of rats. Similarly (Hassanpour *et al.*, 2017) reported that fennel also contains limonene like Spearmint that can increase ovarian weight of rat. In contrast, a research on dill, a plant related to spearmint in the lamiacae family, found no change in ovary weight. Ovarian congestion, which causes blood vessels to enlarge and blood to accumulate in ovarian vessels, is one potential cause of weight gain.

Vascular congestion was caused by an elevation in pyruvate transaminase in serum that was released from the liver, which in turn caused spearmint to have negative

Table 1: Mean ± SEM values of ovarian parameters and serum progesterone level of rats belonging to different groups

Groups	PARAMETERS				
	Ov. WT (g)	Cap. Th (µm)	Stroma (%)	CL (µm)	Prog
A	0.19±0.02^{C}	31.25±2.1^{A}	71.98±6.18^{A}	123.24±9.27^{A}	53.75±8.01^{A}
B	0.36±0.09^{B}	29.54±3.5^{A}	62.21±4.13^{B}	102.71±5.32^{B}	35.87±4.97^{B}
C	0.49±0.05^{A}	33.63±7.2^{A}	52.42±5.86^{C}	88.62±6.43^{C}	23.64±4.96^{C}

Table 2: Mean ± SEM values of morphometric parameters renal architecture of fetuses delivered from different groups female rats during experiment

Groups	Weight of fetus grams (g)	Crown-Rump Length (mm)	Cortex (µm)	Medulla (µm)	Diameter of renal corpuscles (µm)
A	7.3±0.7 ^{A}	44.76±12.43^{A}	146.62±13.26 ^{A}	165.63±11.26 ^{A}	72.34±6.18 ^{A}
B	4.9±0.8 ^{B}	40.76±9.39^{A}	100.56±10.45 ^{B}	157.69±8.25 ^{A}	61.71±3.61 ^{B}
C	4.2±0.6^{AB}	33.20±10.87^{B}	89.38±12.43 ^{C}	140.57±13.60 ^{B}	49.52±9.41 ^{C}

Means sharing different superscript in a column are statistically different at P<0.05

Table 3: Mean ± SEM values of histological parameters of the pregnant uterus of different groups of female rats at the end of the trial.

Parameters	Groups		
	A	B	C
Endometrium	122.4±21.3^{A}	102.6±14.2^{B}	99.2±22.3^{B}
Uterine Glands	38.9±3.12^{A}	23.3±2.24^{B}	17.3±4.2^{C}
Myometrium	613.43±31.2^{A}	526.27±38.1^{B}	448.65±19.7^{C}
Stratum Vasculosum	Normal lumen, not dilated with blood	Mildly engorged with blood	Congested and engorged with blood

Means sharing different superscript in a row are statistically different at P<0.05

Table 4: the number of fetuses delivered from different rats showing graded histopathological changes in the liver of different groups.

Parameter Score	Group A				Group B				Group C			
	-	+	++	+++	-	+	++	+++	-	+	++	+++
Hepatocyte Degeneration	9	0	0	0	0	5	3	1	0	2	4	3
Central Vein Congestion	9	0	0	0	0	7	2	0	0	1	5	3
Hemorrhages	9	0	0	0	0	4	2	3	0	3	1	5
Disrupted hepatocyte radiating pattern	9	0	0	0	0	1	2	6	0	0	2	7

-; No change, +: Mild , ++: Moderate, +++: Sever

effects on it. Through the dilatation of blood vessels, phytoestrogens that affect the metabolism of oestrogen can also increase ovarian weight (Malihezaman *et al.*, 2012). The corpus luteum was normal in control group. A but atrophied corpora lutea were formed in experimental groups B and C. This outcome is consistent with a study that employed Carum Carvi and Carum Longa to examine the hormonal and reproductive parameters of female rats and found a reduction in the size of the corpora lutea (Thakur *et al.*, 2009). Findings of (Malihezaman *et al.*, 2012), are contrary to the present results who observed effects of Dill on oocytes and fertility of adult female rats and found hypertrophied corpus luteum. There is no explained mechanism underlining this pattern of spearmint treatment especially in pregnancy because of the dearth of literature in this aspect. The possible reason for the atrophy of corpus luteum is the shrinkage of luteal cells. The medulla of the ovaries in the control group A showed no abnormality however the medulla of both experimental groups B and C showed dilated blood vessels. There might be possibility that these observations can be due to decreased progesterone level in Group A and B. The luteal cell shrinkage can be attributed to the disrupted superoxide dismutase enzyme which plays an vital role in defense mechanism along with elevated lipid per oxidation (Guney *et al.*, 2006).

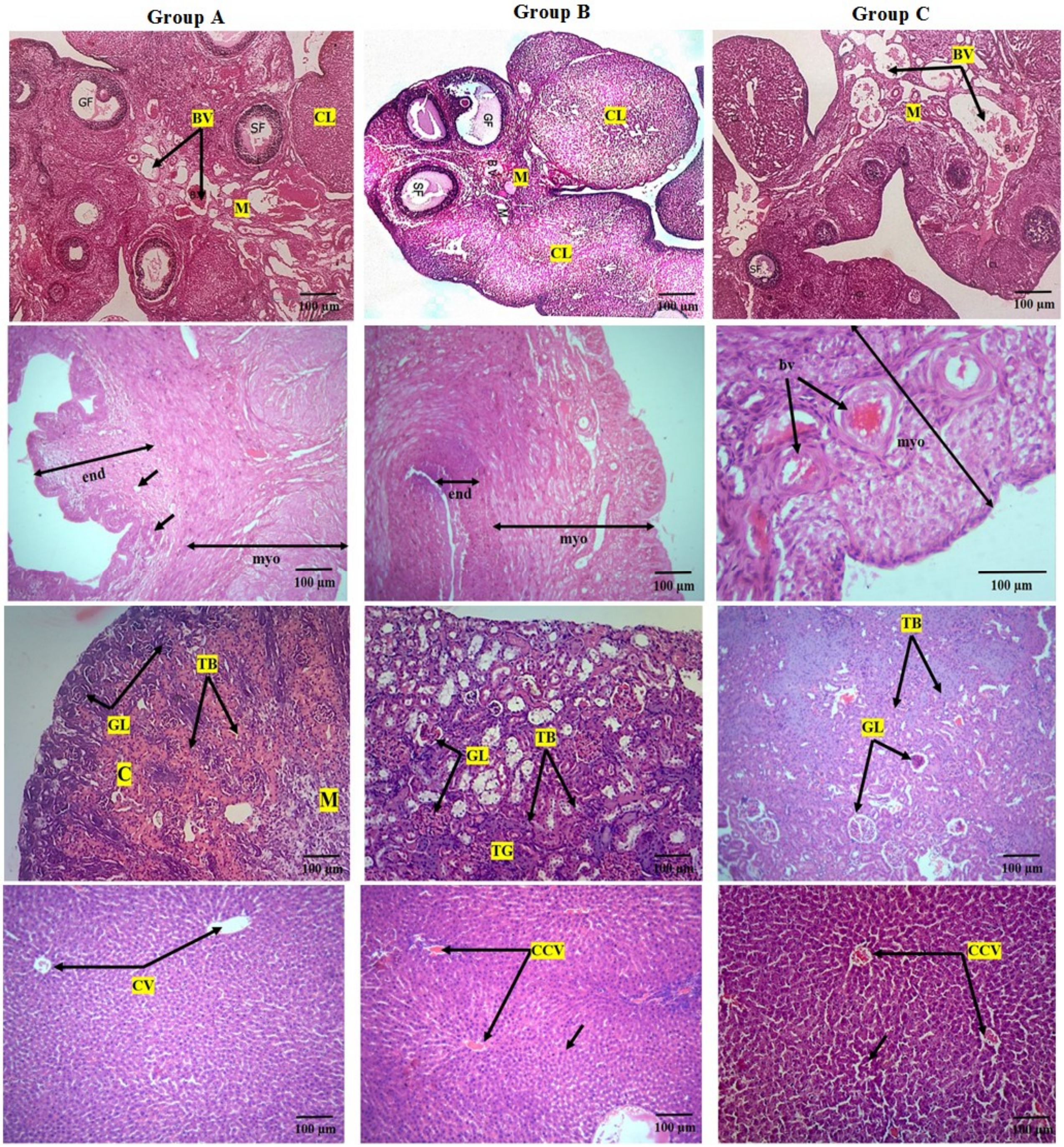

Fig. 2: Panoramic photomicrograph of Ovaries (First row), Uteri (Second row), fetal kidneys (Third row) and fetal liver (Fourth row) collected from groups A, B and C. Ovaries of group A indicated well-developed graffian follicle (GF), the secondary follicle (SF) and corpus luteum (CL) in the cortical region. The medullary region (M) contained the blood vessels (BV). MT treatment caused histological changes in ovaries group B & C in the form of congestion of BV, vacuolation and shrinkage of CL, GF and SF (H&E, 100X). Second Row: The uterine sections of Group A represented the intact uterine epithelium with evenly distributed uterine glands (small black arrow) in the endometrium (end) and myometrium (myo) layers composed of two muscle layers namely inner circular and outer longitudinal smooth muscle layer and stratum vasculosum (bv), blood vessels between these layers, with the normal lumen. The MT treatment reduction in the thickness of endo and myo of the uteri collected from Group B and C. Mild dilated blood vessels in the stratum vasculsoum were found in the group B while this effect became more severe in high dose treated group in the form of congested blood vessels engorged with blood.

To date, this is the first report that describes the dose dependent toxic effect on the cellular architecture of maternal uteri, fetal liver and kidneys. Many reports are available that explained that M. spicata elevate the lipid peroxidation which ultimately increase the free radicals (Akdogan *et al.*, 2004). Histological finding of uteri in current study is supported by this lipid peroxidation.

Same degenerative changes were reported by (Guney *et al.*, 2006) but in non-pregnant rats. The literature is silent about explaining the chronic effect of the M. spicata on the pregnant uteri. However, (Kumar *et al.*, 2008) and (Nozhat *et al.*, 2014) reported long term used MT can damage the male reproductive system through disrupting the hypothalamic-pituitary- of gonadal axis (HPG). May be in this experiment, the ovarian and uterine histology altered due to disturbance in the HPG system which is supported by the decreased progesterone level. Production of free radicals may add up the severity of MT treatment on ovarian and uterine histology. Similar findings were observed in a study conducted by (Güney *et al.*, 2006) in which effects of phenols (constituent of spearmint) were observed on the ovaries of common carp and reduction in the levels of serum progesterone was found. The literature is salient about addressing the long use of spearmint on the pregnant uteri. These perturbative effects of long-term use of spearmint may be the possible reason behind the deceased fetal weight during pregnancy and dams' metabolic disturbances.

There is a study that shows the effects of spearmint phenolic extracts on adult female rats and noticed increased the levels of serum progesterone, in contrast to the reduction in the levels of serum progesterone. Normally, cholesterol is turned into pregnenelone in the cells that make steroid hormones and subsequently, through the action of p450 -hydroxylase, it is changed into progesterone. Phenols can lower steroidogenesis by preventing the p450-hydroxylase enzyme from working.The ability of phenols to lower GnRh from the hypothalamus and impede follicle formation, which ultimately lowers serum progesterone, is the other explanation for the drop (Das *et al.*, 2013).

CONCLUSION

The research showed that the dried spearmint leaf extract has detrimental effects on the histology of the female reproductive system during pregnancy, which ultimately affects the fetus's liver and kidney tissue. The weight of the body as a whole and the paired ovaries were also impacted during the prolonged application of spearing. The rats' ovaries displayed dilated and congested blood vessels, as well as a reduction in the size of secondary and Graafian follicles. Serum progesterone levels were found to be lower as well. The use of spearmint should be limited, especially during pregnancy, as it lowers blood progesterone levels, even if these effects were seen at large doses (equivalent to 4 to 8 cups of spearmint tea daily).

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