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The Protective Effect of (*Andrographis Paniculata*) on 4-Vinylcyclohexene Diepoxide Induced ovarian Toxicity in Female Albino Rats

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Abstract

Protective effect of (Andrographis Paniculata) on 4-Vinylcyclohexene Diepoxide.A group of 55 female albino rats undergo experiment on the outcome effect of 4-vinylcyclohexene Diepoxide injection for a priod of two weeks, as well as treatment effect of methanolic leaf extract of *Andrographis paniculata* oral administration for four weeks. Injection concentrations (160 and 320mg/kg. B.W) also two different doses of treatment (100 and 200mg/kg. B.W) have been applied.The results on body weight and ovary weight were more evident when higher concentration dose was applied. Whereas, both induced dose were effective and no significant difference was recorded. Histological examination of ovary induced clearly the effect in various ovary tissues, and accumulation of connective tissue collagen fibers around corpus luteum where as a higher dose of treatment found to be behind sign of re-normalizing the within four weeks of treatment. More detailed study proposed to findout the exact right dose for treatment and may the further study extend to include other medicinal plant that might be more efficient.

Keywords: 4-Vinylcyclohexene Diepoxide(VCD), *Andrographis Paniculata* Leaf, Ovarian Toxicity, Rats.

1. Introduction

The use of medicinal plants for treatment of both chronic and acute diseses ,infact , goes back to quite long ago, as its found to be fairly cheap, effective and has no to very little side effect [1,2], *Andrographis paniculata* is an example of medicinal herbaceous plants. Extracts of various parts of this plant found to contain enough amount of andrographolide the compound that is found to have major bioactive role as pharmaceutical agents. This compound is used for various sorts of treatment such as antibacterial, anti-viral, anti-tumor, anti-cancer, etc [3, 4]. However, it also

known that such extracts has a role in inhibiting pro-inflammatory responses as well as an acute brain damage, cardiac toxicity infertility and ovarian failure in rats and mouse [4, 5, 6]. Coon and Ernst, 2004 [7] showed that almost 55% of the entire andrographolide administrated to rats and mouse will be dispersed to different tissues and organs, its effect on central nervous system as neuroprotective matter have been refered to [8] as well. The significance role of *A. paniculata* extract against tumor since it prevent angiogenesis and cell proliferation have shown [9] beside that their application for treating several different types of cancer. In fact, using a dose of 200mg/kg of methanolic extract of *A.paniculata* as a protective agent have been demonstrated against arsenic-induced toxicity [8,10].

Infections and environmental factors such as 4-vinylcyclohexene Diepoxide (VCD) is known to damage basic constituents of ovary, that will lead to premature ovarian failure (POF) which is the stoppage of ovarian function. All these will end up with infertility [11, 12, 13]. Furthermore, POF may result in to oxidative stress, histological damages, primary ovarian insufficiency (POI) and in many other complications [14, 15, 16] gave an evidence in each physiological control in female rats.

The aim of the current investigation is to administrate the effect of application of VCD on female rats as well as the outcome of the treatment with methanolic leaf extract of *A.paniculata* on body weight and ovary weight as well as forwarding histological evidence on such organs for a period of four week treatment.

2. Materials and methods.

2.1.Plant Extraction.

20 grams of *A. paniculata* were soaked in absolute methanol for 24 hours, after that the solution filtered and centrifuged. Concentrated extract was stored at 4 C° [17]. For oral toxicity test alcoholic leaf extract of the plant was prepared by dissolving in 2% dimethyl sulfoxide (DMSO) and normal saline to exactly (100 and 200 mg/kg .B.W) this was performed according to [18, 19].

2.2.Animal Husbandry and Breeding.

Healthy albino rats, *Rattus norvegicus* were inbred and kept in plastic cages in Biology Department, Faculty of Science, Soran University-Soran. A total of 55 female albino rat weighing around (200-250) grams were housed under standard conditions of 12hrs light and dark cycle at 25 C°. They had a free access to standard diet ad libitum and clean tap water. All experimental protocols were approved by the Ethical Review Committee ERC-22-110, Soran University, Soran-Iraq.

2.3.Acute Toxicity, Lethal Dose LD_{50} Determination

20 female wistar rats divided into 4 groups (n=5, each group) were used for an acute oral toxicity test. Alcoholic leaf extract of *Andrographis paniculata* was prepared according to [20, 21]. Each extract solution was administered to five female rats at 48 hrs interval, in a single dose through oro-gastric tubes, whereas control group received normal saline only.

The present experiment had conducted in accordance to the procedure of Organization for Economic and Cultural Development (OECD, 2001). Survivors and mortality ratio were observed and calculated after 48 hrs as LD_{50}, any toxicological sign and symptoms of each group was followed and recorded.

2.4.Experimental Design of the Study

An average of 55 female albino rats were used only 35 rats for experimental srudy, all were treated in order to follow up the role of methanolic leaf extract of *A. paniculata* against 4-vinylcyclohexene Diepoxide. The extract was dissolved in 2% DMSO and N.S to 100 mg and 200 mg/kg .B.W concentration [22].The chemical agent VCD is used for toxicity induction as Primary Ovarian Insufficiency (POI) model, was performed with two different doses (160 and 320) mg/kg B.W. The duration of plant extract administration had carried out for 4 weeks, in each group 5 rats used (n=5). Table (1).

Table 1. Experimental Design

Groups	Treatment
Group I (control)	Standard diet *ad libitum* + clean tap water
*Group II (VCD/POI model)	160mg/kg VCD injected intraperitoneally for 2 weeks
Group III (VCD+ *A.paniculata*)	100mg/kg plant leaf extract to VCD induced POI rats
Group IV (VCD+*A.paniculata*)	200mg/kg plant leaf extract to VCD induced POI rats
*Group V (VCD/POI model)	320mg/kg VCD injected intraperitonally for 2 weeks
Group VI (VCD+*A.paniculata*)	100mg/kg plant leaf extract to VCD induced POI rats
Group VII (VCD+*A.paniculata)*	200mg/kg plant leaf extract to VCD induced POI rats

(n=5 rat) For each group

2.5. Body and Ovary Weight Determination

Body weight of the rat in each group as well as fresh ovary were determined and measured. All weights were expressed as mean as well as weight have been recorded for each group using an accurate balance (Electronic Percision Balance, Aohuasi- China). All measurements were performed after treatment with extract also before and after intoxication including (160mg/kg .B.W and 320mg/kg .B.W) groups. Body weight of each rat was measured and the mean of sll five rats in each set were expressed as mean value for the group.

2.6. Histological Tissue Processing

Histological examination for rat ovary were carried out in order to follow up any alterations that were induced by VCD and the adverse effect of methanolic leaf extract of *A.paniculata* as treatment. Routine tissue processing including control , VCD induced toxicity and treated groups were performed according to [23, 24]. Subsequently stined with hematoxylin and eosin (H and E). Finally, they were mounted with D.P.X.

2.7.Statistical Analysis

All data were expressed as standard error of means (±SEM) data were analyzed by GraphPad Prism9 (GraphPad Software ,USA). In addition to ±SEM comparison between mean groups performed using One-Way analysis of variance (One-way ANOVA) and a post hoc (Tukey's test). P-value ($P<0.05$) considered statistically significant in present study.

3. Results

Results of the present experiment on the weight of rat body and ovary, were expressed as mean value before and after injection of experimental rats with two doses (160 and 320mg/kg. B.W) of 4-vinylcyclohexene Diepoxide VCD for two weeks, also after treatment for a period of four weeks with two doses of *Andrographis paniculata* (100 and 200mg/kg .B.W).

A-Rat body weight:

The results were forwarded in the current study as the standard error mean ±SEM values of estimation of five animlas in each group (sets of experiment). figure (1) A, B illustrate the outcomes of the experiment, it was founded that rat weight is reduced from 241g to 234g after VCD injection of low concentration, whereas the weight to 224g when it was injected with 160mg/kg .B.W VCD figure (1). However, still statistical variation in body weight was not significant. Results of body weight of the female rat after treatment for four weeks with both doses of extract (100 and 200mg/kg .B.W) showed that the weight after high dose treatment returned back to around normal in case of 160mg/kg .B.W. injection. Whereas the results were more evident incase of the high dose of injection (320mg/kg .B.W) figure (1B), demonstrate the results of

treatment with both high and low doses of plant extract, gave nearly the same results but the variation was not linear. Statistically in spite of apperant of, variation in animals body weight pre and post injection as well as before and after treatment with couple of doses applied throughout the present experiment still the variation was not linear and clear significant results were not observed.

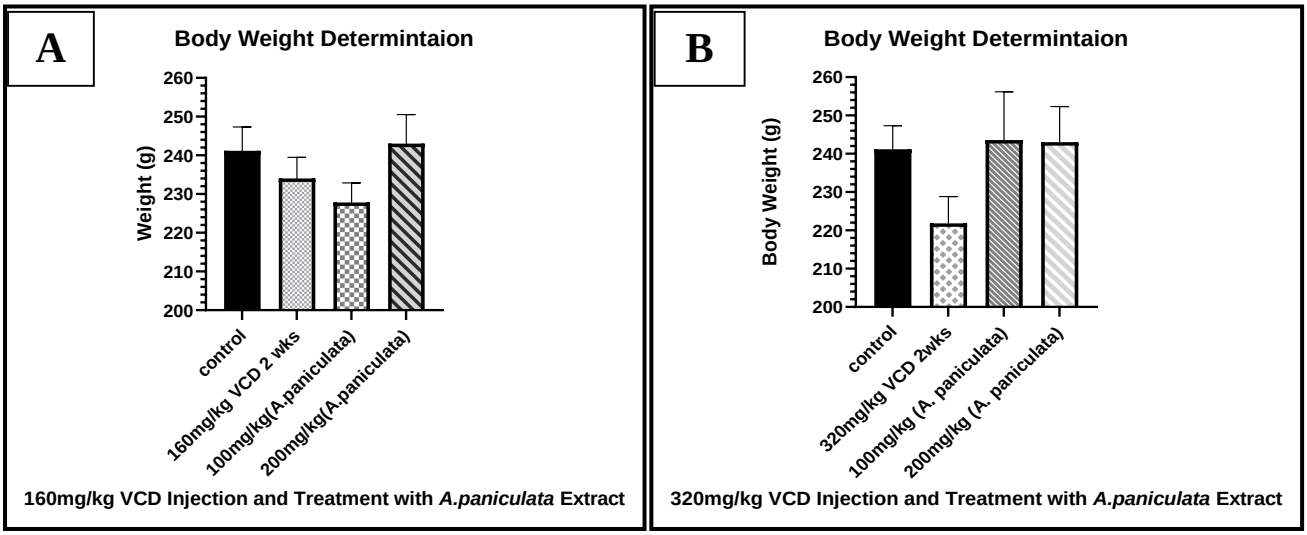

Figure 1. Rat body weight (g) determination, VCD injected in different doses and treatment with A. paniculata extract. A: rats injected with VCD 160mg/kg .B.W. for 2 weeks. B:rats injected with VCD 320mg/kg B.W. for 2 weeks. All groups contain (control, determined dose of VCD, 100mg/kg B.W. *A. paniculata*, 200mg/kg B.W. *A.paniculata*).

B- Ovary Weight Dtermination

Mean weight of fresh ovary of female wistar rats was estimated throughout present project. Ovary weight have been recorded for rats before and after injection with 160mg/kg .B.W for a group and 320mg/kg .B.W for second group for two weeks then after treating rats with (100 and 200mg/kg .B.W, *A.paniculata* leaf extract) of ovary weight measurement took place after four weeks of treatment. The results of figure (2 A+B) indicate presence of slight difference in ovary weigt throughout various steps of the test, when model group injected with 160mg/kg.B.W. However, treatment with high dose (200mg/kg .B.W) of *A. paniculata* extracts showed significant ovary weight elevation at ($P<0.05$) to (53.08mg) as compared to model group ovary weight (44.10mg), figure (3A).

Model group showed non-significant decrease in ovary weight as compared to control group when VCD injected at 320mg/kg B.W. for two weeks. In contrast, the ovary weight significantly increased in both groups of rats treated with (100 and 200mg/kg .B.W) plant extract to 49.94 and 49.92mg respectively, at ($P<0.05$) as compared to model group which was 40.06mg only.

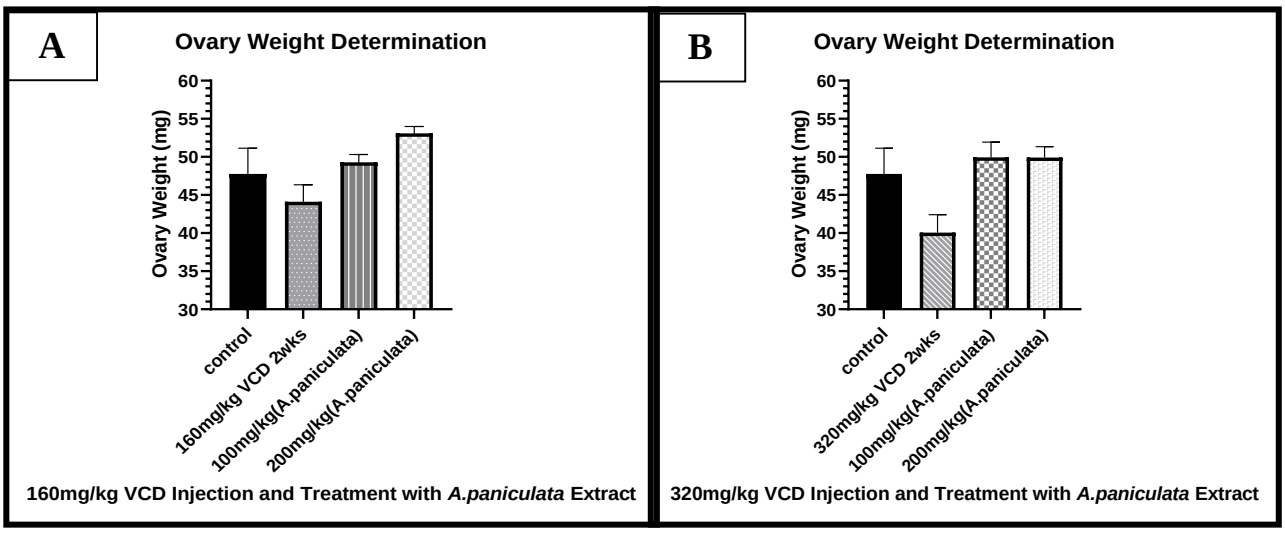

Figure 2. Rat ovary weight (mg) determination, VCD injected in different doses and treatment with A. paniculata extract. A: rats injected with VCD 160mg/kg .B.W. for 2 weeks. B:rats injected with VCD 320mg/kg B.W. for 2 weeks. All groups contain (control, determined dose of VCD, 100mg/kg B.W. *A. paniculata*, 200mg/kg B.W. *A. paniculata.*

C- Histopathological Analysis

Results of histological investigation of rat ovary before and after accused oral toxicity test as well as pre and post treatment with methanolic leaf extract of *A.paniculata* throughout present study are illustrated in figures (3+4**)**. Normal healthy ovarian tissue of the control animal is illustrated in figure (3A), which shows different stages of developing follicles, primordial, primary and atretic follicles. It also illustrates an intact zona pellucida status surrounding the oocyte.aroud layer, theca interna as well as theca externa layer is clear.

The states of ovary with 160mg/kg VCD are illustrated in figure (3B), that shows damages and accumulation of connective tissue collagen fibers which is fibrosis around corpus luteum. An increase of the number of atretic follicles with pre-antral follicles were observed . figure (3C+D) illustrates the tissues of rat ovary after treatment with 100 and 200mg/kg leaf extract of *A.paniculata*, the degeneration of zona pellucida around oocyte and different developing stage of follicles. In figure (3D) the status of the ovary tissue after treatment with high dose (200mg/kg) reflect an increase of number of different stages of growing follicles as primary and secondary is quite evident.

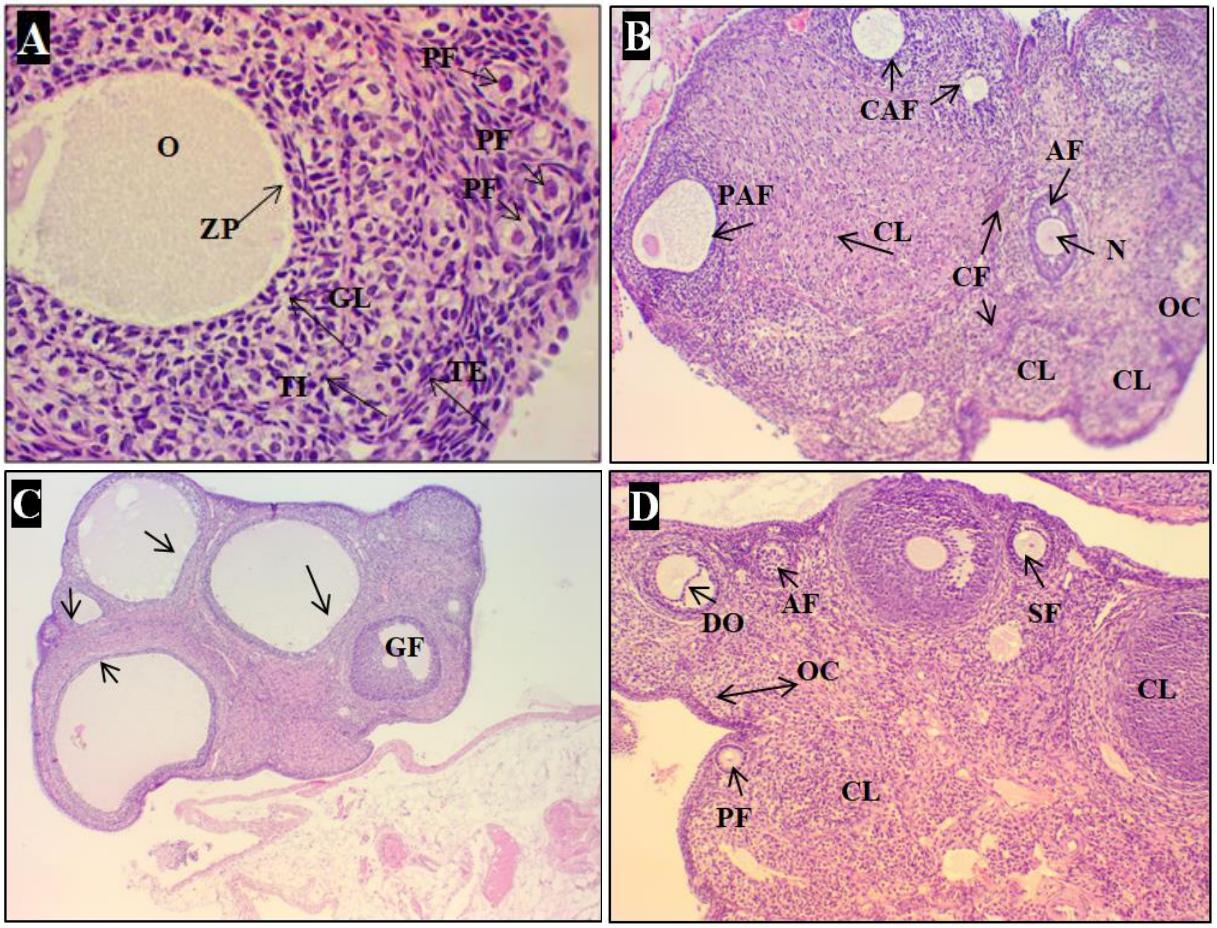

Figure 3. Photomicrograph Sections of ovary control (Healthy), VCD (160mg/kg) induced toxicity and treated with 100 and 200mg/kg (A.paniculata leaf extract) stained with (Hematoxylin-Eosin). A. Shows the control normal ovary with oocyte(O), zona pellucida(ZP), primordial follicles(PF), granulosa layer(GL), theca interna(TI), theca externa(TE) and atretic follicle(AF), 400x. B. Shows fibrosis around corpus luteum(CL) due to VCD injected with 160mg/kg, atretic follicle (AF), cortex of the ovary(OC), cystic atretic follicles, nucleus (N), pre-antral follicles (PAF) and collagen fibers(CF) mainly around the corpus luteum. C. Is an ovary treated with 100mg/kg A.paniculata and the arrows show degenerated zona pellucidum and decreased numbers of growing follicles, graafian follicles (GF). D. Rat ovary treated with 200mg/kg A.paniculata extract illustrating cystic follicle with degenerated zona pellucidum , corpus luteum (CL), degenerated oocyte, primary follicle (PF), secondary follicle(SF), atretic follicle (AF) and the two sided arrow show cortex of the ovary. 250x.

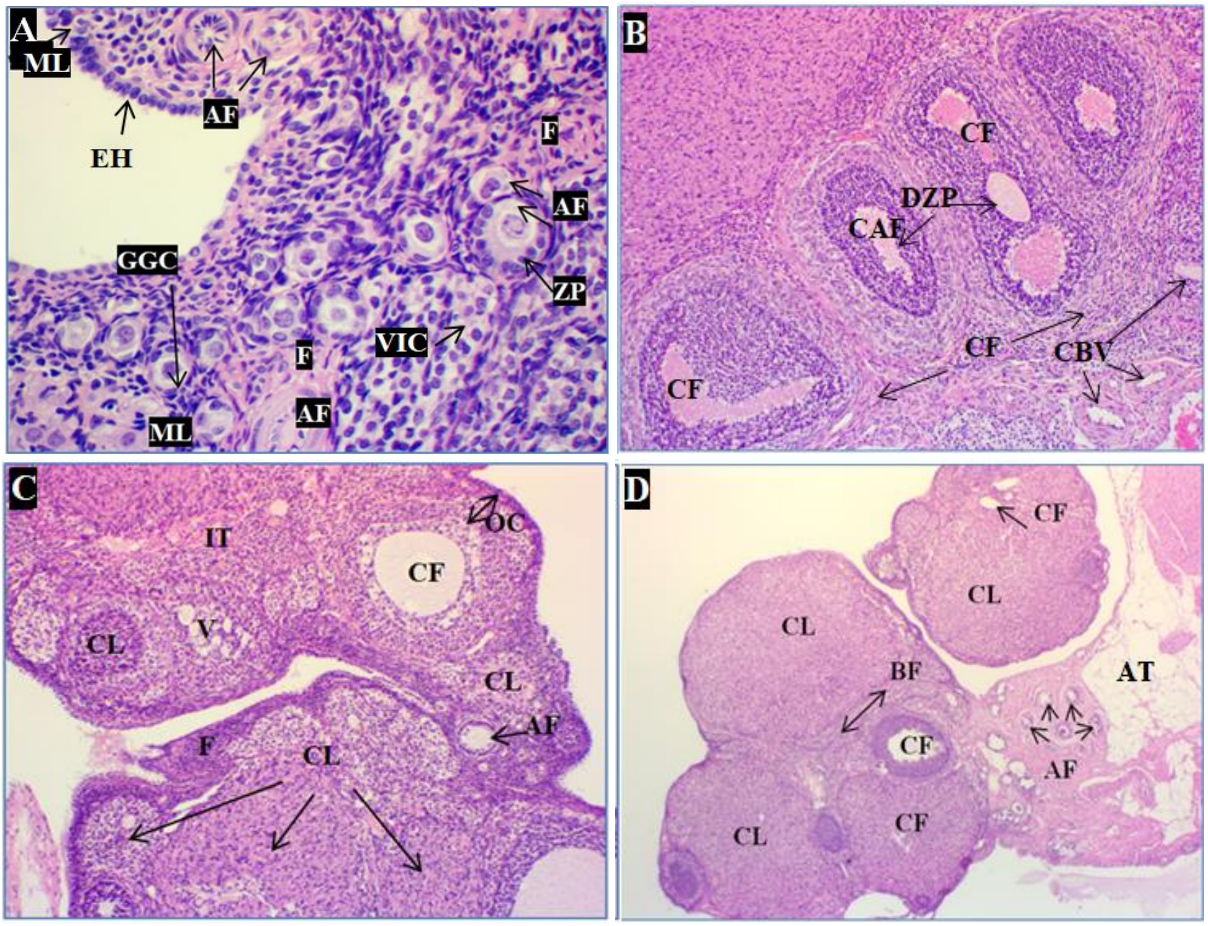

Figure 4. Ovarian cross section stained with (Hematoxylin-Eosin) of rtas injected with 320mg/kg VCD for 2 weeks and treatment with 100 and 200mg/kg of *A.paniculata* methanolic leaf extract. **A & B** Illustrates ovary injected with 320mg/kg VCD for 2 weeks, the damage is indicated by fibrosis (F) (accumulation of collagen fibers), Atretic follicles (AF), degeneration of primary follicles or atretic follicles(AF), vasculozation of interstitial cells (VIC) and hypertrophy of granulosa cells, some of granulosa cell growing and remain as it is (Growing Granulosa Cell- GGC), zona pellucida (ZP), epithelial hypertrophy (EH) and multi-layering of simple cuboidal epithelial layer papillary projection(ML), **A** (400x). Reduction of ovarian follicle's number, degeneration of growing follicles as well as primordial follicles, corpus luteum newly formed damage the cystic atretic follicles(CAF) aslo collagen fibers around the corpus luteum (CF), growing follicles took elongated shape and the degeneration of zona pellucidum (DZP), collagen fibers surround congested blood vessels (CBV). **C.** Is the treated rat ovary with 100mg/kg *A. paniculata* extract and shows the ovarian cortex (OC), fibrosis (F), atretic follicle (AF), corpus luteum (CL), cystic atretic follicle (CF) and interistitial tissue (IT). **D.** Is the rat ovary section treated with 200mg/kg *A.paniculata* and shows the corpus luteum (CL), atretic follicles (AF), adipose tissue (AT), bundle of fibrosis (F), cystic follicle (CF). 250x.

In figures (4A+B) which is the status of ovary tissue after have been injected with 320mg/kg VCD mang histopathological changes can be observed as reduction of different stages of all types of ovarian follicles and an increase in collagen fiber accumulation around cystic atretic follicles along with congestion of blood vessels. However, figure (4C) reflect obvious changes of multi-layering of cuboidal cells after treating with 100mg/kg. figure (4D) shows an increased

corpus luteum after treated with 200mg/kg leaf extract of *A.paniculata*, however the quantity of atretic follicles and vaculozation was decreased and the ovary tissues give the impression to the control rat ovary.

4. Discussion

Result of application of VCD on female albino rats and the outcome after treatment with leaf extract of *A. paniculata* on whole body weight and ovary as well as throughout present experiment have been forwarded . Almost all studies on VCD ability on destroying prenatal follicles and its effect on body weight and ovary in both young and adult rats has been well documented by many authors [7, 9,19]. However Muhammed *et al*., (2009) [9, 25] so far two concentrations VCD doses seems to be common to rats (80 and 160mg/kg) that was administrated via intramuscular injection. In fact, same sort of injection was applied in current investigation but with higher dose also (320 mg/kg). However the effect was quite evident in almost all parameters concerned in the present study . While body weight of rat reduced to almost 230 gram when it was injected with 160 mg/kg VCD whereas when the dose doubled the body weight fell to less than 220. In other Words a reduction of about 20mg of the weight was recorded there for result indicate that high dose result in more loss of the weight. In fact such reduction also was evident in ovary weight. Higher dose led to more lose in the weight this phenomena have been also shown too, by [11,26 , 27].

The effect of VCD on damages and attention of tissues was evident after application of 160 mg/kg dose whereas the variation was tremendous as shown figure (3+4) on application of higher dose (320mg/kg) of VCD . Such variation extend to histological variation also figures (3, 4) Such phenomena have been observed in rats by [24,28, 29] and others. The use of Andrographolide compound from the plant *A. Paniculata* as anti- proliferate as well as pro-apoptotic is used by Yunos *et al*., (2013) as well as its application have been the attention of many scientific particularly in the field of medicinal plants as treatment of anti cancer patellid [9] . Medicinal plant *A.paniculata* is a well known medicinal plant in various parts of the world [30, 31] whereas its application is quite new in Iraqi Kurdistan or Iraq as a whole. Leaf extract of *A. paniculata* used in the present investigation as a treatment agent in female albino rats because of their known antibiotic and anti-oxidation potential activity [17]. In the current investigation the results outcome indicate its effect as treatment with both doses 100, 200 mg/kg on both body and ovary weight of treated female rat. However, the effect on whole body weight did not show any significant difference between the two group of animals that was injected with 160 and 320 mg/kg VCD. In case of treating with higher dose 200mg/kg *A.paniculata* where the weight was almost similar (293gm) figure (3 b)**.** Response in case of 100mg dose reflect significant difference between the

two doses and treatment as the weight raised up to by about 15 mg (227 to 243gm) in correspondent between the doses after 4 weeks, such phenomena was reflected in ovary weight also. However such variation on ovary weight and whole body weight have also been refered to [24] in present study the ovary weight raised up by about 4mg when the dose of *A.paniculata* doubled 100 to 200mg within 4 weeks .

VCD application to induce ovarian failure, known to be the cause of infertility that lead to reduction in follicle cell numbers and amenorrhea, its application may also promote atresia also an increase in immature and mature ovarian follicles [25,32, 33]. Thenafter, ovarian insufficiency in rats may appear in case of rat model of (POI) which was used in current study. Results indicate that outome of two week daily injection of VCD at low dose (160mg/kg.B.W) was infact a reduction in the primary and primordial follicle quantity figure (3 A) was quite evident as similar consequences shown by [14, 26].

In case of high dose injection (320mg/kg.B.W), results showed reduction in ovarian follicles with congestion of blood vessels figure (4, A+B), however degeneration of interstitial cells and some follicle pre-ovulatory follicles was evident. Still an increase of atretic follicle and noticeable increase of corpus luteum with various sizes and in various stages of growing follicles did appear figure (4 B), such results come in accordance to [11, 14, 27].

Infact ovulatory follicles had showed oocyte with micronucleus formation that had been appeared through enclosed thin irregular zona pellucida figure (4 B). Morever, as was detected by [31] most of the granulosa cells in theca interna and externa have degenerated. However, vaculoziation in ooplasm and granulosa cell enclosed by thin layer, disruption and thinning of zona pellucida had been found. In present experiment all of such variation was the result of application of high (320mg/kg.B.W) dose of VCD.

The application of leaf extract of *A. paniculata* as a treatment was applied for four week daily oral dose in present investigation. The result reflect in various tissues in ratt ovary throughout present study. However, two different doses (100 and 200mg/kg. B.W) *A.paniculata* were applied. The result reflect the effect high dose is more effective figure (4 D).

In reference to Halicioglu *et al*., (2021) [31] who reported that so far no any study exist to show histopathological changes such as hemorrhage, congestion, vaculozation and fibrosis caused by VCD exposure in ovarian tissues. In contrast present investigation is forwarding quite clear evidence on VCD effect as its illustrated and forwarded in current study on female albino rat ovary tissues. Furthermore, the variation and changes in ovarian tissues affect treatment with

A.paniculata bot high and low doses was found and illustrated perumably for the first time figure (4 C+D).

Finally, one may propose more detail investigation in this respect using different doses referring to VCD as well as to *A.paniculata* extract. In order to achieve the optimum and most effective dose for treatment present study hopefully will encourage more study in meanings of knowledge in this field.

5. Conclusion

1. Higher dose treatment with *A.paniculata* (200mg/kg) showed the same effect as body weight on female albino rats regardless at toxicities 4-vinylcyclohexene Diepoxide regardless to the injected concentration.
2. Histological alteration and damages was evident in various ovary tissues, and accumulation of connective tissue collagen fibers around corpus luteum after toxicity dose with both 160 and 320 doses.
3. The status of ovary tissues after treatment with high dose of *A.paniculata* reflect an increase in number of follicules of growing follicles.
4. More detail study in this respect may well lead to point out the right dose of treatment and may indicate the exact optimum time required for treatment .
5. Present study is a step to apply the results on other mamals that may lead to its use as a pharmaceutical agent for POF treatment.

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