

Evaluation of the effect of flavonoids isolated from *Spinacia oleracea* leaves on pituitary-adrenal ovarian axis in mice treated with doxorubicin

Huda F. Hasan

Physiology and Pharmacology, College of Veterinary Medicine, University of Baghdad, Iraq.

Correspondence: Huda F. Hasan, Physiology and Pharmacology, College of Veterinary Medicine, University of Baghdad, Iraq. E-mail: Dr.hudaalqaraghuli@yahoo.com.

ABSTRACT

This research was done to evaluate the effect of flavonoids isolated from *Spinacia oleracea* leaves on the pituitary-adrenal ovarian axis in mice treated with doxorubicin. Forty female mice were distributed into four groups; the first group (G1) was given a daily orally dose of *Spinacia oleracea* leaves extract (100 mg/kg), the second group (G2) was given a daily oral dose of flavonoids (100 mg/kg), the third group (G3) was given distilled water, and the fourth group (G4) was left without any treatment. At the beginning of the first week, G1, G2, and G3 were intra-peritoneally injected with doxorubicin (0.15 mg/kg) twice weekly. G1 and G2 showed a significant reduction ($P < 0.05$) in ACTH, Cortisol, FSH, and LH levels with a significant increase ($P < 0.05$) in the levels of glutathione peroxidases, superoxide dismutase, and an enhancement in the fertility index compared to G3. Histopathological section of the ovary in G3 showed degeneration of epithelial cells, ovary section in G2 showed a presence of multiple mature follicles contained oocyte. Ovary in G1 showed the presence of mature follicle. The histopathological section in the adrenal cortex of G3 showed the congestion of blood vessels. The adrenal cortex of G2 showed moderate vacuolization and mononuclear cells infiltration. The adrenal cortex of G1 showed a mild vacuolization. From this study, we concluded that the flavonoids isolated from *Spinacia oleracea* leaves have the ability to attenuate the side effects of chemotherapy drugs by enhancing antioxidant and improving fertility.

Keywords: *Spinacia oleracea*, flavonoids, doxorubicin, adrenal ovarian axis.

Introduction

Some drugs such as chemotherapy drugs can adversely influence fertility and may lead to ovarian insufficiency at therapeutic doses [1]. Anthracycline (Doxorubicin) is commonly used to treat various types of cancer including breast cancer and leukemia and it has been demonstrated to generate toxicity in the reproductive system. In addition, the use of traditional medical herbs has increased among the general public to remove reproductive system dysfunction [2]. *Spinacia oleracea* is a vital plant rich in numerous vigorous antioxidant constituents such as

flavonoids [1]. *Spinacia oleracea* is also considered a source of iron, apocynin folate, fatty acid (Omega 3), as well as vitamins A and C, which are antioxidants with the ability to prevent and scavenge free radicals lead to boost fertility [3]. Flavonoids are complexes that act as antioxidants via the oxidative stress scavenging possessions of their hydroxyl group [4]. Moreover, they have anti-thrombotic, anti-allergic, antiviral, anticarcinogenic and anti-inflammatory effects. *Spinacia oleracea* flavonoids have a good percentage of quercetin, which plays an important role in promoting female fertility by inducing ovaries folliculogenesis [5, 6]. Furthermore, flavinoids of *Spinacia oleracea* include Kaempferol and 7-tri-hydroxyflavone that are able to regulate female hormones and fertility [7]. Thus, the aim of this research was to evaluate the effect of flavonoids isolated from *Spinacia oleracea* leaves on the pituitary-adrenal ovarian axis in mice treated with chemotherapy drugs.

Materials and Methods

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Extraction and isolation of flavonoids from *Spinacia oleracea* leaves:

Spinacia Oleracea was extracted according to the method recorded by Shaheen *et al.*,^[8] with mild modification. The leaves were washed and dried at room temperature, after which the leaves were ground then sifted. Then approximately every 300g of the powdered plant was soaked in about 1500 ml of 90% methanol and then placed on a magnetic stirrer at 45 °C for 72 hours. Subsequently, the total mixture underwent a coarse filtration using a clean white cotton material. After that, the extract was filtered by using Whatman filter paper. Alcohol was removed from extract via rotary evaporator at 50 °C for 4 hours. The concentrated extract was poured into a petri dish and put in the incubator at 45 °C until complete drying. The dried extract was stored at -20 °C until use. The extraction process was repeated several times to obtain sufficient crude extract for testing. Prior to concentrating, the crude extract was extracted using diethyl ether, ethyl acetate, and petroleum ether (PE) to isolate flavonoids using the methods documented by Yadav and Kumar^[9]. The PE method was excluded because it became rich in fatty materials. Ether was used to extract free flavonoids. As well as, ethyl acetate was supplemented and hydrolyzed additionally within H₂SO₄ (7%) for 24 hours and then the extraction was repeated using ethyl acetate. The obtained flavonoids were repetitively rinsed with distilled water and dried.

Doses and administration of *Spinacia oleracea* leaves and flavinoids:

The crude extract of *Spinacia Oleracea* was orally administered to mice at a dose of 100 mg/kg B.W, which was prepared by dissolving one gram of crude extract in distilled water until it reached the volume of 100 ml according to Luka *et al.*^[10]. Flavonoids were administered to mice at a dose of 100 mg/kg and the concentration was 10 mg/ml according to Zhenna *et al.*^[11].

Experimental design:

Forty female mice were equally distributed into four groups. The period of treatment was four weeks (February 2018). The 1st group (G1) was treated with *Spinacia oleracea* leaves extract at a daily dose of 100 mg/kg administered orally, the 2nd group (G2) was orally given a daily dose of flavonoids 100mg/kg, the 3rd group (G3) was daily treated with distilled water (positive control group), while the 4th group (G4) received no treatment (negative control group). At the first week of treatment, G1,

G2, and G3 were treated with 0.15 mg/kg of doxorubicin given (I/P) two times per week.

Adrenocorticotrophic hormone (ACTH), Cortisol, follicles stimulating hormone (FSH) and luteal hormone (LH):

Hormones magnitudes were prepared according to steps founded in commercial Radioimmunoassay (RIA) kits. The analysis was done in the clinical laboratory of the radioactive isotope.

Antioxidant SOD and GTP:

Superoxide dismutase and Glutathione peroxidase levels were performed following the niceties founded in Bio-diagnostic kit instructions^[12], whereas GTP was determined spectrophotometrically following the technique of Paglia and Valentine^[13].

Fertility index:

This parameter was calculated according to Oberlander *et al.*^[14]. Fertility index: number of pregnant mice/the total number of mated female mice × 100.

Histopathological changes (ovaries and adrenal glands):

At the end of the treatment period, mice were sacrificed. The adrenal glands and ovaries were impassive the fat and connective tissues were separated and then they were kept in 10% formalin until histologically evaluation according to Luna^[15].

Statistical analysis:

Data were subjected to analysis using SAS software^[16]. One-way ANOVA and least significant difference (LSD) post-hoc test were used to assess the significant differences between the means obtained. Also, the Chi-square test was used to assess differences between fertility index percentages. p < 0.05 was considered significant.

Results

The mean levels of the ACTH (7.90 pg/ml), Cortisol (16.75 µg/dl), FSH (1.27 U/L), and LH (1.94 U/L) in the were significantly lower (P < 0.05) than the corresponding means (115.71 pg/ml, 20.57 µg/dl, 2.09 U/L, and 2.82 U/L, respectively) of the G3 as shown in Table 1. Similar results were obtained for G2 and G4 as all means of the previous parameters were significantly lower (p < 0.05) in G2 and G4 compared to that of G3.

Table 1: Effect of *Spinacia oleracea* leaves extract and flavonoids of *Spinacia oleracea* on some parameters of female mice treated with doxorubicin.

Para Groups	ACTH (pg/ml)	Cortisol (µg/dl)	FSH (U/L)	LH (U/L)	Glutathione peroxidases (mmol/mg)	Superoxide dismutase (u/ml)
G1	70.90±2.99B	16.75±0.28 B	1.27±0.10B	1.94±0.13 B	8.07±0.51 A	10.92±0.73A
G2	69.03±3.81B	14.06±0.21 B	1.25±0.20B	1.99±0.17B	9.03±0.69 A	11.99±0.90A
G3	115.71±4.92 A	20.57±0.30 A	2.09±0.19A	2.82±0.14 A	6.01±0.79 B	8.09±0.85B
G4	68.22±3.18B	15.41±0.29B	1.23±0.17B	1.95±0.16 B	8.04±0.87 A	11.16±0.98A

* Means with a different letter in the same column significantly different (p < 0.05).

The levels of glutathione peroxidases in G1 (8.07 mmol/mg), G2 (9.03 mmol/mg), and G4 (8.04 mmol/mg) were significantly ($p < 0.05$) higher than in the G3 (6.01mmol/mg) (Table 1). A similar trend was shown concerning the superoxide dismutase as the means were 10.92, 11.99, and 11.16 u/ml in G1, G2, and G4, respectively while the mean in G3 was 8.09 u/ml. As shown in Table 2, the differences between the fertility indexes (FI) were significant ($P < 0.0001$). The highest FI was detected in G4 (100%), followed by 80% for G1 and G2. The lowest FI was shown in G3 (10%).

Table 2: The effect of *Spinacia oleracea* leaves extract and flavonoids of *Spinacia oleracea* on Fertility Index (%) of female mice treated with doxorubicin.

Groups	Parameter	Fertility index%
G1		80%
G2		80%
G3		10%
G4		100%
	Chi-square value	21.31
	p	<0.0001

The histopathological variations in the ovary of G3 revealed the occurrence of atretic follicles, which had a thin wall of atrophied and degenerated lining epithelial cells (granulosa cells) as shown in (Figure 1), while the ovarian segment in G2 showed the presence of several mature follicles that contained oocyte in addition to the presence of corpus luteum and proliferation progression in their lining epithelial cells with regeneration of atretic follicles (Figure 2). Concerning the histopathological section of the ovary in G1, the results showed the presence of mature follicle and fewer deteriorated follicles with a delicate wall (Figure 3). The histopathological section of the adrenal cortex of animals treated with distilled water and doxorubicin showed necrosis, pyknotic nuclei, and congestion of blood vessels in the cortical adrenal gland (Figure 4). Whereas, the histopathological section in the adrenal cortex of animals treated with flavonoids and doxorubicin showed a moderate vacuolization of adrenal cortex and mononuclear cells infiltration (Figure 5), while histopathological section of the adrenal cortex of animals treated with *Spinacia oleracea* leaves extract and doxorubicin showed the mild vacuolization of adrenal cortex (Figure 6) as compared with adrenal gland of mouse from negative control group (Figure 7).

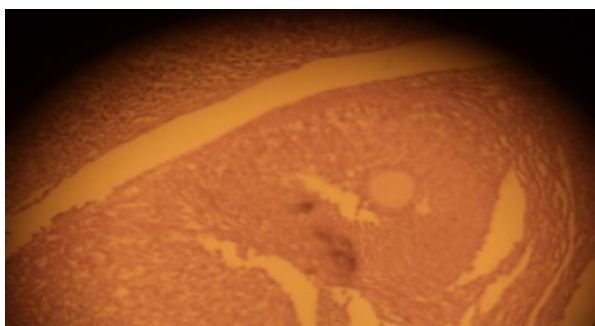


Figure 1: Histopathological section of the ovary of a mouse treated with distilled water and doxorubicin (H&EX40).

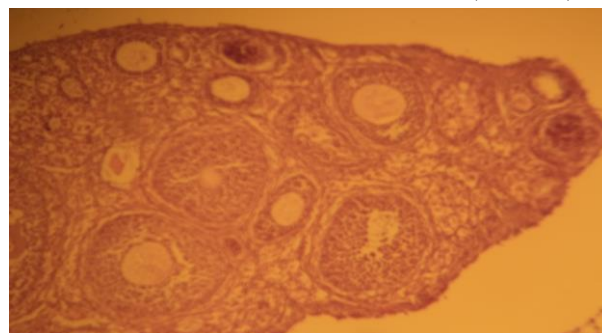


Figure 2: Histopathological section in the ovary of a mouse treated with flavonoids and doxorubicin (H&EX40).

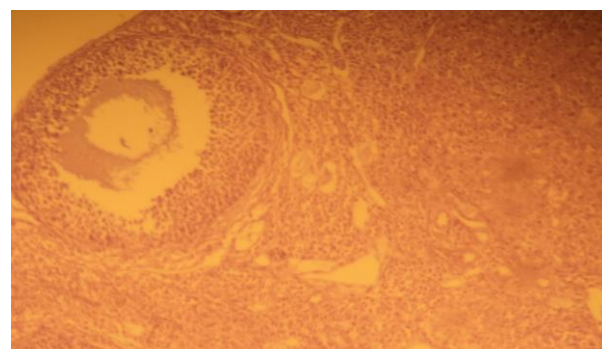


Figure 3: Histopathological section in the ovary of a mouse treated with *Spinacia oleracea* leaves extract and doxorubicin (H & E stain, 40X).

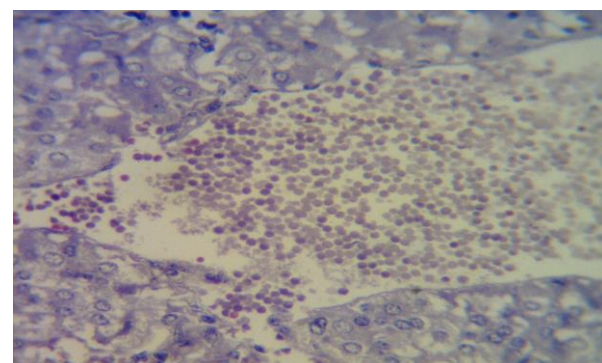


Figure 4: Histopathological section in the adrenal cortex of animal treated with distilled water and doxorubicin showed necrosis, pyknotic nuclei, and congestion of blood vessels in the cortical adrenal gland (H&Estain 40X).

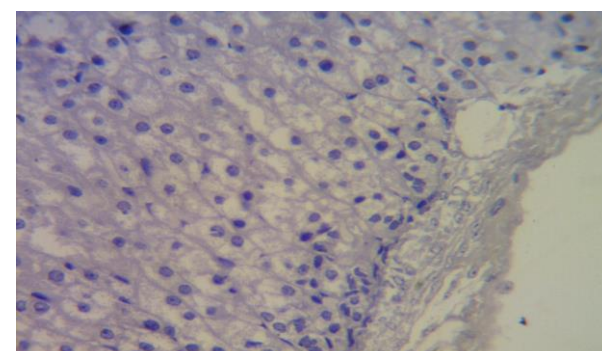


Figure 5: Histopathological section in the adrenal cortex of the animal treated with flavonoids and doxorubicin showed moderate vacuolization of the adrenal cortex and mononuclear cells infiltration (H&Estain 40X).

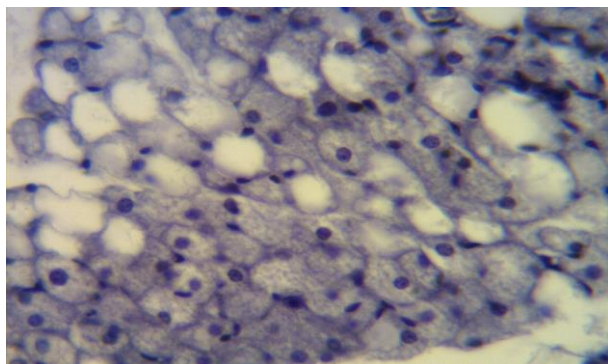


Figure 6: Histopathological section of the adrenal cortex of the animal treated with *Spinacia oleracea* leaves extract and doxorubicin showed mild vacuolization of the adrenal cortex (H&Estain 40X).

Discussion

Increased levels of ACTH, cortisol, FSH, and LH levels and also decreasing levels of glutathione peroxidases, superoxide dismutase, fertility index, and increased atretic follicles with degenerated lining epithelial cells (granulosa cells) in G3 may be due to doxorubicin, leading to creation of hydrogen peroxide and superoxide resulted in free radicals creation and exhaustion of tissue antioxidants by its ability to inhibit topoisomerase II and cooperation with DNA topo-isomerase, produced DNA strand disruptions and lead to damage of the oocyte straightly and ovarian toxicity in addition to change various types of cells at this organ. Furthermore, cortisol is commonly mentioned as the “stress hormone” and exists in the organs exposed to stress. Moreover, the increase in ACTH and cortisol levels are slightly correlated to the reduction in total antioxidant concentrations and little total antioxidant capacities [17]. Furthermore, doxorubicin, an anthracycline agent, induces apoptosis in the oocyte. Anthracyclines are able to induce ovarian injury, endovascular damage, germ cell damage, ovarian toxicity, and desensitization to LH and FSH as well as increasing their levels in the circulation. These consequences are in agreement with the results mentioned by Codacci-Pisanelli *et al.* [18].

Inhibitory effects of flavonoid on the production of ACTH, cortisol, FSH, and LH and the enhancement of glutathione peroxidases, superoxide dismutase, fertility index and the presence of several mature follicles that contain oocyte as compared to G3 may result from as antioxidants via the oxidative stress scavenging possessions of its hydroxyl group. The high conjugation through the flavonoids molecules and various groups of hydroxyl was enhanced their antioxidant assets such as hydrogen, reducing mediators or electrons providing agents [19]. Antidepressant effects of flavonoids decrease ACTH and cortisol levels also increase the antioxidant concentration. Moreover, flavonoids of *Spinacia oleracea* have a

good percentage of quercetin, which plays an important role in enhancing female fertility by increasing folliculogenesis in the ovaries [6]. Furthermore, flavinoids of *Spinacia oleracea* include Kaempferol and 7-tri-hydroxyflavone, which has the ability to regulate female hormones and fertility [7].

Decreased levels of ACTH, cortisol, FSH, and LH along with increased levels of glutathione peroxidases, superoxide dismutase, fertility index, and the presence of several mature follicles compared to G3 could be attributed to *Spinacia oleracea*, which has numerous active antioxidant constituents such as uridine, flavonoids, and coumaric acids that can act synergistically [4]. Moreover, vitamins C and E could positively act as complements for attenuating the adverse effects of chemotherapy. *Spinacia oleracea* has excessive nutritious substances such as saponins, terpenes, steroids, folic acid, iron, vitamins A, resins, and folate. The extract of this plant is considered as a source of zinc, which has an important role during differentiation and division of cells and is vital for growth of tissues [20].

Conclusion

The results of the present study confirmed that the flavonoids isolated from *Spinacia oleracea* leaves have the ability to attenuate the side effect of chemotherapy drugs by enhancing antioxidant and improving fertility.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Conflict of Interests

The author declares no conflict of interests.

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