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### Evaluation of the Antifertility Activity of the Hydroalcoholic Extract of the Leaves of *Plumbago Zeylanica* L. (Plumbaginaceae) in Female Wistar Rats Vishnukanta\* and A.C. Rana

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#### Abstract

There is a need to develop safe and effective agents such as plant-based contraceptive agents. Hydroalcoholic extract of Plumbago zeylanica Linn leaves were prepared using cold extraction process (maceration). Antiimplantation activity was studied in adult female wistar rats, by administering the extract for 1-7 days of post-coitum. Total number of implants in both uteri and total number of corpora lutea in both ovaries were counted on  $10^{th}$  day of pregnancy. Estrogenic/antiestrogenic activity was studied in immature ovariectimised female wistar rats.

It was found that the extract has very significant antiimplantation activity, in the doses of 200mg/kg and devoid of estrogenic activity, but showing antiestrogenic activity causing overall structural and functional changes in uterus.

Key words: Plumbago zeylanica, antifertility, antiimplantation, estrogenic, corpora lutea

#### INTRODUCTION

Rapid rise in population has caused serious problems in the economic growth and all-round human development in developing countries like India. Family planning has been promoted through several methods of contraception, but due to serious adverse effects, produced by synthetic steroidal contraceptives, attention has now been focused on indigenous plants for possible contraceptive effect<sup>1,2</sup>. *Plumbago zeylanica L*. commonly known as white chitrak (family:Plumbaginaceae) is a perennial herb that is grown in most parts of India and is used in the traditional system of Indian medicine against a number of ailments including skin diseases, diarrhoea and leprosy<sup>3</sup>.

The pharmacological studies carried out by several workers indicate that *Plumbago zeylanica* L. possesses antibacterial, antifungal, anticarcinogenic<sup>4</sup> and radiomodifying properties <sup>5</sup>. It is also reported to have antitumor activity<sup>6</sup>. The roots of this plant has been reported to be a powerful poison when given orally or applied to ostium uteri, causes abortion<sup>7</sup>. Earlier workers

reported the anti-implantation and abortifacient activity without any teratogenic effect<sup>8</sup>.

50% alcoholic extract of roots of *Plumbago zeylanica L*. has been reported for 100% antiimplantation activity<sup>9</sup> and no antiimplantation activity was present in the petroleum ether, alcoholic and aqueous extract of roots<sup>10</sup> but none of the work is reported on the leaves of this plant. So the aim of the present study is to evaluate the antifertility activity of hydroalcoholic extract of leaves of *Plumbago zeylanica* L.

#### **MATERIALS AND METHODS**

**Plant collection and preparation of extract:** Fresh leaves of *Plumbago zeylanica L*. were collected from Rajasthan Agriculture College Campus, Udaipur Rajasthan, in the month of March. The plant was authenticated by Dr. S. S. Katewa, Dept. of Botany, College of Science, MLSU, Udaipur. Leaves were dried in shade, moderately grinded and macerated with hydroalcoholic solvent (70:30) for 7 days with intermittent shaking. On 8<sup>th</sup> day the macerate was filtered through muslin cloth and solvent was evaporated at room temperature. The residue obtained, was lyophilized (lyophilizer-Step Origin Electric, Lonavala) and freeze-

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dried (Freeze dryer, Allied Frost) to provide dry hydroalcoholic extract of *Plumbago zeylanica L*. leaves (HEPZL) with the practical yield of 17 % W/W.

**Animals:** Female wistar rats were used for the study. Mature females, weighing between 150-200 g were used for antiimplantation activity and female, weighing between 50-60 g were used for estrogenic/antiestrogenic activity. Institution Animal Ethic Committee approved all experimental procedures (approval no. 03/ACR/BNCP-06/IAEC). All the animals were maintained under standard husbandry conditions with food (Chakan mill, Sangali, Maharashtra) and water ad libitum.

Acute oral toxicity: It was determined using OECD/OCDE guideline<sup>11</sup> 425, main test was performed and LD50 was found to be 5000mg/kg the dose selected was  $1/50^{\text{th}}$  and  $1/25^{\text{th}}$  of LD50.

Antiimplantation activity: For this study, vaginal smear of each rat was monitored daily, those rats showing regular estrus cycle were included in study. They were left overnight with proven fertile male in the ratio of 2:1 (female: male), in proestrous phase and examined the following morning for evidence of copulation. Those rats, which showed thick clumps of spermatozoa in their vaginal smear, were separated for the experiment and that day was designated as day 1 of pregnancy. They were divided into three groups containing six rats in each group. The first group was control group received vehicle {1% Carboxy Methyl Cellulose (CMC)}, second and third groups were drug treated, received HEPZL at the dose of 100 mg/kg and 200 mg/kg respectively. All the treatment were given orally from 1 to 7 days of pregnancy, then laparotomy under light ether anesthesia and semi sterile condition was performed on day  $10^{th}$  of pregnanacy<sup>12,13</sup>.

The uteri were examined for implantation sites (I) and total numbers of corpora lutea (CL) on both the ovaries were counted. The abdominal wound was sutured in layers and animals were allowed to go to term. After delivery, the litters (L) were counted. Percent Preimplantation loss (CL-I/CL\*100), percent postimplantation loss (I-L/I\*100) and percent antiimplantation activity (CL-L/CL\*100) were calculated<sup>14</sup>.

**Estrogenic/ antiestrogenic activity:** female albino wistar rats (50-60 g) were ovariectimised<sup>15</sup> under light ether anaethesia and semi sterile conditions. They were

divided into six groups, containing six rats in each. The first group received vehicle (1% CMC p.o.) the second group received estradiole valarate (Trade name Progynone depot, German remedies) in groundnut oil in the dose of  $0.1\mu$ g/rat/day, subcutaneously. The third and fourth groups received HEPZL at the dose of 100 mg/kg and 200 mg/kg, p.o. respectively. Fifth and sixth group received both, the doses of extract along with standard estradiole valarate. All the treatment was given for seven days. On eighth day, premature opening of vagina is noted and vaginal cornification is observed, then all the rats were sacrificed by decapitation; the uteri disse.

One uterine horn was stored in deep freeze for glycogen estimation<sup>18,19,20</sup> and another horn was fixed in bouin's fluid and embedded in paraffin wax. The paraffin sectioncted out, surrounding tissues were removed and weight was taken immediately, then uteri were slightly pressed between the folds of filter paper and again weight was taken<sup>16,17</sup>s were cut at 5 micron and stained with haematoxylin-eosin for histological observations. The diameter of uterus, thickness of myometrium, endometrium and height of epithelial cells were measured using trinocular optical microscope (Labomed, Ambala).

**Statistical Analysis:** The data was analysed by using oneway ANOVA followed by Tukey Multiple comparision test. A p value <0.05 was considered to be significant. **RESULTS** 

Antiimplantation activity: In the present study, hydroalcoholic extract of *Plumbago zeylanica L*. leaves were tested for anti-implantation activity. Both the doses of extract showed highly significant (p<0.001) anti-implantation activity. At the dose of 100 mg/kg and 200 mg/kg, extract showed more preimplantation loss than post implantation loss and was significant with control group. At 100 mg/kg and 200 mg/kg extract showed 58.03 % and 95.16 % antiimplantation activity, respectively, this was in dose dependent manner (Table No.1)

**Estrogenic/ antiestrogenic activity:** To explore the mechanism of anti-implantation activity Estrogenic/ antiestrogenic activity was carried out, estrogen treated group showed highly significant (p<0.001) increase in the weight and glycogen content of uterus. All the animals showed premature opening of vagina and cornified cells in vaginal smear. Administration of hydroalcoholic

extract in rats at doses of 100 and 200 mg/kg did not show any increase in uterine weight and glycogen content. The vagina remains closed and cornification was not induced. Co- administration of estradiole valarate and extract showed highly significant (p<0.001) decrease in uterine weight as compared to estradiole valarate group in dose dependent manner, vaginal opening and cornification were also reduced as compared to estradiole valarate group. There was significant (p<0.01) decrease in glycogen content of animals receiving 100 mg/kg of extract along with estradiole valarate and highly significant (p<0.001) decrease in glycogen content with 200 mg/kg of extract along with estradiole valarate (Table No. 2).

**Histological studies:** The uterine lumen in control rats was wide and pits and folds were present. Endometrium was lined by a single layer of cuboidal ciliated epithelial cells. The glands appeared very prominent and dilated. In estradiole valarate treated group, there was increase in blood supply and significant increase in diameter, thickness of myometrium, endometrium and height of epithelial cells as compared to control group (Table No. 3). Uterine lumen was wider and numerous pits and folds were present.

In drug treated groups, there were no significant changes in histoarchitecture of uterus but co-administration of extract with estradiole valarate showed highly significant decrease in diameter, myometrium thickness, epithelial cell height and endometrium thickness as compared to standard estrogen treated group. There was complete structural and functional change in uterus. In these animals as compared to estradiole valarate treated group, uterine lumen is thin and reduced in size with very less or disappearance of pits and folds. Continuity of endometrial cells was also interrupted. Decreased in the number and size of uterine glands was observed and there was functional disparity of endometrial gland, indicated by necrosis of gland tissue. (Fig: 1-6).

#### DISCUSSION

In majority of animals, implantation takes place at a fixed interval of time after ovulation when the corpus luteum is fully formed. The hormonal control ensures maximum endometrial sensitivity when the mature blastocyst is present in uterus. A reaction, than takes place between the trophoblast and the uterine epithelium so that the endometrium is stimulated to undergo a chain of reactions leading to the formation of the placenta<sup>21</sup>.

Implantation in rat depends on the completion of the basic sequence of the events occurring both in fertilized egg and endometrium. Normally, estrogen and progesterone act synergistically upon the uterine endometrium to prepare it for nidation. The endometrium needs 48 h period of progesterone preparation and presence of the estrogen at the end, leading to the formation of high sensitive decidualised endometrium. It is therefore assumed that a substance, which can impair the synthesis, secretion and function of ovarian steroids, may block the implantation process by hindering the development of oocycte, graffian follicle as well as the endometrial epithelium<sup>22</sup>.

It is reported that the passage of the ovum down the uterine tubes is controlled by the same ovarian hormones that sensitise uterus for implantation, so that any experimental alteration of hormones level during this time would affect the transport of blastocyst. There is increased permeability of the blood vessel supplying the uterus at the blastocyst implantation and estrogen is necessary for this effect, so drug which antagonizes the estrogen, decreases the blood supply at this point hence affect the implantation of blastocyst.

Preimplantation loss is a parameter used to establish a correlation between the number of released ova, which after fertilization, manage to implant in the uterus. The rate of postimplantation loss which establishes the correlation between the number of implanted embryos and those, which manage to develop normally. The increased preimplantation loss could be associated with a relaxant effect of the extract on the oviductal motility, interfering with transport towards the uterus and consequently with its implantation. In relation to malformations, the preimplantation period in mammals has been considered as an all or none period i.e. in which the maternal exposure to chemical agents may cause embryolethality or may interefere with the embryo normal development<sup>23</sup>.

In present histological studies extract alone although, does not produce much change in structure and function of uterus but along with estradiole valarate it antagonizes the estrogen induce uterotropic changes. Continuity of endometrial cells was interrupted by desquamation of epithelial cells from stroma. Vacuolation of cytoplasm and functional disparity of endometrial gland was indicated by necrosis of gland tissue, characterized by disorganization of epithelial cells and loss of cellular character leading to functional aberration of the uterus<sup>24</sup>.

In the present study, extract at 200mg/kg body weight shows highly potent (95.167%) antiimplantation activity. This is because of anti-estrogenic activity of extract which antagonizes the action of estrogen, causes structural and functional changes in uterus. The antiestrogenic effect is also supported by decrease in glycogen content, diameter, thickness of endometrium, myometrium, reduced uterine lumen with decreased pits and folds, decreased in the number and size of the uterine glands, vaginal opening and cornification, overall the extract directly affect the target organ i.e. uterine horns.

Although plumbagin is reported in whole plant<sup>8</sup> but in

present findings the hydroalcoholic extract of leaves of the plant does not contain plumbagin as conformed by HPTLC analysis at M/s Anchrom HPTLC specealists Mumbai. The earlier workers reported that the plant contains glycoside, tannins, flavonoids, betasitosterol and steroids, which may be responsible for antifertility activity; since the phytosterols, polyphenols<sup>25</sup>, alkaloids and glycosides<sup>21</sup> are, reported to posses the antifertility activity.

#### CONCLUSION

In our study we found that the hydroalcoholic extract of *Plumbago zeylanica L*. leaves retarded the preparation of the uterus for nidation, by virtue of its antiestrogenicity thereby blocking the implantation.

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# Table No.1- Antiimplantation activity of hydroalcoholic extract of leaves ofPlumbago zeylanica L.

Group	Treatment	% Pre implantation loss	% Post implantation loss	% Anti implantation activity
I (Control)	Vehicle (1 % CMC) p.o.	$6.750 \pm 3.703$	4.697±2.105	11.06±4.203
II (Drug treated)	HEPZL (100mg/kg) p.o.	47.52±5.697***	17.95±6.3	58.03±3.757***
III (Drug treated)	HEPZL (200mg/kg) p.o.	88.457±3.982***+++	41.667±20.069	95.167±3.371***+++

Data are analysed one-way ANOVA followed by Tukey multiple comparison test, a P<0.001 is considered to be significant. Values are mean $\pm$  SEM. N=6.\*\*\* p<0.001, when compared with control group, +++ p<0.001, when compared with the group<sup>2 nd</sup>

	)	)	•		)	
Group	Treatment	Uterine weight With fluid (mg/100g BW)	Uterine weight With fluid (mg/100g BW)	Premature opening of vagina	Vaginal cornification	Glycogen content of uterus (µg/100mg uterus)
Ι	Vehicle (1%CMC) p.o.	$41.43 \pm 1.73$	38.96±1.92	9/0	I	$42.4 \pm 6.156$
Π	Estradiol valarate (0.1 µg/ml/rat) s.c	125.2±8.67***	98.96±6.62***	6/6	Cornified cells (+++)	74.39±4.821***
Ш	HEPZL (100mg/kg) p.o.	47±2.407	38.58±2.766	0/6	1	44.73± 1.266
IV	HEPZL (200mg/kg) p.o.	51.45±2.702	44.03±2.867	9/0	1	$36.97 \pm 1.143$
Λ	Estradiol valarate + HEPZL(100mg/kg) p.o.	$82.3 \pm 5.44$ ***	73.51±1.494 ***	3/6	Cornified cells (++)	54.07±1.249 <sup>++</sup>
VI	Estradiol valarate + HEPZL(200mg/kg) p.o.	62.75±1.176 ****	55.71±1.576 *** #	2/6	Cornified cells (+)	47.57±0.91 <sup>+++</sup>

Table No. 2 Estrogenic/antiestrogenic activity of hydroalcoholic extract of leaves of *Plumbago zeylanica L*.

Data are analysed one-way ANOVA followed by Tukey multiple comparision test. Values are mean± SEM. N=6.

\*\*\*p<0.001, when compared to other groups, +++ p<0.001 when compared to gp 2, <sup>++</sup> p<0.01 when compared to gp 2, <sup>++</sup> p<0.01 when compared to gp.V

	Tabl	e No.3 Histological s	studies of immature rat ut	erus	
Group	Treatment	Diameter	Thickness of	Thickness of	Height of endometrial
		of uterus (um)	myometrium (µm)	endometrium (µm)	epithelialcells (µm)
Ι	Vehicle (1%CMC) p.o.	$228.38 \pm 23.32$	<b>41.86</b> ± <b>6.78</b>	$52.21 \pm 11.3$	$3.41 \pm 0.32$
Π	Estradiol valarate (0.1 ? g/ml/rat) s.c.	$430.97 \pm 13.8 * * *$	80.29± 5.74***	$162.35 \pm 25.18^{***}$	$10.625 \pm 0.77 * * *$
III	HEPZL (100mg/kg) p.o.	211± 2.9	42.8± 1.4	<b>55.55</b> ±15.88	$3.96 \pm 0.40$
IV	HEPZL (200mg/kg) p.o.	220± 4.8	$38.34 \pm 1.62$	$50.27 \pm 5.11$	$3.31{\pm}0.36$
	Estradiol valarate HEPZL (100mg/kg) p.o.	$329{\pm}6.36$	$62.79 \pm 1.67$ $^+$	$98.43{\pm}3.09$ $^{+}$	$6.56\pm.216$ <sup>+++</sup>
ΙΛ	Estradiol valarate HEPZL (200mg/kg) p.o.	$290\pm 6.69$ ***	$52.89\pm1.74$ ***	$89.61{\pm}2.4$	4.75±.24 ***

 $2^{nd}$  ,  $^{^+}p{<}0.05$  when compared to gp  $2^{nd}$ Values are mean± SEM. N=6 \*\*\* p<0.001, when compared to control group,  $^{\rm +++\,pc}$ 0.001 when compared to gp  $2^{nd}$ , <sup>++</sup> p<0.01 when compared to gp Fig:1 Section of immature rat uterus treated with vehicle (1% CMC) p.o. (50X)



Fig:3 section of immature rat uterus treated with HEPZL (100mg/kg) p.o. (50 X)



valarate + HEPZL(100mg/kg) p.o. (50 X)

Fig:5 section of immature rat uterus treated with Estradiol



Fig:2 section of immature rat uterus treated with Estradiole valarate ( $0.1\mu g/ml/rat$ ) s.c. (50 X)



Fig:4 section of immature rat uterus treated with HEPZL (200mg/kg) p.o. (50 X)





Fig:6 section of immature rat uterus treated with Estradiol valarate + HEPZL(200mg/kg) p.o. (50 X)

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