Estrogenic Properties of Phyllanthin and Hypophyllanthin from *Phyllanthus amarus* against Carbofuran Induced Toxicity in Female Rats

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Summary

The present study has been undertaken to know the effect of a carbamate insecticide, carbofuran, on estrous cycle and follicular growth in virgin Wister rats as well as recovering from the damaged estrous cycle with treatment of *Phyllanthus amarus* lignans *viz.* phyllanthin and hypophyllanthin, the two major lignans of this important herb and generally know as phytoestrogen. Phytoestrogens are non-steroidal plant molecules with estrogen-like activities whose structure differs from gonadal hormones**,** but with an estrogen-type activity: they are capable of interacting with estrogen receptors, showing both agonist and antagonist methods of action. Since, phyllanthin and hypophyllanthin have been found to be systemically transformed into enterolignan(s), which is known to be responsible for augmenting estrus cycle in rats.

Introduction

The reproductive cycle of female rats is called estrous cycle and is characterized as proestrus, estrus, metestrus (or diestrus I) and diestrus (or diestrus II)[1] . The ovulation occurs from the beginning of proestrus to the end of estrus. From the onset of sexual

maturity up to the age of 12 months, the mean cycle length in the female rat is 4 days[1] and this short cycle length makes the rat an ideal animal for investigation of changes occurring during the reproductive cycle[2,3].

During the estrous cycle, prolactin, LH and FSH remain low and increase in the afternoon of the proestrus phase. Estradiol levels begin to increase at metestrus, reaching peak levels during proestrus and returning to baseline at estrus. Progesterone secretion also increases during metestrus and diestrus with a decrease afterwards. Then the progesterone value rises to reach its second peak towards the end of proestrus[2].

Carbofuran (2, 3-dehydro-2,-2-dimethyl-7 benzofuranyl methylcarbamate), is a systemic N-methyl carbamate pesticide with predominantly contact and stomach action. It is mainly used as a soil applied chemical to control soil dwelling and foliar feeding insects and nematodes on a variety of agricultural crops, including maize, corn, rice, potatoes, alfalfa and grapes[4].Carbofuran is a potent cholinesterase inhibitor and is highly toxic to humans and wildlife through the oral and inhalation routes of exposure[5]. It has been shown to affect the thyroid system in ewes, resulting in increased thyroxine concentrations[6].Yousef; *et al.* has reported that the carbofuran decreased libido and sperm number in rabbits. The carbamate pesticides also have been recently reported in the induction of gonadal toxicity to female rats after chronic exposure to mancozeb and the administration of the sodium N-methyl dithiocarbamate inhibits the secretion of luteinizing hormone thus affecting the ovulation in rats[7].

Phyllanthus amarus (PA) is a well known herb for its pharmacological activities such as anti-diabetic,anti-cholesterol properties, anti-cancerous and cellular protective actions, liver protective and detoxification actions, antiviral actions, antispasmodic, pain-relieving anti-inflammatory activity and normalizes elevated urinary calcium levels in calcium stone forming patients. Furthermore extracts of PA possess antiparasitis, antibactereial and antimicrobial activity. It is also used for its wound healing properties. But the effect on female reproductive system has not been carried out.

Since, phyllanthin and hypophyllanthin have been found[8] (Islam *et al*; unpublished observation) to be systemically transformed into enterolignan(s) (Fig. 1), which is known to be responsible for augmenting estrus cycle in rats, the title was undertaken.

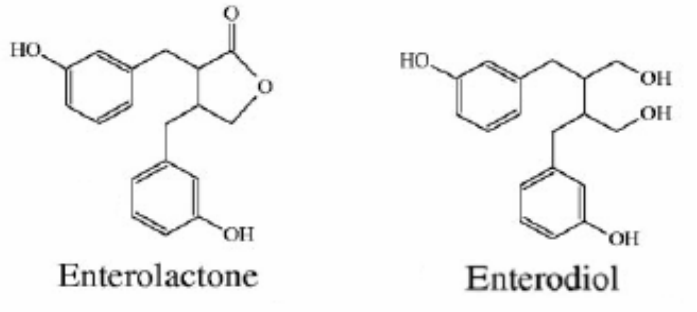


Fig.1.

Materials and Methods

Chemicals and Drugs used:

Technical grade Carbofuran was (from Rallis India Ltd.) dissolved in olive oil as a vehicle for oral administration. The dose of the carbofuran was 1mg/kg body weight. For the estimation of estrogen level we followed ELISA method.

Animals:

Female Wistar rats (*Rattus norvergicus*), three months old, weighing 150 to 200 g were used. The animals were housed in standard cages, six per cage, in a controlled temperature room (22ºC), with a 12 h light: 12 h dark cycle, lights on at 6:00 a.m. Standard laboratory chow and tap water were available *ad libitum.* The study was conducted in accordance with Good Laboratory Practice (GLP) Regulations of the WHO (WHO Document, 1998). The "Principles of laboratory animal care" (NIH Publication # 85-23, 1985) were also followed in this study. The 'Institutional Animal Ethics Committee' approved the present experimental protocol. Animals were dividing into five groups having 6 animals in each. PAL were administered in 3 different doses (25mg/kg, 50 mg/kgand 100mg/kg body weight to respective groups). The details of the experimental groups are mentioned here:

Table 1.

Groups	Treatment	Doses
Group I	Vehicle Control	Olive oil (0.9%) (w/w)
Group II	Only Carbofuran	(1mg/kg)
Group III	(P + HP) (1:1) + Carbofuran (%)	25mg/kg (P=12.5mg + HP = 12.5mg) + Carbofuran (1mg/kg)
Group IV	(P + HP) (1:1) + Carbofuran (%)	50 mg/kg (P = 25mg + HP = 25mg) + Carbofuran (1mg/kg
Group V	(P + HP) (1:1) + Carbofuran (1mg/kg)	100 mg/kg (P=100mg + HP=100mg) + Carbofuran (1mg/kg

P - Phyllanthin; HP- Hypophyllanthin.

Determination of different phases of Estrous Cycle:

During one month, every morning between 8:00 and 9:00 a.m. each animal cage was carried to the experimental room. Vaginal secretion was collected with a micro pipette with blunt ended microtip filled with 10 µL of normal saline (NaCl 0.9%) by inserting the tip into the rat vagina, but not deeply. Vaginal fluid was placed on glass slides. A different glass slide was used for each group of animals. One drop was collected with a clean tip from each rat. Unstained material was observed under a light microscope, without the use of the condenser lens, with 10 and 40 x objective lenses. Three types of cells could be recognized: round and nucleated ones are epithelial cells; irregular ones without nucleus are the cornified cells; and the little round ones are the leukocytes. The proportion among them was used for the determination of the estrous cycle phases. Round and nucleated ones are epithelial cells; irregular ones without nucleus are the cornified cells; and the little round ones are the leukocytes. A proestrus smear consists of a predominance of nucleated epithelial cells (Figs.2.a); an estrous smear primarily consists of anucleated cornified cells (Figs.2.b); a metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells (Figs.2.c); and a diestrus smear primarily consists of a predominance of leukocytes (Figs. 2.d).

Hormonal assay:

In order to determine the variation of plasma estrogens level in different experimental groups at the end the experiment. After the last administration ,while the animals were in estrous phase,each rat was anesthetized using diethyl ether and blood samples were taken by dorsal aorta puncture (approximately 500µl).The blood samples were centrifuged for 15 minute (2000 rpm) and serum portions were separated. The estradiole concentration were measured by ELISA method.

Statistical analysis:

Experimental results were expressed as mean ± SEM analysis of variance was performed by one way ANOVA procedures (SSPS 10.0 for Windows). Significant differences between means were determined by Dunnett's post hoc test. $p<0.05$ implies statistically significance.

Results

It is cleared that the control mice exhibited regular 4–5 day estrous cycle. Rats of Group V (treated with Carbofuran + 100mg/kg/PAL) the number of estrous cycle and the duration of proestrous and estrous remain unaltered which is comparable to the normal control rats (Group I).Treatment with 50mg/kg PAL + Carbofuran (Group IV) causes moderate decrease in the number of estrous cycle and the duration of proestrous,estrous,metestrous with moderate increase in the diestruos phase.Lickwise rats of Group III (treated with Carbofuran +25 mg/kg PAL) causes a significant decrease in the number of estrous cycle and the duration of proestrous,estrous,metestrous with the concomitant significant increase in the diestrousphase.Group II rats were treated with only Carbofuran (1mg/kg served as negative control) also causes significant cessation of the number estrous cycle and the duration of proestrous, estrous, metestrous with the simultaneous increase in the diestrous phase.

Diestrous index was also decreased dose dependently in all the PAL treated groups following the administration of Carbofuran.

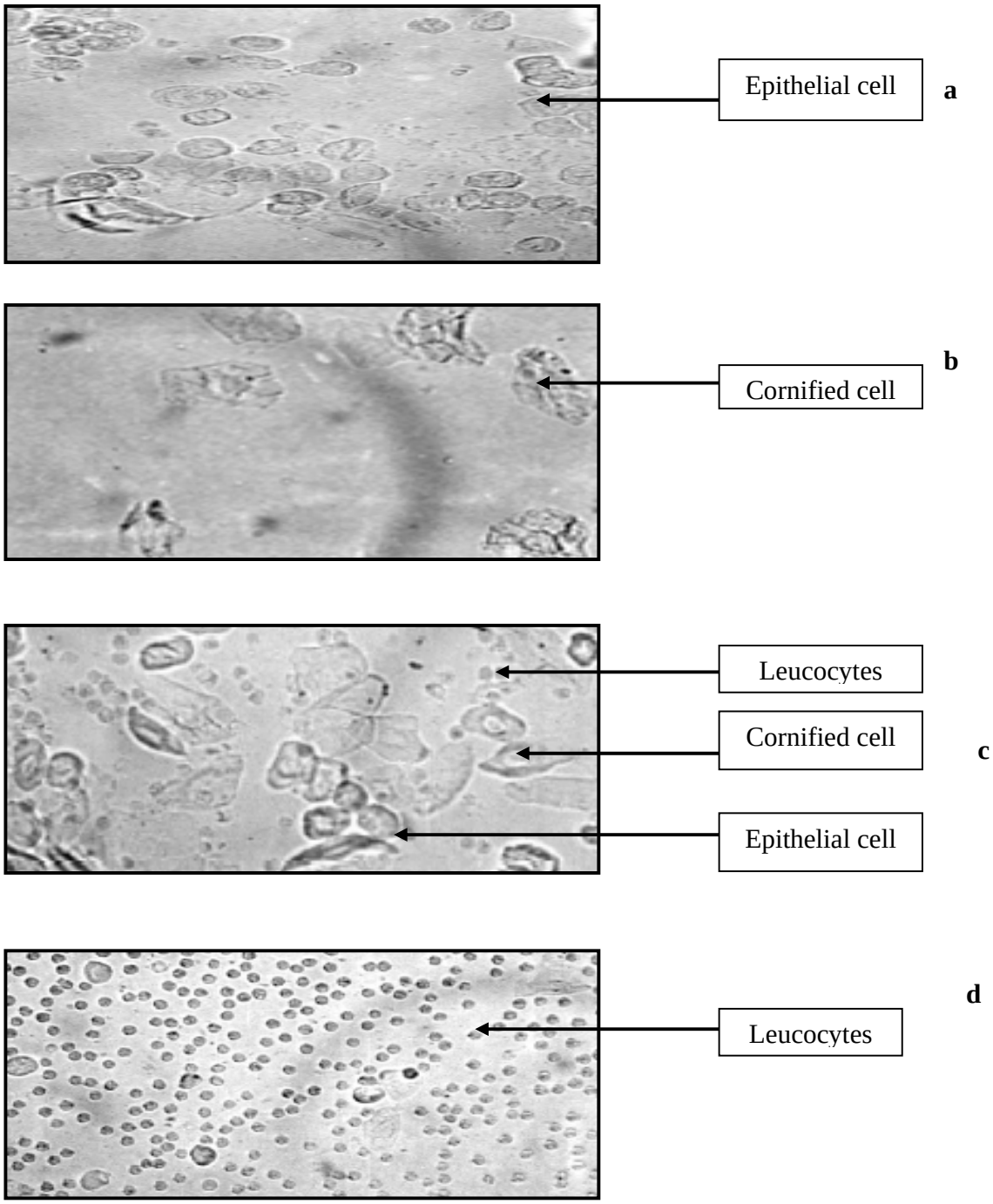


Fig. 2. Photomicrographs of unstained vaginal smear from female rats of different phases (proestrus(a),estrus(b),metestrus(c)and diestrous (d).

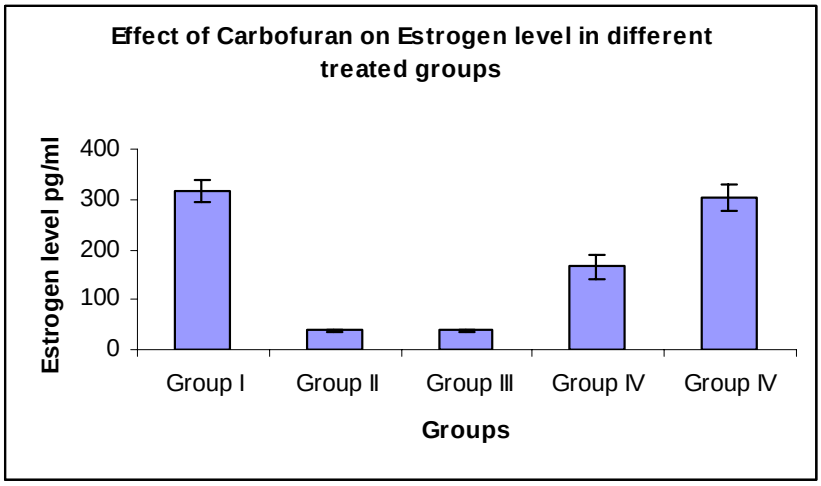
Table.2.

Groups	No.of Rats	No.of Cycles	Duration in days (M±S.E)				Diestrous Index
			Proestrous	Estrous	Metestrous	Diestrous	
Group I	6	6.1±0.21	4.7±0.27	7.5±0.23	5.6±0.21	12.1±0.21	40.33
Group II	6	4.31±0.29	2.1±0.33	5.5±0.3	3.4±0.23	18.2±0.48	60.66
Group III	6	4.12±.19	2.8±0.31	6.3±.31	4.6±0.22	15.8±0.51	52.66
Group IV	6	5.41±.19	3.5±0.51	7.11±0.25	5.1±0.17	12.9±0.35	43
Group V	6	5.9±0.25	4.63±.38	7.6±0.25	5.3±0.23	12.11±0.43	40.36

Effect of PAL on Carbofuran Impaired Estrous Cycle
Six cycles means 24 phases of estrous cycles.

Low dose of the drug produces significant change in serum estradiol and in moderate dose there was also significant alteration of estradiol but comparatively high than that of low dose. The high dose converted the estradiol level more or less normal. Thus it can be concluded that the estradiol level altered in dose dependent manner.

Fig .3.



Effect of Carbofuran on Estrogen level in different treated groups

Discussion

The results obtained in the present study indicate that the control mice exhibited regular 4–5 days estrous cycle. Rats of Group V (treated with Carbofuran + 100 mg/kg/PAL) the number of estrous cycle and the duration of proestrous and setrous remain unaltered which is comparable to the normal control rats (Group I).Treatment with 50 mg/kg PAL + Carbofuran (Group IV) causes moderate decrease in the number of estrous cycle and the duration of proestrous, estrous, metestrous with moderate increase in the diestruos phase. Lick wise the rats of Group III (treated with Carbofuran 25 mg/kg PAL lignans) causes a significant decrease in the number of estrous cycle and the duration of proestrous,estrous,metestrous with the concomitant significant increase in the diestrousphase.Group II rats were treated with only Carbofuran (1mg/kg) served as negative control) also causes significant cessation of the number estrous cycle and the duration of proestrous, estrous, metestrous with the simultaneous increase in the diestrous phase.

Cyclic changes of the vaginal smear observed in the estrous cycle gives a reasonable index of the ovarian activity and its hormonal synthesis of estrogen and progesterone. The levels of these hormones are controlled by hypothalamus releasing gonadal hormones and pituitary gonadotropins.

Diestrous index was also decreased dose dependently in all the PA lignans treated groups following the administration of Carbofuran.

Recently, similar results have been reported that the rats treated with a carbamate fungicide mancozeb causes a significant decrease in the number of estrous cycle and the duration of proestrus, estrus, and metestrus with a concomitant significant increase in diestrus phase[9]. Similar results have also been reported with other organophosphate pesticides treated animals[10,11]. In contrast, organochlorin pesticides like DDT, chlordecone, methoxychlore and dicofol showed a capacity to induce the persistent vaginal estrus, thereby affecting the number of estrous cycle resulting from the hormonal imbalance and prolonged estrus[12,13]. In the present study it has been shown that treatment with carbofuran showed prolonged diestrus and hence, carbofuran may not have estrogenic activity as it has been shown in the chlorinated pesticides treated animals.

It has been reported that carbamate fungicide sodium N-methyl dithiocarbamate is shown to block the ovulation by inhibiting the secretion of LH in rat[14]. Since carbofuran is a carbamate pesticide, it may be possible that it would act on the level of hypothalamus to adversely affect the ovary, which in turn affects the estrous cycle and folliculogenesis due to the hormonal imbalance in estrogen progesterone ratio.

Plowchalk *et al*[15] (1993) have reported that the quantitative assessment of follicle number is an indicator of the normal function as well as toxic responses in the ovary. Follicles are the principle functional units of the mammalian ovary. The most important controllers of follicular development are follicle stimulating hormone (FSH) and luteinising hormone (LH) produced from the pituitary and the ovarian steroid estradiol produced by granulosa cells. Treatment with carbamate fungicide sodium N-methyl dithiocarbamate is shown to block the ovulation by inhibiting the secretion of luteinizing hormone in rats[14]. Recently it has been reported that rats treated with carbamate fungicide mancozeb, showed a decrease in the number of healthy follicles with increased atretic follicles[7,9]. In the present study, there is also a possibility that the decreased healthy follicles with concomitant increase in atretic follicles in mice may be due to affecting gonadotropin secretion via central nervous system mechanism, as it was observed in the rats with the following administration of dithiocarbamates[16]. In the present study there is also a possibility that the disruption in the estrous cycle, decrease in the healthy follicles with concomitant increase in the atretic follicles may result from the damage by toxicants at the level of hypothalamo-pituitary gonadal axis. It has been reported that the insecticides may destroy endocronologic Homeostasis, by suppressing GnRH release, may act directly on the gonadotropins to alter the gonadotropins synthesis and secretion or indirectly by altering the pituitary cell responsiveness to GnRH or gonadal steroids which result in the alterations in the levels of FSH and LH affecting the feed-back mechanisms[17-18]. Further evidence of hormonal imbalance is corroborated with as the mice shows continuous diestrus. Therefore, the reason may be due to the hormonal imbalance in any of the stages in hypothalamo-hyphophysial ovarian axis or by insensitising the follicular receptors to the available gonadotropins thereby led to the retardation of further development of surviving follicles into next successive follicular stages and also arrest of estrogen production which affects the estrous cycle or directly on

the ovary by causing fibrosis[19].However, further investigation in this regard is essential to know the mechanism of action of carbofuran on follicular development and estrous cycle. Treatment with carbofuran showed dose related toxicity in terms of body weight. There is a significant decrease in the body weight gain in high dose of carbofuran treatment, as there may be suppression towards food and water intake. Although food and water intake has not been measured in this study, this may be one of the reasons for low weight gain and alteration in the estrous cycle. The ovary weight was decreased significantly with high doses of carbofuran treatment. Similar observations were made in rats treated with monocrotophos and have reported that decrease in weight and size of the ovaries is due to extensive fibrosis and atretic follicles[18].Treatment with carbofuran in different dose groups did not alter the weights of uterus kidney, adrenal, liver, spleen, thymus and thyroid. Similar results have been reported in other pesticide treated rats. The alteration in the estrous cycle with prolonged diestrus and decrease in the healthy follicles with an increase in the atretic follicles in carbofuran treated mice may be due to the reduced synthesis of steroids in the ovary, causing imbalance in the estrogen: progesterone ratio. Whether the observed toxicity occurred as a result of direct effects upon the ovary or indirectly through the action on the hypothalamus and/or pituitary, or by desensitizing the ovary to gonadotropins cannot be ascertained from this study.

Conclusion

It may be concluded that *Phyllanthus amarus* lignans can prevent the lethal effect of the carbofuran by regularizing the hormonal secretion to maintain the normal life process.

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