

PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES ON IPOMOEA DIGITATA

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UNIVERSITY OF BOMBAY

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IN
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SUBMITTED BY
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UNDER THE GUIDANCE OF
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IPOMOEA DIGITATA

A

THESIS

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For The Degree Of

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CHAPTER - I

1. a) Introduction
- b) Review of the Literature of Ipomoea
- c) Review of estrogenic plants and their active principles
- d) Biological activities of estrogens
- e) Therapeutic uses of estrogens

CHAPTER - II : PHYTOCHEMISTRY

2. a) Review of literature of Ipomoea digitata
- b) Review of separation of steroids
- c) Review of the physical methods in organic chemistry for elucidation of structure of a compound
- d) Materials and Methods
- e) Results and discussion

CHAPTER - III : PHARMACOLOGY

3. a) Review of Biological activities of steroids
- b) Materials and Methods
- c) Results and Discussion
- d) Conclusion

References

Synopsis

Statement

CHAPTER I - INTRODUCTION

Introduction:

Natural products were once the source of all drugs, and purification of medicinal substances from the plants became a major concern of medicinal chemistry during the 19th century. In the recent years the limitations of imaginations of synthetic compounds have now been seen in many diseases particularly in malignancy. Progress in analytical and preparative methods stimulated a recurrence of interest in this field (Bonhoeffer and Pieroni, 1966). Natural products and their derivatives still represent nearly half of all the drugs in clinical use (Gosselin, 1962).

Occasionally, folk-lore provides clues to plants with pharmacologic activity (Kroig, 1964). Digitalis, opiates and the cinchona alkaloids were introduced into modern medicine by this route. Curare was obtained from a south American plant long used by natives to prepare arrow poisons. Cardiac glycosides from *Strophanthus* seeds and physostigmine from calabar beans exemplify useful drugs employed as poisons in the native habitat. Rauwolfia serpentina was used for centuries in India, as a native remedy for variety of illnesses. Only in recent years its tranquilizing and blood pressure lowering properties were recognized in western medicine and the active principle, reserpine, isolated. Atropine, pilocarpine, nicotine, ephedrine, cocaine, theophylline and numerous other drugs were obtained by purifying extracts of plants alleged to have medicinal qualities.

Steroid hormones can be isolated from animal sources, but not in sufficient amounts or at low enough cost to supply the mass market. The practical advances in the therapeutic use of anti-inflammatory glucocorticoids and the introduction of oral contraceptives, rest entirely upon the systemic purification of steroids from various natural sources and their use as starting materials for new organic syntheses.

The first practical impetus to research in this area came during World War II, when a rumor circulated that Germany had perfected an adrenal cortical hormone capable of protecting airmen from the effects of high altitude. As a result, American pharmaceutical firms were requested by government to investigate the possibility of large scale production of steroid hormones. Eventually, a complicated 31-step process was developed for production of steroid hormone cortisone, from deoxycholic acid. Following a discovery in 1949 of a dramatic therapeutic effect of cortisone in patients with rheumatoid arthritis (Hench *et al.*, 1949), there began an intensive competitive search on the part of drug manufacturers for an inexpensive way of producing synthetic steroid hormones (Appelzweig, 1962). At that time, the possible starting materials consisted of cholesterol, the bile acid deoxycholic acid, the plant sterols like stigmasterol, the sapogenins like diosgenin or hederagenin or a closely related aglycone sarmogegenin.

Stigmasterol seemed to be a promising starting material,

especially because of the double bond in its side chain, which made it easier to modify than cholesterol. But stigmasterol, too, was in relatively short supply and expensive. The situation precipitated an almost frantic search for plant sterols, that would be cheap, plentiful and readily modified in the chemical laboratory. The years 1949 and 1950 were marked by a "great Strepantus search" (Kreig, 1964), an effort to find a reputed source of sarmentogenin in a variety of strepantus species in Africa. Sarmentogenin might well have provided a satisfactory starting material, but an adequate source was never discovered.

At last, Mexican Yams (*Dioscorea*), were found to contain the sterol diosgenin, which could be converted by an economic process to progesterone (Feisar and Fieser, 1959). The final breakthrough was the discovery in 1952, to use progesterone in the synthesis of adrenal cortical hormones. This required introduction of an oxygen atom in position 11. A strain of *Rhizopus* was isolated that could carry out an enzymic hydroxylation of progesterone to 11- α -hydroxyprogesterone (Peterson et.al., 1952)

A curious by product of the developments was the accidental discovery, that the halogenated steroids were highly potent with respect to glucocorticoid activity, with relatively little mineralocorticoid action. In the conversion of 11- α -hydroxy-steroids produced by the microbial reaction to biologically active 11- β -hydroxy compounds, halogen was introduced in the 9th position. The halogenated intermediates were found to be

surprisingly potent. It was found, that 9- α -fluorohydrocortisone was more than 10 times potent as cortisol with respect to anti-inflammatory activity (Fried and Sabo, 1954). The end result was the introduction into clinical use of such fluorinated anti-inflammatory steroids as, dexamethasone, triamcinolone and fluocinolone acetonide.

Literature review of Ipomoea :

Convolvulaceae consists of about 50 genera and about 1000 species. About 400 species belong to genus *Ipomoea*. The members of this genus are either herbs or shrubs and frequently climbing. A number of species are used for their ornamental purposes. The common morning glory (*Ipomoea Purpurea*, *Ipomoea violacea*), are well known for their showy flowers of different colours. One of the most useful species of this genus, *Ipomoea batatas* (sweet potato vine) is cultivated on account of the edible tuberous roots. Almost all the species contain resin, which have purgative action.

Ipomoea purga, contains glycosidal resin (8-12%). 10% of the resin is ether soluble and is identical commonly of *Ipomoea* resin, while the 90% is ether insoluble and is called as jalap resin or convolvulin. The rhizome enters in the purgative mixtures (Callis, 1953). The resin of *Ipomoea Orizabensis* contains 6% fatty substances. 64.6% is not similar to jalap resin, which on alkaline hydrolysis gives, ipuranol, metutyric acid, tiglic acid and product A. Product A on acid hydrolysis yields jalapinic acid and methyl jalapinate. The resin has

high purgative activity (Vallie, 1953).

Green, 1946 studied the phytochemistry of Ipomoea carnea. He showed the absence of the alkaloids. A new carbohydrate called ipomose was found, which gave positive tests for ketoses and aldoses. Extraction of leaves yielded a glycoside, whose aglycone contained anthracone. The gum on hydrolysis gave jalapin and saponins containing considerable proportions of sapotoxin. Two toxic principles were indicated but not identified, one water soluble caused haemolysis, when given intravenously, but not when given orally. It reduced the blood pressure. The other was soluble in ether, caused depression of central nervous system and cardiac regulatory centre when given intravenously. Given orally it acted only as a mild purgative.

Altman, 1956 investigated few plants for steroidal saponins. He found that, Ipomoea fistulosa contains 0.29% and Ipomoea dioscorea contains 1.65% of steroidal saponin on dry base percentage.

Chaudhary et.al., 1957 showed that the seeds of Ipomoea palnata, contained 11.5% oil containing palmitic, stearic, arachidic, behenic, oleic, linolic acids and β -sitosterol. They extracted the oil free alcoholic extract with benzene. From the benzene extract curicatin A and curicatin B were isolated. They also studied the pharmacological activities of this drug. Alcoholic extract of seeds, its fixed oil and curicatin A were given in albino rats. The extract in dose of 2 mg/kg

showed mild purgative action while, muricatin A showed marked purgative action in dose of 0.5 mg/kg. The fixed oil did not show any purgative action. Muricatin A, showed hypotensive activity in the doses of 20-40 mg/kg intravenously while, muricatin B did not show any activity.

Sinha, 1953 showed that seeds of Ipomoea caerulea contained 16% of yellow oil. The oil contained palmitic, arachidic, oleic, linoleic and linolenic acid. β - sitosterol was obtained from the unsaponifiable matter. The defatted residue yielded mucilage, which on acid hydrolysis gave xylose, arabinose and galactose.

In further studies Sinha, 1960 studied a sterol from seeds of Ipomoea caerulea. The seeds were extracted exhaustively with petroleum ether, the oil obtained was hydrolysed with alcoholic potassium hydroxide (20%) under reflux. The unsaponified matter was chromatographed over Brockman alumina, using benzene : chloroform (3:2) as an eluent. The sterol was isolated having melting point 135° , $(\alpha)_D^{25} = -37$ in chloroform. The molecular weight (414), established the molecular formula as $C_{29}H_{50}O$. The sterol acetate, benzoate, digitonide, acetate-dibromide and dinitrobenzoate were prepared and their melting points and specific rotations were shown as follows, 125° , $(\alpha)_D^{25} = 41.5$; 144° , $(\alpha)_D^{25} = 14.5$; 221° , 116° and 201° respectively. The sterol was identified as β -sitosterol.

Shellard, 1961 described the similarity of Morronia tuberosa and oporeculina morrocarnae resin with veraecus resin.

Akacawa, 1960 isolated ipomeamarone from fungus affected sweet potato roots (Ipomoea batatas), by silica gel column chromatography, using chloroform : ethyl acetate as an eluent. He also studied the physical constants of ipomeamarone.

Tabor et.al., 1963 found the presence of clavine and lysergic acid alkaloids in the seeds of morning glory. Ergine, isocergine, ergometrine, ergometrinine, olivoclavine, penniclavine and chanoclavine were identified by using thin layer and paper chromatography.

Carmen et.al., 1967 found that alcoholic and ethyl acetate extracts of Ipomoea carnea, showed three spots on thin layer and paper chromatography. The spots were treated with Van Urk's reagent. The Rf values of 2 spots coincided with that of lysergic acid amide and isolysergic acid amide.

Gourley et.al., 1969 isolated ipalbine the hexahydro-indolizine alkaloid, melting point 118° , (α) D^{23} 32.5 (ethyl alcohol) and ipalbidine, melting point $147-3^{\circ}$ from the basic extract of seeds of Ipomoea alba.

Lacoutat, 1970 described a volatile fraction from Ipomoea pes-caprae, which antagonised the contractile effect of histamine on guinea pig ileum as compared to benadryl and antazoline methanesulphonate. The fraction also antagonised the contractile effect of jelly fish toxin as effectively as did these antihistamines.

Nikolin and Nikolin, 1971 identified the clavine and ergot

alkaloids in the seeds of 12 samples of morning glory. The identification was done by thin layer chromatography on silica gel plates using, chloroform : methanol (17:3), and detecting with Van Urk's reagent. They also determined the alkaloid content in the seeds spectrophotometrically after extraction with the Van Urk's reagent. They showed the alkaloid content in the following species as follows;

<u><i>Ipomoea purpurea</i></u>	0.008 - 0.015
<u><i>Ipomoea albonia</i></u>	0.0055
<u><i>Ipomoea bayerianna</i></u>	0.0035

Clavine alkaloids were detected in many species of *Ipomoea*. Tuttobello et.al., 1971 extracted the clavine alkaloids with chloroform at alkaline pH and the detection was done by thin layer chromatography on silica gel.

Nikolin and Nikolin, 1972 separated the ergot and clavine alkaloids by gel filtration from seed extract of *Ipomoea violacea*. They separated the alkaloids, ergosclavine, ergometrine, ergometrinine, penniclavine and lysergic acid amide on Sephadex LH-20 and Sephadex G-25, using 96% ethanol, acetone, N-N-dimethyl formamide and water as eluents.

Isolation of muristerone A, and it's structure determination from the chemical and spectral data has been carried out by Canonica et.al., 1972.

Stanescu et.al., 1973 carried out a qualitative chemical

study on the seeds of *Ipomoea violacea* and showed that, the seeds contained glycerides, phytosterols, saponins, sterols, free amino acids, indole alkaloids, tannins, starch and pectins. The saponin fraction was formed by 5 saponosides belonging to two saponinic aglycone with sterol structure. In alkaloid fraction chanoclavine, olivoclavine, peniclavine and isolysergic acid amide were identified chromatographically.

Singh et.al., 1973 treated the convolvulin (the ether insoluble portion of purified jalap resin), with sodium methoxide in methanol, which yielded a new β -D-quinovoside from *Ipomoea purga*.

Serin et.al., 1973 isolated a new glycoside ipolecoroside from *Ipomoea leari*, with anticancer activity.

Rubmani et.al., 1974 studied *Ipomoea fistulosa* for its chemical constituents. They isolated norellin ($C_{56}H_{112}O_2$) 1-triacontanol ($C_{30}H_{62}O_2$), tritri acetane ($C_{33}H_{68}$), and β -sitosterol ($C_{29}H_{50}O$).

Villalobos et.al., 1974 described the presence of acetylcholine, from leaves, stem and flowers of *Ipomoea abutiloides*, and an acetylcholine hydrolysing enzyme from the seeds.

Der Hadergjian et.al., 1974 isolated 39 ergoline alkaloids from *Ipomoea mulleria*. Twenty alkaloids were identified by combined column and thin layer chromatography. Setoclavine, isopomni clavine, α -dihydrolysergol, isopomniclavine and

molliclavine were found for the first time in the genus *Ipomoea*.

Indole alkaloids were detected by Banerjee and Bhatnagar, 1974 in the seeds of 16 *Ipomoea* species. Nine of these species contained ergoline and clavine alkaloids.

C. Review of estrogenic plants and their active principles

Historically a number of plants have been used as sex hormones in native medicine. The pomegranate *Punica granatum* (F. Punicaceae), was a symbol of immortality, fertility and love in oriental religions and its legendary powers date back to Greek and Judeo-Christian mythology. In Egypt, the pollen grains of the date palm, *Phoenix dactylifera* (F. Palmae), were used to induce fertility in women.

Phytochemical interest in plant estrogens however, remained relatively dormant until the 1950. The increased interest in phytoestrogens was, according to Biggers, 1959 due to

- 1) The recognition that infertility in animals and humans could follow excessive ingestion of plants rich in compounds possessing estrogenic activity.
- 2) The known existence of "spring flush" (increased yield of improved milk) in dairy cattle ingesting certain rapidly growing grasses that contained estrogenic substances.
- 3) The possibility of improvement in carcass quality by the feeding of plants rich in estrogens.

- 4) The possibility of obtaining the estrogenic substances economically from plant sources.

The occurrence of substances in plants capable of inducing animal estrus was first demonstrated by Dohrn et.al., 1926. The first isolation of an estrogen from plants was reported by Butenandt and Jacobi in 1933, when they succeeded in isolating 13 mg of estrone from 50 kg of a botanically unspecified palm kernel press cake. In the same year Skarzynski reported the isolation of 7.5 mg of estriol from 65 kg of female willow catkins. Thirty years elapsed before any concerted efforts were made in the phytochemical investigation of these compounds.

Jacobsohn et.al., 1965 raised the question as to the validity of claims for the presence of estrogens in plants, when they failed to isolate estrone, from any of four geographic varieties of the African oil palm, Elaeis guineensis (F. palmas). Elaeis guineensis may or may not have been the plant investigated by Butenandt and Jacobi in 1933, although by deductive reasoning Bradbury and White, 1954 thought that it certainly must have been.

The question of the presence of estrogenic hormones in plants raised by Jacobsohn et.al., 1965 has been answered by work since 1965. The date palm, Phoenix dactylifera (F. Palmas), was demonstrated to contain estrone by TLD. Subsequently, the compound was isolated by Heftmann et.al., 1966.

Estrone has been isolated from number of plants e.g. Avena sativa, Oryza sativa, Triticum aestivum (P. Gramineae) by Pellner, 1925. Elaei guineensis, Hypbaene thebaica, Phoenix dactifera Var. saman (P. Palmae), Punica granatum Var. nana. (P. Punicaceae), Malus sylvestris (P. Rosaceae), Salix caprea (P. Salicaceae), Cleistanthus bruguieri (P. Sterculiaceae).

Estriol has been isolated from Glycyrrhiza glabra (P. Leguminosae) Costello and Lynn, 1950 Kopcevicz, 1971 has isolated estriol and 17- α -estriol from Phaseolus vulgaris (P. Leguminosae).

Biological and chemical interest in nonsteroidal estrogenic substances was spurred by the work of Bennetts and his coworkers, who reported in 1946 that the serious decline in lambing rates in Australia was due to their grazing on Dalganup strain of subterranean clover (Trifolium subterraneum) and the subsequent isolation of the isoflavone, genistein from this plant (Biggers and Curnow, 1954). They also showed that this compound has estrogenic activity. Since then, other isoflavones having estrogenic activity have been isolated. Farnsworth et.al., 1975 have listed many such isoflavonoids which have been reported by various authors to possess estrogenic activity.

In addition to the steroids and isoflavones, another group of chemical compounds found in forage plants that have estrogenic activity are the coumestans. The best known

compound of this series is coumestrol. Its isolation and estrogenic activity were first reported by Bickoff et.al., 1957. They isolated coumestrol from ladino clover, which is 1000 times less potent as estradiol. Coumestan derivatives have been isolated from various plant species by various workers. Farnsworth et.al. 1975 have listed many such compounds from their respective plant species.

Synthetic stilbene derivative, diethylstilbestrol is a prototype of nonsteroidal estrogenic hormones. Rhaponticin is the only naturally occurring stilbene derivative which possesses estrogenic activity. The activity was demonstrated by Knorr et.al., 1956.

d. Biological activities of estrogens

The estrogens are largely responsible for the changes that take place at puberty in girls; and they go a long way toward accounting for the intangible attribute called femininity. The development of reproductive tract in the female and the development of female secondary sexual characters, such as breast, body contour, hair, skin and voice. They are also responsible for the psychological and emotional set up, typical of femininity.

Reproductive tract

a) Ovary :

Estrogens enhance the response of ovary to both endogenous as well as exogenous gonadotrophins. The region of the median eminence of hypothalamus is stimulated by estrogens, which is responsible for releasing the Gonadotrophin-releasing factor (GRF). The GRF is then carried via the hypophyseal portal system to the pars distalis where it influences the synthesis and release of gonadal-stimulating substances. The gonadotrophin is released via the anterior hypothalamic-preoptic-suprachiasmatic region thereby bringing about cyclic changes in ovarian-uterine function.

b) Cyiduct :

Estrogens produce an increase in muscle and mucosal growth. More recent studies show that estrogens increase ciliary and tubal motility. They also cause an increase in pH,

sodium content and a decrease in lactate, glucose, and pyruvate. This is associated with increase in the number of ribosomes and in the alkaline phosphatase activity.

c) Uterus :

The principle effect of estrogens upon the uterus is growth. Astwood, 1933 showed that administration of estrogen to immature or castrate rodent is associated at first with a striking uptake of water followed by an increase in dry weight of the uterus. Imbibition of water occurs as a result of vasodilation as well as by a change in the capillary permeability. Estrogens induce a release of histamine which alters the permeability of the capillaries. Estrogens induce a hyperemia of the uterus with an associated increase in secretion of luminal fluid. The sudden increase in fluid distends the lumen of the uterus and this forces the increased amount of fluid into the intracellular areas.

The change in dry weight is due to an increased rate of synthesis of protein and nucleic acids by both the endometrium and myometrium. Estrogens activate the enzymes involved in the synthesis protein as well as fatty acids, cholesterol and in the formation of adenosine monophosphate. Ribonucleoproteins located in the cytoplasm of the glandular elements are increased. The glands proliferate as straight tubule. Alkaline phosphatase content increase with slight increase in uterine glycogen.

Estrogens enhance or increase the motility of the uterus

of the rhodent. The increased contractibility is accentuated by the addition of posterior pituitary pitocin and is depressed by the progestins.

d) Cervix :

Estrogens increase the tortuosity of the cervical glands. There is an enhancement of the luminescence of the epithelium. The pH of the cervical area is increased to 5.2. The physical properties of cervical mucus are changed by a reduction of the free simple sugars, glycoproteins, aminoacids, lipids and cholesterol. The sodium chloride, ascorbic acid and glucose content of the cervical cells characteristically increases.

e) Vagina :

Estrogens primarily affect the epithelial layers of the mucosal lining of the vagina and cause an increase in number of mitotic figures in the cells of the basal layers, followed by increase in cell thickness. In addition, the number of epithelial papillae along the basement membrane is also increased. Estrogens cause decrease in numbers of leucocytes, and the decrease in the pH due to degradation of glycogen to lactic acid by Doderlein bacillus.

Breast :

Estrogens not only cause duct growth but also enhance the development of the lobule-alveolar system. High doses of estrogens are effective in preventing postpartum lactation due to direct pituitary inhibition by the action of steroid

on the hypophysis or via hypothalamus. Estrogens cause increased pigmentation of the areolae and accentuate Montgomery's tubercles.

Endocrine glands

a) Pituitary gland :

Estrogen acts in a feed back mechanism, influencing the production of follicle stimulating hormone (FSH) from the pituitary gland. The FSH in turn promotes the development of the immature ovarian follicles, which increases the production of estrogen from the ovary. In large amounts, estrogens inhibit the production of FSH and stimulate the production of luteinizing hormone (LH) from the pituitary.

b) Thyroid gland :

Administration of estrogens to the human cause an increase in the plasma-protein-bound-iodine level without significant change in the radioactive iodine uptake (Selye, 1942). Estrogens cause enlargement of thyroid at puberty, menstruation and during pregnancy (Neumann, 1937).

c) Adrenal glands :

Estrogens have no known effect upon adrenals.

Cardiovascular system

a) Blood lipids :

Estrogens apparently diminish blood cholesterol and phospholipids if these are elevated.

b) Blood vessels :

Estrogens influence particularly the capillary bed of skin due to relaxation of the arteriolar tone. This is followed by increased blood flow and a definite rise in temperature.

c) Skin and Hair :

Estrogens increase the amount of elastic elements in the skin. Estrogens do not increase sebaceous activity. Administration of estrogens to patients with acne, diminish the severity of acne.

Estrogens may actually enhance hair growth on the scalp where hair loss have taken place because of increased androgenic activity.

d) Bone :

Estrogens cause calcium deposition and epiphyseal closure.

Therapeutic uses of estrogens :

Amenorrhoea:

In amenorrhoea due to pituitary failure ideal treatment would be to use gonadotropins. However, estrogens can be used to produce cyclic withdrawal bleeding in both primary and secondary amenorrhoea. Usually 0.05 mg of ethinyl oestradiol is given daily for 24 days with addition of an oral progestin during the last 8-10 days of estrogen therapy. This is followed by 'withdrawal bleeding' within 3-5 days.

Therapy is restarted five days after the onset of bleeding. Such therapy may be necessary almost for a life time in patients with marked ovarian failure.

Estrogens used at the appropriate time, in patients with prepuberal ovarian failure, bring about the growth, the genital development and secondary sex characters in almost normal fashion.

Menopausal syndrom :

As the age advances, the ovarian function decreases resulting in menopause. The menstrual irregularity is observed just prior to the menopause is due to anovulatory cycles which can be treated by cyclic administration of progestins. Occurrence of menopause is sometimes followed by symptoms like hot flashes, muscle cramps, anxiety, insomnia, vertigo, myalgias and arthralgias. Fainting and syncope can also occur. Estrogens are specific in relieving these symptoms. Diethyl stilbestrol 0.5-2 mg or ethinyl estradiol 0.02-0.05 mg daily for 4 weeks, followed by no treatment for one week, is usually recommended. Estrogens also help to clear senile vaginitis and Kraurosis vulvae, a condition associated with itching and infection of the vulva.

Vulvo-vaginitis in children :

In this condition, the infection persists because the vaginal pH at this age is alkaline, and the epithelium is columnar. Following estrogen therapy the vaginal epithelium cornifies with deposition of glycogen. Glycogen is converted

into lactic acid by the Doderlein bacilli, resulting into acidic pH, which prevents growth of bacteria. Ethinyl estradiol in doses of 0.02-0.05 mg. daily for 6-7 days along with proper antibiotic is usually recommended. The combination can also be used topically.

Functional uterine bleeding :

Usually oral progestins are preferred in this condition. However, estrogens are useful when a rapid haemostatic effect is desired. Large doses of estrogens, 4-5 times of the therapeutic dose, are necessary.

Functional dysmenorrhea :

Lower abdominal discomfort and pain before or during menstruation, when this condition is severe and incapacitating, menstruation can be rendered painless by inhibiting ovulation either by estrogen alone, given orally daily from 5-25 day of the cycle or by giving estrogen-progestin combination.

Osteoporosis :

This condition is characterized by thinning and weakening of the bones. It is usually seen in postmenopausal women. The most effective treatment of this condition is believed to be the increase in daily calcium uptake to 2-2.5 gm. Estrogens and Vitamin D in therapeutic doses may be added.

Oral contraceptives :

Estrogen alone can inhibit ovulation, but is not suitable as it causes profuse irregular bleeding. However, estrogen-

progestin combination increases the certainty of contraceptive effect and prevents the break-through bleeding.

Prostatic cancer :

Carcinoma of the prostate is probably androgen dependent and beneficial effects are obtained by administration of oestrogens. Diethylstilbestrol is given 5 mg thrice daily for several days and then reduced to 1 mg. thrice daily.

Estrogens are also useful in the treatment of carcinoma of the breast, in males and in postmenopausal females.

Acne and hirsutism :

Estrogens are sometimes used to control excessive acne in both sexes. They probably act because of their antiandrogenic action. However, their serious side effects should be borne in mind before prescribing these drugs in males.

Estrogens can also be used to treat hirsutism in females, where no endocrine cause is detected. Usually, diethylstilbestrol in doses of 2 mg. daily is given in cyclic fashion over a long period.

Miscellaneous uses :

Estrogens are sometimes used to suppress lactation and relieve the engorgement of the breast. Stilbestrol in doses of 5 mg. thrice daily for 5 days followed by 5 mg. daily for 5 days is usually recommended.

Estrogens have been used to reduce blood cholesterol levels in patients with coronary insufficiency. They are also used in the treatment of orchitis in mumps.

a) Review of Literature of I. digitata

The species Ipomoea digitata is perennial herb with large ovoid or elongated tuberous roots. The stems are long, thick twining tough and glabrous. This plant is mentioned by the early Sanskrit writers on medicine under the names of Vidari and Bhumi-bhuchanda. In the Nighantas it bears numerous synonyms, such as Payas-vini, "abounding in milk"; Vrikshavalli, "tree-creeper"; Ikshu-valli and Kohira-vidari.

The large tuberous root is considered as a tonic, alterative, aphrodisiac, demulcent, and lactagogue. In the emaciation of children with debility, and want of digestive power, the following diet is recommended: "Take of Vidari, wheat flour and barley equal parts, and make into a confection with milk, clarified butter, sugar and honey". Susruta gives several prescriptions for its use as an aphrodisiac. The simplest one is as follows: "Macerate the powder of the root in its own juice, and administer with honey and clarified butter. Vidari enters into the composition of several diuretic and demulcent mixtures".

In konkan the root is peeled and cut in small pieces and dried in the shed, it is then powdered and the powder repeatedly moistened with the juice of the fresh root and dried. Half a tola of this preparation may be taken daily in honey or milk as an aphrodisiac. From this powder a Pauchtik is made by frying it in butter with equal parts of almonds, quince seeds, cloves, cardamoms, nutmegs, catuwar, and

gokharoo, seed of Lacuna pruriens, small and making the whole into a conserve with sugar. This conserve is taken dissolved in milk in doses of half a tola or more, as an aphrodisiac. In gonorrhoea the juice is given with cumin and sugar, and as a lactagogue it is combined with coriander and Fenugreek.

Rheede says :- "Radix in oleo coccata, trita, in pulvorem redacta, cum saccharo et butyro decocta et assumpta, mollescent fortius reddere et obesem; sed et immodicum mendum potit fluxum, et in febribus osium confort".

(Dymock,

Pharmacographia Indica.

Part II

pp. 334-5.

Aurveda, states that the root is sweet, cooling, tonic, aphrodisiac, galactagogue, diuretic, stimulant, alterative; useful in leprosy, burning sensation, vomiting, diseases of blood; improves the voice and complexion;

Charaka and Sushruta recommend the drug as a remedy for snake and scorpion bites.

Yunani system mentions the root sweetish bitter; heating and dry; carminative, expectorant, anthelmintic, stomachic, appetitor; useful in syphilis, gonorrhoea, and inflammations. The leaves enrich the blood.

Elotra and Dutta, 1962 studied the preliminary pharmacology of Ipomoea digitata. They carried out investigation

on ether insoluble and alcohol soluble part designated as fraction D. The fraction gave positive tests for monosaccharides and negative tests for alkaloids. Tewari et.al., 1964, isolated a neutral compound of melting point 72°C, D - citosterol, a pale yellow fixed oil, a glycoside, free reducing sugars and mucilage. D - citosterol was obtained by chromatographing the alcoholic extract on alumina using petroleum ether: benzene (3:2). The glycoside was separated by extracting petroleum ether extract residue with ether. The ether extract was dissolved in alcohol and evaporated. The residue was extracted with boiling water. The aqueous solution was evaporated under reduced pressure to dryness and the product was crystallised from alcohol.

The ether soluble fraction possessed hypotensive and muscle relaxant activity. On injection it slowed heart (frog) beat. The average blood pressure of dogs was decreased by 15-20 mm of mercury. The amplitude of contraction of rabbit jejunum was decreased. It also caused relaxation of rat uterus.

Matin et.al., 1969, studied the pharmacological effects of paniculatin, a glycoside isolated from Ipomoea digitata. They extracted the tubers with 95% ethanol. After recovery of solvent and treatment of the residue with ether, a glycoside named paniculatin having melting point 134°C was isolated. The glycoside elevated the blood pressure and showed a stimulant action on myocardium and respiration. It also showed vasoconstrictor and bronchoconstrictor

activities. A spasmogenic effect was observed on smooth muscles of gut. It also showed oxytocic activity. The LD_{50} (48 hours) was found to be 867.4 mg/kg intraperitoneal in mice.

Ratin et.al., 1969, studied the pharmacodynamic actions of an other soluble compound after the isolation of glycoside. The extract produced hypotensive effect in anaesthetised dogs, which was not modified by adrenergic, cholinergic, ganglionic blockade, antihistaminic drugs, spinal transection or bilateral cervical vagotomy. The extract produced relaxant effect on blood vessels and other smooth muscles of guinea pigs, rats and frogs.

b) Review of separation of steroids

Accounts of the early history of chromatography are given by Coll and Williams, 1950; Ferradene, 1951; and Zechmeister, 1951. Russian botanist Tswett was the first to be aware of the great possibilities of chromatography. He described in detail the separation of pigments by filtration through columns, followed by development with pure solvents.

The first paper of Tswett, published in 1903, contains a study of more than 100 adsorbents used with several different solvents and a comparison of the efficiency of column and batch adsorption. The chromatography of a leaf extract on a column of inulin is described as follows.

"The adsorption phenomena observed during filtration through the powder are particularly interesting. The liquid emerging from the lower end of the funnel is at first colourless, then yellow whilst at the top of the column of inulin a green ring is formed below which there soon appears a yellow band. When pure ligroin is filtered through the column, both bands start spreading and move down the column".

Further descriptions of chromatographic techniques are contained in a paper by Tswett in 1906 and in his book 'The chromophyllus in the plant and animal world'. Tswett died prematurely in 1920, and his method was used only very rarely during the following years.

It was only in 1931, when Kuhn and Lederer separated the carotenes and xanthophylls on a preparative scale on

columns of alumina and calcium carbonate, that the possibility of Tswett's method were fully realized. Rapid development of the new field by workers such as Brockmann, Karrer, Winterstein, Seelmeister, etc; soon enabled the organic chemist to apply this technique to the separation and isolation of a wide variety of compounds.

In 1933, Reichstein introduced the liquid or flowing chromatogram, thus extending the applicability of the method to colourless substances. During the years 1940-1943, Pictet worked out the techniques of frontal analysis and displacement development.

Partition chromatography on silica gel, introduced by Martin and Synge in 1941, further extended the scope of chromatography to a great range of biologically important substances. Paper chromatography was first described Gonadon, Gordon and Martin in 1944 and has become a major tool for biochemical analysis and research.

The development of gas-liquid chromatography (GLC), by James and Martin in 1952, has opened a new field in analytical chemistry which promises to have wide application, both in research and industry.

Adsorption Column Chromatography :

This technique is particularly well suited for preparative purpose. The mixture, dissolved in a suitable solvent is passed down a vertical column. If the constituents of the mixture have different adsorption co-efficients, the individual substances will separate according to their varying mobilities.

The structural and stereoisomerism of steroids does not give rise to any great difference between the separating powers of adsorption chromatography. Even of strongly polar steroids are easily separated on weak adsorbents.

Adsorbents : The most suitable adsorbents for chromatography of steroids are hydrophilic materials like alumina, magnesium silicate, silica gel, silicic acid, kieselguhr, magnesium carbonate, aluminium silicate or hydrophobic charcoal. The activity of adsorbents decreases in the above order. A finer gradation of properties can be achieved by mixing adsorbents with different activities or especially by stepwise activation or deactivation of an adsorbent. Activation of hydrophilic adsorbents is usually achieved by heating (removal of water) and deactivation by the addition of definite quantities of water. Smaller the particle size of adsorbent better is the adsorption.

In selecting a suitable adsorbent one has to consider the polarity and lability of the steroids being separated. On hydrophilic adsorbents highly saturated compounds and

compounds containing more polar groups are strongly adsorbed. Therefore weakly-polar compounds are chromatographed on strong adsorbents- and strongly polar compounds on weak adsorbents. On hydrophobic adsorbents, weakly-polar compounds are strongly adsorbed and the strongly polar ones are most weakly adsorbed.

When silica gel is used as an adsorbent, the chromatographic behavior on the column can be worked out from TLC layer. Duncan, 1962 has applied the relationship, $r = a/(b + 0.1 a)$, where a is the R_f - value of faster migrating compound, b is the R_f - value of slower migrating compound. If $r \geq 1$, separation is possible in column also, provided that the ratio of silica gel to substance is 500 - 1,000 and the flow rate of eluent sufficiently small (20-40 ml/hour). On the other hand, the properties of alumina used for column chromatography and that for TLC, vary so much that similar conclusions about conditions in the column can not be drawn from the results on TLC layers.

Disadvantages of adsorbents

Alumina : Activated and alumina for certain purposes is alkaline and causes various alterations in structures of the compounds to be chromatographed on it.

- 1) 17-Hydroxy-20-ketones, especially those of the 17 α - progesterone series can be rearranged to 9-ketosterones.
- 2) $\Delta^5 - 3$ - ketones are rearranged to $\Delta^4 - 3$ - ketones.
- 3) Free hydroxyketones (α -ketols) are partly decomposed even by alkali free alumina.

- 4) Breakage of the lactone ring of cardenolides causing loss of biological activity.
- 5) Splitting of acetic acid from cardenolides.
- 6) Replacement of bromine by hydroxyl group in allyl compounds.
- 7) Dehydration of steroid cyanhydrins, depending upon the kind of adsorbent used.
- 8) Saponification of steroid benzoates have been observed on alumina.

Silica gel : Side reactions have hardly been proved to occur on more weakly active adsorbents like silica gel.

- 1) Isomerisation of steroids have been reported by Trappe, 1943 on silica gel in halogen containing solvents.
- 2) Hutton and Mason, 1956 have recently reported the destruction of α -ketols on silica gel.

Separation of estrogens : Adsorption chromatography on alumina has been used relatively seldom. Keller, 1953 used alumina for a rough fractionation of urinary estrogens into estrone, estradiol and estrial fractions. Brown, 1955 has separated the methyl ethers of estrone and estradiol on alumina with petroleum ether: benzene (6:4) as an eluent. Glumshits and Sandberg, 1956 fractionated a mixture of estrogens on florisil with benzene: acetone. Mori, 1959 has described separation of estrial, estradiol and estrone, by non ionic adsorption on Amberlite IRC-50.

ii) Partition Chromatography :

Martin and Synge, 1941 introduced the method of partition

chromatography, which is based on counter-current distribution method of Craig. The distribution co-efficient of a substance is more independent of its concentration within wider limits than the adsorption coefficient. Particular advantage of partition chromatography lies in the fact that the solvent systems can be varied over a wide range and hence adapted to the most varied requirements. Two phases of limited miscibility and a hydrophilic supporting medium is chosen for the purpose. The supporting medium binds the more strongly polar phase, rendering it stationary. The less polar phase is mobile and passes from top to bottom. It is sometimes possible to constitute stationary and mobile phases out of two completely miscible solvents, if one is preferentially bound to the solid support.

In counter-current distribution the best results are achieved by collecting the phases such that at equal volumes the average distribution coefficient of the sample is approximately 1. In column the solvent system must be so chosen that the distribution coefficient

$$\frac{\text{concentration in mobile phase}}{\text{concentration in stationary phase}}$$

lies between 0.2 and 0.8. Another possibility is to make the supporting medium hydrophobic, whereupon the nonpolar solvent becomes the stationary phase, and the polar solvent becomes the mobile phase. This procedure with reversed phase, is particularly useful if no common solvent system can be found for a particular substance which gives a

distribution coefficient of approximately 0.1.

Supporting media : Neutral, porous, finely-grained materials which adsorb only slightly or not at all, are suitable media for stationary phase. Silica gel, kieselguhr and cellulose have proved especially suitable; various silicates (diatomaceous earth), florisil are also employed occasionally. The particle size of the supporting media should be 100-500 mesh, in order to permit the fluid to run freely, but without the formation of channels.

In reversed phase chromatography hydrophobic kieselguhr or in some cases hydrophobic or acetylated cellulose, or rubber powder is used as a supporting medium.

Separation of estrone : Wyer and Braunschweig, 1951; Stern and Wyer, 1952; Bitman and Sykes, 1953 separated estradiol from estrone using celite-545 as a supporting medium, 2.5 N. sodium hydroxide as a stationary phase and benzene as a mobile phase. They further showed that estradiol can also be eluted if carbon dioxide is passed through the column.

Macmillan et al., 1953 separated estradiol, α -dihydroequilin and dihydroequilenin using celite-545 as a supporting medium, 0.4 N sodium hydroxide as a stationary phase and benzene as a mobile phase. Estrone, estradiol and estradiol were fractionated on celite-545, using 3.1 N sodium hydroxide as a stationary phase and benzene: petroleum ether (4:1) and chloroform: butanol (17:3) as mobile phases (Braunschweig et al., 1954).

Rye et.al., 1951 showed that, estradiol can be separated on rubber powder using benzene as a stationary phase and methanol: water (2:3) as a mobile phase. Later on Beech, 1955 separated estrone on rubber powder, with benzene and methanol: water (6:4), as stationary and mobile phases respectively. Bauld, 1955 used 0.5 N sodium hydroxide as a stationary phase and benzene as a mobile phase on celite-555, for the separation of estrone; separation of estradiol was achieved by changing the mobile phase to benzene: ethylene chloride (1:3). Harrison and Bauld, 1955 isolated estradiol using celite 555 as a supporting medium, methanol: water (7:3) and ethylene chloride as stationary and mobile phases respectively.

(iii) Thin layer chromatography (TLC):

The special advantages of thin layer chromatography are, versatility, speed and sensitivity. Versatility is due to the fact that number of adsorbents may be spread onto a glass plate or other support and employed for chromatography. Although silica gel is most widely used, aluminium oxide, celite, calcium hydroxide, magnesium phosphate, polyamide, cephalex, polyvinylpyrrolidone, cellulose and mixture of two or more of the above materials is used for TLC. The speed of the TLC is due to the more compact nature of the adsorbent, when spread on a plate and is of advantage when working with labile compounds. Sensitivity of TLC is such that separations can be achieved on ug amounts of the material.

For preparation of plates for TLC, the glass plates are cleaned with acetone to remove grease. The slurry of silica

gel (or other adsorbent) is shaken vigorously and spread on glass plates by hand or by spreader. Finally, the plates are air dried and then activated by heating in an oven at 100-110°C for 30 minutes or as required. In some separations, the properties of adsorbent are modified by addition of an inorganic salt (silver nitrate or sodium chloride), when the plate is being spread.

The solution of a mixture of compounds to be separated is applied as a spot with the help of micro bulb pipettes, graduated micropipettes or by micro syringes. The sample may be applied in form of a band. After drying of the spot or band of the sample on the plates the plates are developed with the solvent or a mixture of solvents.

A wide range of solvents can be applied to TLC. The plates may be developed ascendingly, descendingly, horizontally or in some cases the plates are developed two dimensionally. In two dimensional development the plate is developed in two directions, the other is perpendicular to the first direction. The development is carried out in a tank, which is paper-lined so that the atmosphere inside is saturated with the solvent phase.

Detection of compounds on TLC plates is normally carried out by spraying the plates with a specific reagent. The chromatograms are viewed in short wave UV light, numerous compounds absorb UV light of particular wavelength and some of them emit it as a visible light. This phenomenon, termed as fluorescence or luminescence, is a very sensitive means of

identifying small amount of substances on a chromatogram. Compounds are also detected either by adding a fluorescent dye to the slurry when the plate is being prepared or also by spraying the developed plate with a similar dye solution.

Preparative TLC is carried out by using thick layers of adsorbent. The separated constituents are recovered by scraping off the adsorbent at the appropriate places on the developed plates, eluting the powder with a solvent and finally centrifuging to remove the adsorbent.

Separation of estrogens : Linbo and Diesfeldt, 1962 have carried out the extensive investigation on the separation and characterization of estrogenic steroids. Diamantstein and Lercher, 1962; Takuchi, 1963; and Starke, 1965 have reported further systematic studies on estrogen separations.

Hexane : ethanol (90:10); Cyclohexane : ethyl acetate : ethanol (45:45:10); and methylene dichloride : ethanol (82.5:7.5) are suitable for the separation of mixtures of estrone, estrane, 17- β -estradiol and estril (16 α , 17 β). Starke, 1965 has shown that, the mixtures of carbon tetrachloride with alcohols, ethers or esters are suitable on alumina.

Luini *et al.*, 1964 claimed better separation in the horizontal HU - chamber using cyclohexane : ethyl acetate (55:65).

Hartelond and Gannon, 1963 stated that separation of four epimeric estrils from one another necessitates methylation of the phenolic hydroxyl groups and use of cyclohexane : ethyl acetate : ethanol (45:50:5) as a developing solvent system.

Lioboa and Dicafealuy, 1962 have investigated a large number of other estrogen derivatives, like 2-hydroxy-, 2-methoxy 6-7-keto, 16-keto and 6- α -hydroxy and 6- β -hydroxy metabolites. They worked out a separation scheme based on initial TLC with cyclohexane: ethyl acetate: ethanol (45:45:10), under standard conditions. A first group of 5 polar estrogens (R_f upto about 0.37) was eluted and resolved into its components by two dimensional TLC in hexane: ethyl acetate: ethanol (15:50:5). The 19 less polar estrogens from the first TLC stage (R_f between 0.37 and 0.80) were separated into 6 subgroups through two dimensional TLC in cyclohexane: ethyl acetate (50:50).

Struck, 1961; and Jacobsohn, 1964 have worked on the quantitative determination of estrogens on TLC, using spectrophotometric and photogram-densitometric methods. Numerous investigations have been carried out on the application of TLC to determine urinary estrogens. Siegel and Dorfman, 1963 replaced the column chromatography in the established method of Brown by TLC, using an added internal tritium standard. Lotis and Chatteraj, 1964 subjected the urine extract to a preliminary separation on acid washed silica gel, using benzene: ethyl acetate (50:50); the following four zones were obtained;

- a) estrone + 2-methoxy estrone;
- b) estradiol + ring D- α -ketols and 17 ketosteroids;
- c) estradiol with 16, 17-trans-glycols;
- d) estradiol with 16, 17-cis-glycols.

Jung et al., 1963, have quoted a shortened procedure with only single TLC stage on silica gel, using benzene: ether (40:60) and Eber- reaction on the eluates. Korman and Dedy, 1963 reported detection of estrogens in sesame oil preparations by TLC.

iv) Gas-liquid chromatography (GLC):

The possible development of gas-liquid chromatography was suggested by Martin and Synge in 1941, but no practical work was reported until 1952, when Martin and James described its application to separation of volatile fatty acids. Valuable pioneer work was carried out in the I.C.I. laboratories at Winnington, Dillingham and Blackley and thereafter a stream of publications began which has already reached flood level.

In GLC the separation of a mixture of volatile organic compounds is effected with a column of porous solid impregnated with a non-volatile liquid, usually referred to as a stationary phase. The column is normally in equilibrium with an inert gas flowing through it, which is known as carrier gas. If a small volume of a mixture of volatile compounds is injected into the gas stream, at a point where it enters the column, the components of the mixture will pass through the column at different rates and will emerge from it separately. As each component passes along the column it is alternately dissolved in, and vaporized from the stationary phase and its rate of travel will depend upon its own vapour pressure above its solution in the stationary phase. The gas emerging

from the column is passed through some device which detects each component of the mixture as it arrives. The detector response is recorded with a potentiometer.

The apparatus required consists of a source of carrier gas, the column, detector and recorder. In addition, valves are required to control the gas pressures at the inlet and outlet of the column, a flowmeter to measure the gas flow through the column and power supplies and regulators for the heaters.

The column consists of an intimate mixture of inert support and stationary phase packed into a tube. Diatomaceous earth is used almost exclusively as an inert support. Considerable use has been made of colite 545. A furnace insulation brick ground to the particle size 30-100 mesh (chromosorb) is also used as supporting medium.

Ideally, the stationary phase should not have vapour pressure at the operating temperature of the column. With a non-polar stationary phase the non-polar components are separated according to their order of boiling point, the polar components pass more rapidly than non-polar vapours of similar boiling point through the column. The commonly used stationary phases are:

Stationary phases	Approx operating temperature
a) Paraffin hydrocarbons	
Squalene	140
Liquid paraffin	100
b) Silicones	
Silicone oil	200
Silicone greases	300

c) Esters:		
	Dicnnyl phthalate	100
	Tricnnyl phosphate	100
	Polyglycol oleate	120
d) Glycols:		
	Polyethylene glycol	100
	Glycerol	100
	Diglycerol	120
e) Miscellaneous:		
	Dimethyl sulphoxide	40
	Diethyl formamide	0
	Ethylene glycol/silver nitrate	40

The column is heated with a thermostatic heater with temperature constant to ± 1 . A inert gas free from impurities is used as carrier gas. Most commonly used carrier gases are nitrogen, carbon dioxide, helium and hydrogen.

Sample injector is attached to a point very close to the beginning of the column. The sample is injected either in the form of liquid or gas by a hypodermic syringe into the inlet port through a rubber septum into the column.

The detector measures the compounds as they sweep through the column. The detector is frequently based on either flame ionization or electrons capture. The detector is linked with to a potentiometric recorder, which produces the results of the separation as a series of peaks of varying intensity. The area under the peaks is directly proportional to the concentrations of the components in the original mixture. The peak areas can be determined automatically by use of an electronic integrator.

The results of GLC can be expressed in terms of retention volume R_v , which is the volume of the carrier gas required to

elute a component from the column, or in terms of retention time R_t , which is the time required for elution of the sample. These parameters are nearly always expressed in terms relative to a standard compound (as R_{rt} or R_{rt}).

Many times GC is combined with MS. These involve GC techniques for separation and estimation purposes and MS techniques for identification purposes.

C) REVIEW OF THE PHYSICAL METHODS IN ORGANIC CHEMISTRY
FOR ELUCIDATION OF STRUCTURE OF A COMPOUND

It is necessary to determine the class of compound during isolation, after isolation, and purification of the compound. After confirmation of it's homogeneity i.e. single spot in several TLC and GC systems, the class of compound is generally clear from it's response to spot tests, and it's spectral characteristics. Complete identification within the class depends upon determination of melting point, optical rotation, elemental analysis, molecular formula and spectral studies such as, ultra violet and visible spectra, infra red spectrum, mass spectrum and nuclear magnetic resonance and then comparing these data with those in the literature. A known plant compound can be usually identified by comparing the TLC spot, overlapping I.R., U.V. etc. However if the authentic material is not available careful comparison with literature data, gives sufficient information for its identification. If a new compound is present, the structure is confirmed through chemical degradation or by preparing the compound by laboratory synthesis and by the knowledge of biosynthesis of the type of compounds in plants.

Elemental analysis and empirical formula:

When a compound has been purified and its molecular homogeneity assured, the first operation necessary in determining its structure is to know how many atoms of each kind are present in its molecule. This is obtained from the percentage composition of the elements present and leads to an

empirical formula. The elements commonly found in organic compounds, are carbon, hydrogen, oxygen, nitrogen, halogens, sulphur and phosphorus.

Carbon and Hydrogen : In quantitative combustion analysis, a weighed portion of a compound is burned in oxygen over hot copper oxide at about 700°C . Carbon dioxide and water are produced and are individually captured and weighed in absorption tubes. This is accomplished by first passing the combustion gases through a tube containing a neutral desiccant, such as magnesium perchlorate (anhydrous), which absorbs the water vapour and converts it to water of hydration. Then by leading them through a tube containing finely divided sodium hydroxide, which absorbs and converts carbon dioxide to sodium carbonate.

Nitrogen : If a compound contains nitrogen, Duma's analysis is frequently used to determine the percentage of this element. The substance is mixed with cupric oxide and brought to a dull-red heat, which results in complete oxidation of the organic material. The resulting gases are passed over a surface of hot copper to reduce nitrogen oxides to nitrogen. The other more reactive gases are chemically absorbed, and the residual volume of nitrogen is measured at carefully determined temperatures and pressures. The weight of nitrogen is calculated and divided by the initial sample weight to give fraction of nitrogen in the original compound.

Sulphur and Halogens : Sulphur and halogens are determined by complete destruction of the organic compound with hot fuming

nitric acid (Carius method) or sodium peroxide (Parr method). The sulphate or halide ions in the residue are precipitated and weighed as barium sulphate or silver halide.

Oxygen : The amount of oxygen in an organic compound is generally determined by difference, i.e. all other elements in the substance concerned are determined quantitatively and the sum of the percentages is subtracted from hundred. The difference gives the amount of oxygen present.

In Ter Meulen's method, oxygen is estimated by heating strongly the substance in a current of hydrogen and passing the vapour over red-hot platinised asbestos and then over finely divided nickel mounted on thorium oxide at 350°C. The oxygen is thus converted into water which is absorbed by fused calcium chloride or concentrated sulphuric acid over pumice stone.

Empirical formula : In order to convert these data into an empirical formula two steps are required.

- a) Weight of each element in the sample and its percentage composition.
- b) The relative weights in the sample are then converted to ratio numbers of atoms by dividing by the atomic weight of that atom.
- c) The ratio of numbers of atoms converted to nearest whole number ratios first by dividing each crude ratio by the lowest of them, so that one number goes to

unity; and then multiplying each by some integer so as to create integers of all ratio numbers.

Melting point :

A pure crystalline organic compound has, a definite and sharp melting point. The presence of small quantities of miscible or partially miscible, impurities usually produce a marked increase in the melting point range and cause the commencement of melting to occur at a temperature lower than the melting point of the pure substance. The melting point is therefore a valuable criterion of purity of an organic compound.

The experimental method in most common use is to heat a small amount (about 1 mg) of the substance in a capillary tube inserted into either a paraffin bath or electrically heated heater. The temperature at which the compound melts completely is recorded as the melting point. The capillary melting point tubes are prepared from soft glass tubes. One end of the capillary tube is sealed by heating in the flame. The substance is crushed to powder and the open end of the capillary tube is pushed into the powder and the powder is then shaken down the tube by tapping gently the sealed end on the bench or by gently drawing the flat side of a file along the upper end of the tube. The procedure is repeated until the length of lightly-packed material is 3-5 mm, and the outside of the tube is finally wiped clean.

Two principle types of melting point apparatus in common

use are those in which heating of the capillary tube is by means of a heated liquid bath and those in which heating is carried out in, or on, an electrically heated metal block.

Optical rotation :

Compounds which rotate the plane of polarized light around its axis, whether they are in gaseous, liquid or molten state, or in solution, are said to be optically active. This property arises from the lack of certain elements of symmetry in the molecule (i.e., a centre, a plane or an n -fold alternating axis of symmetry) with the result that the molecule and its mirror image are non-superimposable.

When a beam of monochromatic light is passed through a crystal of Iceland spar, two beams are transmitted, each vibrating in one plane which is perpendicular to the other. A Nicol prism is composed of two sections of Iceland spar cut, and again sealed with Canada balsam, that one of these rays is refracted to the side so that the light passing through the prism is vibrating in one plane only. This light is said to be plane polarized. This light is allowed to pass through another Nicol prism similarly oriented and the light viewed from a point remote from, but in line with, the light source. On rotating the second prism the field of view appears alternately light and dark and the minimum of brightness follows the maximum as the second prism is rotated through an angle of 90° . The prism by which the light is polarized is termed

as polariser and the second prism, by which the light is examined is called as analyser.

Polarimeter : In half shadow polarimeter (Lippich type), two polarised rays are produced by means of the main Nicol prism and a small prism. The later covers half the field of the main Nicol prism and its plane of polarisation is slightly inclined to that of main Nicol prism. The angles between the planes of polarisation may be altered by a slight rotation of the main Nicol prism.

Upon rotating the analyser, a position is found at which one beam is completely extinguished, while the other only partially extinguished. Therefore one half of the field of view appears dark, while the other remains light. However, when, the analyser occupies an intermediate position, the field of view appears of uniform brightness.

For determination of the optical rotation the zero position of the instrument is determined with the empty polarimeter tube. The solution to be examined is filled in the polarimeter tube free from air bubbles. The tube is placed in polarimeter, and field of even brightness is obtained by turning the analyser clockwise or anticlockwise. When the analyser has to be turned clockwise in order to get a field of uniform brightness, the optically active substance is said to be dextrorotatory or (+). In laevorotatory or (-), the analyser has to be turned anticlockwise. The reading

on circular scale (using vernier scale) is noted.

The magnitude of the optical rotation depends upon,

- 1) nature of the substance.
- 2) length of the column of liquid through which the light passes.
- 3) wavelength of the light employed.
- 4) temperature.
- 5) concentration of the optically active substance.

The specific rotation for a neat active liquid at temperature t , is given by,

$$(\alpha)_D^t = \frac{\alpha}{ld} \quad \text{where, } \alpha \text{ is the angular rotation, } l \text{ is the length of the}$$

column of liquid in decimeters and d is the density at temperature t .

The specific rotation for a solution of an optically active substance is given by,

$$(\alpha)_D^t = \frac{100\alpha}{lc} = \frac{100\alpha}{l\rho d}$$

where l is the length of the column of liquid in decimeters, c is the number of grams of substance dissolved in 100 ml. of the solution and d is the density of solution at temperature t .

Ultraviolet and visible spectra :

When a molecule absorbs ultraviolet or visible light of frequency ν or wavelength λ , an electron undergoes a transition from a lower to higher energy level in the molecule.

The energy difference ΔE is given by the expression :

$$\Delta E = h\nu = \frac{hc}{\lambda} \quad \text{where, } h \text{ is Planck's constant and}$$

c is the velocity of the radiation. Saturated molecules (and groups in molecules) are transparent to ultraviolet and visible light. In unsaturated molecules there are two major kinds of electronic transitions.

i) $\pi \longrightarrow \pi^*$ transitions; these are usually associated with the multiple bonds of carbon with carbon, nitrogen, oxygen, sulphur etc. and they generally give rise to high intensity absorption.

ii) $n \longrightarrow \pi^*$ transitions; these are usually associated with groups such as carbonyl, thiocarbonyl, nitroso, etc; and generally the intensity of absorption is very much lower than that arising from $\pi \longrightarrow \pi^*$ transitions.

In the spectra of simple molecules the absorption due to $n \longrightarrow \pi^*$ transitions lie at longer wavelengths than those arising from the $\pi \longrightarrow \pi^*$ excitations, because lower energy is required for $n \longrightarrow \pi^*$ transitions. The absorption occurs over a range of wavelengths about discernible maxima leading most frequently a broad absorption curve. This is because the spacing between the rotational and vibrational transitions of a polyatomic molecule are relatively small and electron transitions occur from range of vibrational-rotational levels in the ground state to a range of such

levels in the excited state.

Changes in the molecular environment of the absorbing electrons cause small shifts in λ_{max} , and hence in ΔE between the two states. Conjugated systems of electrons are stabilised by resonance and the stabilisation is greater in the excited state than the ground state. Conjugated electron systems are called as chromophores. Major shifts to longer wavelength occur with increased substitution on the chromophore skeleton.

Spectrophotometer : A generalised instrument for obtaining spectra is a double-beam spectrophotometer. The light source (hydrogen lamp) generates a continuum of wavelengths which are sparged by a prism (Quartz prism) and then selected by slits such that rotation of the prism causes a continuously increasing series of wavelengths to pass through the slits for recording purposes. The selected monochromatic beam is then split into two beams one for passage through the unknown sample dissolved in a solvent and the other for passage through solvent alone. The two beams are each measured for intensity by a photoelectric cell. The later is subtracted electronically from the former to give an intensity (absorbance or transmission) signal which is characteristic of compound alone. This signal is recorded automatically on a graph as a function of the wave length being utilised.

The absorption spectra can be measured in very dilute

solution against a solvent blank using an automatic recording spectrophotometer. For colourless compounds, measurements are made in the range 200 to 400 nanometers (nm); for coloured compounds, the range used is 200 to 700 nm. The value of ultraviolet and visible spectra in identifying unknown compound is related to the relative complexity of the spectrum and to the general position of the wavelength maxima. If a compound shows a single absorption band between 250 and 260 nm, it could be any one of a considerable number of compounds (e.g. a simple phenol, a purine or pyrimidine, an aromatic amino acid and so on).

Intra red spectroscopy :

The intra-red region includes radiation at wavelengths between 0.7 and 500 μm or, in wavenumbers, between $14,000\text{ cm}^{-1}$ and 20 cm^{-1} . When the energy of radiation coincides with the energy difference between the excited and ground states of molecule, then the radiant energy is absorbed by the molecule, causing the increase in its natural vibration and thus bringing a change in its electrical dipole moment. Infra-red spectroscopy involves the twisting, bending, rotating and vibrational motions in a molecule.

Stretching vibrations involve changes in the frequency of the vibration of bonded atoms along the bond axis.

a) Symmetrical stretching : In symmetrical stretching mode there is no change in the dipole moment as the two atoms

move equal distances in opposite directions from the central atom. Such vibrations are infrared inactive.

b) Asymmetrical stretching : There is a change in the dipole moment, since during these vibrations the centers of highest positive charge and negative charge, move in such a way that the electrical centre of the group is displaced from the carbon atom. These vibrations are observed in the infrared spectrum of the methylene group.

When a three-atom system is a part of a larger molecule, it is possible to have bending or deformation vibrations. These vibrations imply movement of atoms out from the bonding axis.

1) Deformation or scissoring : The two atoms connected to a central atom move toward and away from each other with deformation of valence angle.

2) Rocking or in-plane bending : The structural unit swings back and forth in the symmetry plane of the molecule.

3) Wagging or out-of-plane bending : The structural unit swings back and forth in a plane perpendicular to the molecule's symmetry plane.

4) Twisting : The structural unit rotates back and forth around the bond which joins it to the rest of the molecule.

Splitting of bending vibrations due to in-plane and out-of-plane vibrations is found with larger groups joined by a central

atom.

The intensity of an infrared absorption band is proportional to the square of the rate of change of dipole moment with respect to the displacement of atoms. The vibrations occur at specific frequencies governed by the atomic masses and strengths of chemical bonds. This can be given as,

$$\bar{\nu} = \frac{1}{2\pi c} \sqrt{\frac{K}{\mu}}$$

where, $\bar{\nu}$ = frequency of vibration; c = velocity of light; K = force constant; μ = reduced mass of atoms involved.

Infrared spectrophotometer : The double-beam infrared spectrophotometer has a single 60° Littrow-mounted rocksalt prism; which can be replaced by other prisms. The grating filters remove the scattered light. By means of combined rotating mirror and light interruptor, the source is flicked alternately between the reference and sample paths. An electric cam maintains constant energy through the spectrum. This cam is a potentiometer tapped and chunted at several intervals about its circumference to yield a voltage distribution nearly proportional to the slit widths required for uniform energy distribution. By balancing the voltage from a potentiometer coupled to the slits, against the cam voltage, a servomotor maintains the slits at the proper value. The beam from a Globar source is chopped at 13 CPS and the thermocouple signal amplified, rectified, and filtered and modulated at 60 cycles to drive the two phase rebalancing servomotor. The spectrum

is recorded directly in transmittance on the charts.

The spectra of the compounds are measured either in chloroform, or carbon tetrachloride, as a mull with furol oil or in solid state, mixed with potassium bromide as a thin disc, or a neat film of the substance.

The spectra give valuable information about the various functional groups or bands due to their individual vibrations in the molecule. The following table shows the infrared frequencies of some functional groups.

Mass spectroscopy :

When bombarded by electrons, every molecule ionizes and fragments uniquely. A molecule may simply lose an electron or it may fragment into two smaller units, an ionized fragment and neutral particle, the sum of whose masses equals their precursor. The rapidly moving ions, after being accelerated by the electrostatic slits, are diverted into circular path by a magnetic field parallel to the slits and perpendicular to the ion beam.

Ions of mass m (in gm), and charge e (in abeculombs), on passage through an accelerating electric field attain a velocity V , which is expressed in terms of the accelerating potential V (in abvolts) and the kinetic energy of the individual atom as it leaves the electric field, $\frac{1}{2}mv^2 = eV$ on solving for the velocity term,

$$V = \sqrt{\frac{2eV}{m}}$$

Equating the centripetal and centrifugal forces to which the ion beam is subjected on entering the uniform magnetic field H (in gauss)

$$\frac{mv^2}{r} = Hev.$$

Each ion follows its own circular trajectory of radius r , which is proportional to momentum and given by,

$$r = \frac{mv}{eH}$$

The radius is also expressed as,

$$r = \frac{1}{H} \sqrt{2V \frac{m}{e}}$$

Only those ions which follow the path which coincides with the arch of the analyser tube in the magnetic field are brought to a focus on the exit slit, where the detector is located. Ions of other mass/charge (m/e) ratio strike the analyser tube (grounded) at some point, are neutralised, and are pumped out of system along with all other unionised molecules and uncharged fragments. Thus, the magnetic field classifies and segregates the ions into beams, each of a different m/e , where,

$$\frac{m}{e} = \frac{H^2 r^2}{2V}$$

To obtain the mass spectrum, the accelerating voltage is varied and each m/e ion from light to heavy is successively brought to a focus on the exit slit. A magnetic analyser also focuses ions that are of same mass and velocity but of different

initial directions.

Mass spectrophotometer :

The sample is introduced into a low vacuum side of the system which is maintained at 10^{-4} to 10^{-5} mm of mercury. The molecules are bombarded with electrons emitted from a heated filament. The direction of the electron stream is controlled by a coarse magnet and the intensity regulated by the emission regulator. Un-ionized molecules are drawn out of the system by an exhaust pump. The positive ions are accelerated by a high voltage and focused by an ion lens into a sharp beam. This high-velocity ion beam passes through a narrow collimating slit into a region free from electric fields, forming a collimated beam of uniform energy. This ion beam passes through a strong magnetic field, and ions of different mass follow circular paths of different radii. The ions having a particular specific mass pass through a narrow collector slit and strike a collector plate. The collector plate current is measured, which gives an indication of abundance of the particular mass, which is recorded as a peak, on a chart paper.

The mass spectra provide an accurate molecular weight and yield a complex fragmentation pattern which is often characteristic of that particular compound.

Nuclear Magnetic Resonance spectroscopy :

Nuclear magnetic resonance spectroscopy concerns radio-frequency (rf) induced transitions between quantised energy states of magnetic nuclei that have been oriented by magnetic fields.

In addition to charge and mass, about half of the known nuclei possess spin or angular momentum. Such nuclei resemble a tiny magnet, and when placed in a powerful, uniform magnetic field, H_0 , tend to assume an allowed orientation with respect to the external field. However, the nuclei do not align perfectly parallel or antiparallel to the field. Increasing the strength of the field makes the nuclei precess faster. They can be made to undergo a transition to a higher energy level by applying a second, much weaker rf field, H_1 , at right angles to the uniform magnetic field, H_0 . When the frequency of the rotating component of this second field reaches the precession frequency, the spinning nuclei absorb energy and flip into higher energy level.

The resonance frequency, ν , that effects transitions between energy levels is derived by equating the planck quantum of energy with the energy of reorientation of a magnetic dipole.

$$\Delta E = h\nu = \frac{\mu H_0}{I} \quad \text{where, } I = \text{spin quantum number in}$$

units of $h/2$; h = Planck's constant; E = energy.

There will be $2I + 1$ possible orientations and corresponding energy levels. Nuclei with $I = \frac{1}{2}$ give the best resolved spectra because their electric quadrupole moment is zero. These nuclei include ^1H , ^{13}C , ^{19}F and ^{31}P .

Chemical Shifts: Shifts in resonant frequency occur due to partial shielding of the nuclei from the applied magnetic field by the electron cloud surrounding the nuclei. The density of the electron cloud varies with the number and nature of the neighbouring atoms. The magnitude of the effective field felt by each group of nuclei can be expressed as follows,

$$H_{\text{eff}} = H_0 (1 - \delta) \text{ where, } \delta = \text{shielding constant.}$$

Thus, the protons at various sites in a molecule are spread out into a spectrum according to the values of their shielding parameters. A field or sweep brings out protons at each particular site into resonance one after another. The more the field induced by the circulating electrons, the more it shields the nucleus and opposes the applied field, requiring higher field to achieve resonance if the field is varied, or lower resonance energy if the frequency is varied.

Because nuclear magnetic resonance spectrometers employing different field strengths it is desirable to

express the position of resonance in field-independent units and with respect to the resonance of reference compound. Tetramethyl silane (TMS), is the reference compound for proton spectra in non aqueous media. The position of TMS is assigned exactly as 0.0 on the scale. The magnitude of the chemical shift is expressed in parts per million.

$$\delta = \frac{H_{\text{sample}} - H_{\text{TMS}}}{\nu_i} \times 10^6$$

where, H_{sample} = position of absorption lines for sample expressed in frequency units (Hz).

H_{TMS} = position of absorption lines for TMS expressed in frequency units (Hz)

ν_i = operating frequency of the spectrophotometer.

Another scale used for chemical shift is Tau (τ), in which $\tau = 10.0 - \delta$.

Recommended reference materials for other nuclei include, carbon disulphide or TMS for ^{13}C , trichlorofluoromethane for ^{19}F , and phosphoric acid for ^{31}P .

Spin-spin coupling : Nuclei can interact with each other to cause mutual splitting of the sharp resonance lines into multiplets, called spin-spin coupling. The strength of coupling, denoted by J , is given by the spacing of multiplets

and is expressed in hertz.

The number of lines in a multiplet is given by $2nI + 1$, where, n = number of nuclei producing the splitting. For protons this becomes $n + 1$ lines. The relative intensity of each of the multiplets as reflected in the integral curve, is proportional to the number of nuclei in the group. Intensities of the peaks within a multiplet are proportional to the coefficients of the binomial expansion. Thus, one neighbouring proton splits, the observed resonance to a doublet (1:1), two produce a triplet (1:2:1), three produce a quartet (1:3:3:1), and so on. The area under an absorption band is proportional to the number of nuclei responsible for the absorption.

d) Materials and Methods :

Plant material:

The species *Ipomoea digitata*, belongs to genus *Ipomoea* of family Convolvulaceae. It is a perennial herb, with large ovoid or elongated roots. The stems are long, thick, twining, tough and glabrous.

About 100 kg of tubers of the plant *Ipomoea digitata* (F. Convolvulaceae), were collected from Dapoli (Dist. Ratnagiri) of Maharashtra, soon after the end of rainy season. The tubers were washed with water to remove the attached clay. They were cut into small pieces and were dried in shed and powdered in a pulveriser.

Extraction: The powdered material was extracted with petroleum ether (40° - 60°), in a soxhlet apparatus exhaustively for 7-8 runs. This was tested for steroids using Liberman-Burchard reaction. The extract gave strong positive test for steroids successive extracts with chloroform and methanol gave weak positive test for steroids. Therefore petroleum ether extract was used for chromatographic studies.

The extract was concentrated under reduced pressure and at controlled temperature (40° - 50°), using flash evaporator (Carter, U.S.A.). The concentrated extract was stored at 4°C until further use.

Thin layer chromatography (TLC) of the petroleum ether extract :

The thin layer plates (20 x 20 cm) of silica gel G (BDH), were prepared by spreading the silica gel G slurry (10 gm of silica gel G in 10 ml. of distilled water), on a glass plate by hand. The plates were dried at room temperature and then activated by heating in an oven for $\frac{1}{2}$ hour at 110°C. The plates were then allowed to cool in a desiccator.

A drop of petroleum ether extract was applied 2 cm. above the lower edge of the plate with the help of a thin capillary tube. The spot was allowed to dry at room temperature. The plate was then put into a chromatographic chamber previously saturated with the mixture of petroleum ether(BDH,AR) : chloroform (BDH,AR) (10:90). After the desirable run of the solvent, the plate was removed from the chromatographic chamber and was allowed to dry at room temperature.

The plates were then sprayed with Liberman-Burchard reagent with a sprayer (Liberman-Burchard reagent was prepared by mixing 5 ml. of concentrated sulphuric acid with 15 ml. of acetic anhydride. The mixture was then added dropwise into 50 ml. of absolute alcohol kept in ice bath). The plates were allowed to dry at room temperature, then they were heated in an oven at 110°C for 10 minutes and were observed for various coloured

spots. The extract showed four spots and were named as IdX_1 , IdX_2 , IdX_3 , IdX_4 above downwards respectively. In few experiments the petroleum ether extract was run simultaneously with β -sitosterol as a reference compound. IdX_4 corresponded to the spot of β -sitosterol. The extract was then subjected to adsorption column chromatography for separation of steroid fractions in large amounts.

Adsorption column chromatography :

The petroleum ether extract of *Ipomoea digitata*, was subjected to the adsorption column chromatography to isolate the steroid fractions in pure form and in larger amounts.

Activation of silica gel : 250 gm of silica gel (60-120 mesh, BDH), was activated by heating in an oven at 110°C for 2 hours. Then it was removed from oven and was allowed to cool in a desiccator.

Packing of column : In a glass column (100 x 2.5 cm), a piece of glass wool was placed at the bottom of the column with the help of long glass rod and solvent mixture petroleum ether : chloroform (10:90), was added to it. It was ensured that no air bubble was entrapped in the glass wool. 200 gm of activated silica gel was placed in a beaker and a slurry was prepared in the solvent mixture, petroleum ether : chloroform (10:90). The slurry

was poured into the column at the constant speed. The excess of solvent mixture was allowed to drain off. The column was lightly tapped so that packing was uniform.

5 gm of concentrated petroleum ether extract was dissolved in 10 ml. of mixture of petroleum ether : chloroform (10:90). It was loaded slowly on the silica gel column, keeping the flow rate of column 30-40 ml/hour.

Fractionation: After complete adsorption of the extract on the column, the column was eluted with the same solvent mixture at the flow rate of 30-40 ml/hour. The 10 ml fractions were collected in the test tubes.

All the fractions were screened using TLC as described earlier, on silica gel G and using petroleum ether: chloroform (10:90), as a solvent system. Fractions showing presence of single spot were pooled together, the solvent was allowed to evaporate at room temperature. The compound thus obtained (IdX, crude) was further purified by rechromatography on activated silica gel.

Rechromatography of IdX, (crude): IdX, was rechromatographed on activated silica gel column as described earlier to remove all colouring matter. 0.5 gm of IdX, was loaded on 200 gm of activated silica gel column and was eluted with the mixture of petroleum ether : chloroform (10:90). The flow-rate was kept constant at 30-40 ml/hour. 10 ml.

fractions were collected. The solvent was allowed to evaporate at room temperature and the compound IdX₁ was further purified by studying the solubility of IdX₁.

Purification : IdX₁ was dissolved in little amount of chloroform (BDH, AR). About 10 ml of acetone (BDH, AR) was added to it and was kept in a deep freezer (Krispcold,) at -35°C. The precipitate was filtered with the filter paper (Watman) at -35°C. The precipitate was washed several times with the chilled acetone (-35°C). The precipitate thus obtained was then dissolved in chloroform. The chloroform was allowed to evaporate at room temperature and the compound IdX₁ was further screened for its homogeneity on TLC on silica gel G layer, using various solvent systems.

Homogeneity of IdX₁ : The homogeneity of IdX₁ was confirmed on silica gel G layer, on TLC in the following solvent systems.

- a) petroleum ether: chloroform: methanol (10:80:10);
- b) heptane: chloroform (10:80), and c) benzene: chloroform (30:70).

In order to determine the class of IdX₁, it was tested for various coloured spot tests of steroids (stahl, 1969). Further it was studied for its physicochemical characteristics and biological activities of steroids.

Test for Phenolic steroids : The compound was dissolved in chloroform and was applied on a strip of filter paper with a thin capillary tube. The spot was allowed to dry at room temperature. The strip of filter paper was dipped in the Ferricyanide-ferric chloride reagent (The Ferricyanide-ferric reagent was prepared by mixing equal volumes of 1% aqueous potassium Ferricyanide solution with 2% aqueous ferric chloride solution just before use).

Elemental analysis : The following qualitative test was performed for detection of nitrogen, halogens and sulphur.

Sodium fusion test : (Lassaigne's test)

In the short tube of hard glass (6-7 x 1 cm), a dry clean piece of sodium was dropped and heated. When fused, a little amount of substance (IdX_1) was added over it and heated gently. Further small quantity of IdX_1 was added and heated to redness for three minutes. The tube was broken in distilled water in a dish. The solution was concentrated by boiling and filtered. The filtrate was divided into portions and tested as follows.

a) Test for nitrogen: 10 ml. of the filtrate was added to 5 ml. of saturated ferrous sulphate solution and one drop of 5% sodium hydroxide solution, and boiled for two minutes. Two drops of saturated solution of ferric chloride were added to the boiling mixture and was cooled and acidified with concentrated hydrochloric acid.

b) Test for sulphur : To 2 ml. of the filtrate, one drop of 5% sodium hydroxide solution was added, followed by addition of four drops of 1% of fresh solution of sodium nitroprusside.

c) Test for halides : 5 ml. of filtrate was acidified with dilute sulphuric acid, boiled for 5 minutes and then added a few drops of dilute nitric acid and silver nitrate.

Physico-chemical studies :

Melting point : The melting point of IdX_1 was determined on electrically heated melting point apparatus.

Specific rotation : The specific rotation of 0.1% solution of IdX_1 in chloroform was determined on polarimeter (Carl Zeiss, Germany).

Spectral studies :

a) Ultra violet spectrum : The ultraviolet spectrum of IdX_1 was recorded by means of ultraviolet-visible spectrophotometer. (Karl-Ziss)

Concentration of IdX_1	: 1 mg/ml (0.1%)
Solvent	: chloroform
Range	: 200 nm to 360 nm.
Cell	: 1 cm breadth

b) Infrared spectrum : The infrared spectrum of IdX_1 was recorded on Perkin-Elmer infrared spectrophotometer.

Results and Discussion

Exhaustive extraction of 10.0 kg of dried powder of tubers of *Ipomoea digitata* with petroleum ether ($40^{\circ} - 60^{\circ}$), produced 2.5 kg (25 %) of the extract. This extract showed strong positive test for the steroids with Libermann-Burchard reagent.

The extract was resolved into four main spots on a thin layer chromatography plate using solvent system petroleum ether: chloroform (10:90). They were named as IdX_1 , IdX_2 , IdX_3 and IdX_4 , above downwards respectively. The rf value of IdX_4 corresponded with that of β -sitosterol, which was taken for reference. This indicates that IdX_4 is β -sitosterol. Tiwari *et al.* (1964) have reported the presence of β -sitosterol in this plant. The rf value of IdX_1 was 0.76 (fig.II).

The crude petroleum ether extract was chromatographed on activated silica gel G column using petroleum ether: chloroform (10:90) as a solvent system. All the four fractions were collected separately and were concentrated at room temperature. The yield of these fractions from this column chromatography was as under

Total extract loaded : 150 Gms.

<u>Fraction</u>	<u>Yield (mg)</u>	<u>%</u>
IdX_1	150	0.1
IdX_2	70	0.046
IdX_3	50	0.033
IdX_4	870	0.58

The homogeneity of these fractions was checked by thin layer chromatography, using different solvent systems, viz;

- i) petroleum ether: chloroform : methanol (10:80:10)
- ii) heptane: chloroform (10:80)
- iii) benzene: chloroform (30:70)

The fraction IdX_1 and IdX_4 showed single spot in all the above systems whereas IdX_2 and IdX_3 exhibited a mixture of two or three compounds. Since IdX_4 is a known compound i.e. β -sitosterol, IdX_1 was taken for further purification.

The fraction IdX_1 was rechromatographed and purified as described earlier. The pure IdX_1 thus collected was yellowish semisolid having little oily odour. It was insoluble in water and methanol but soluble in chloroform, petroleum ether and partially soluble in acetone. It gave strong positive test for phenolic steroids indicating that it may be a phenolic steroid, like estrogen.

The elemental analysis is as follows:

<u>Element</u>	<u>%</u>
Carbon	75.76
Hydrogen	11.81
Oxygen	12.33
Nitrogen	Nil
Sulphur	Nil
Halides	Nil

indicating a probable empirical formula $C_{10}H_{18}O$.

Fig. — Thin layer chromatography of petroleum-ether extract of *Ipomoea digitata*.

DETECTION:

Lieberman-Burchard reagent.

SOLVENT SYSTEM:

Petroleum ether: Chloroform (10:90)

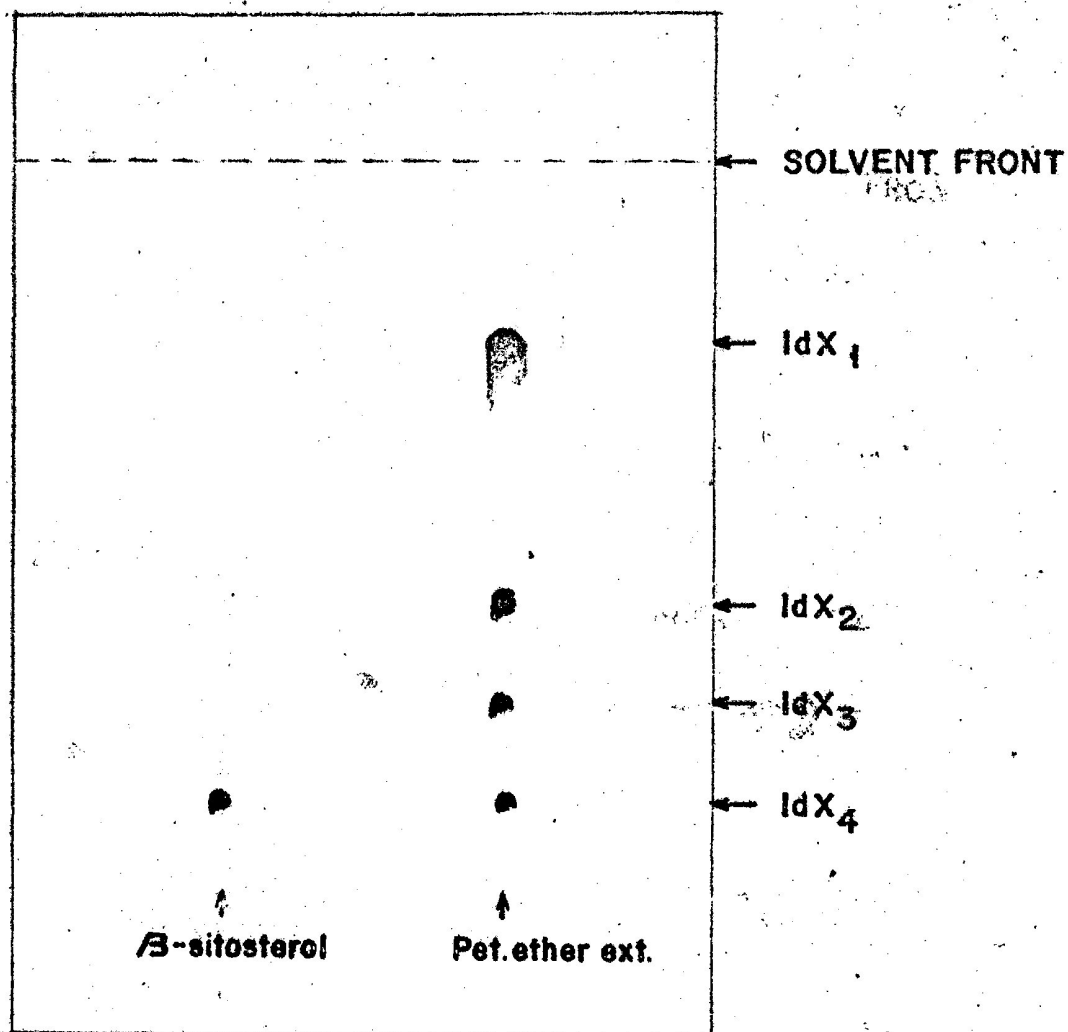
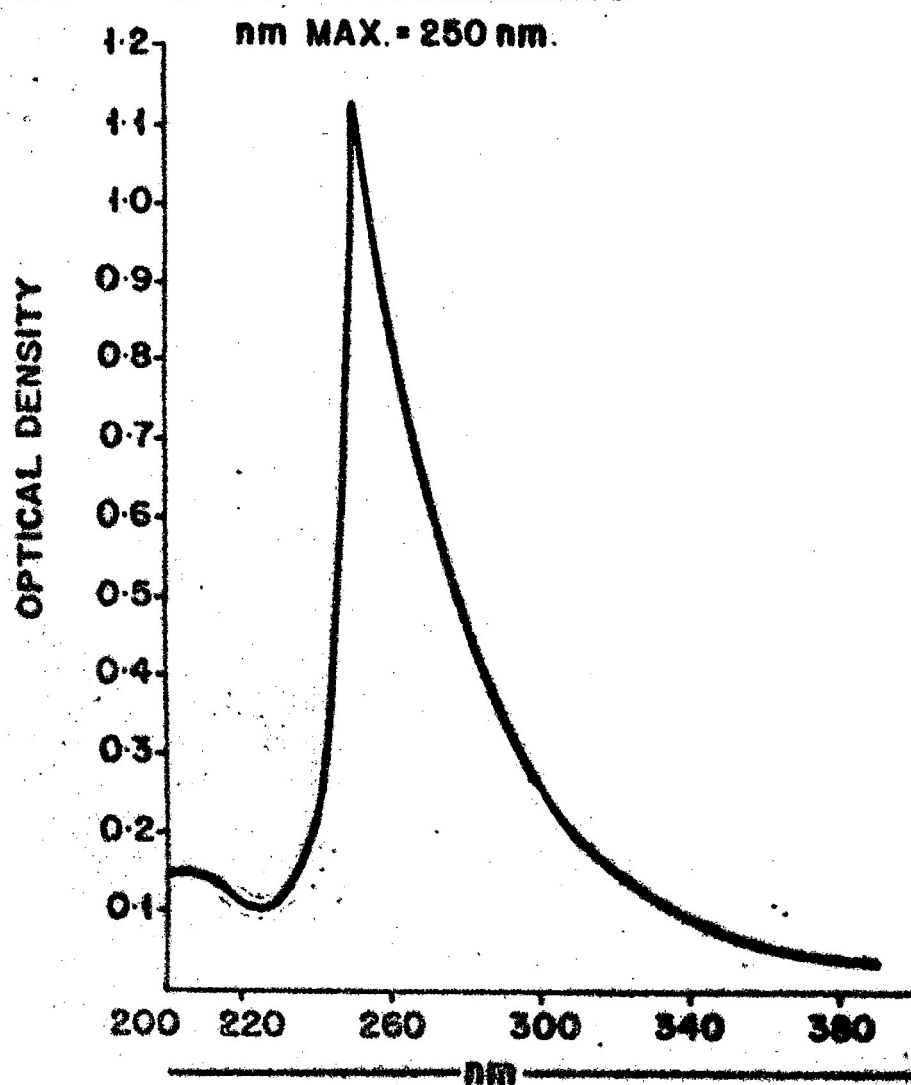
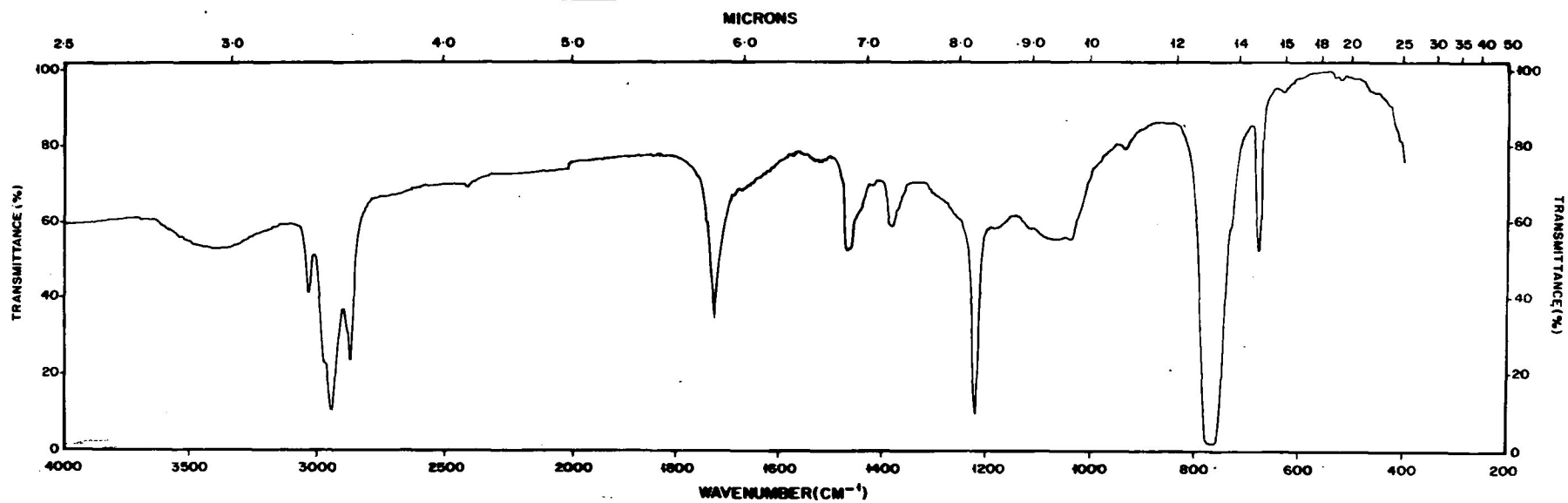


Fig - U.V. Spectrum of IdX₁



INFRA RED SPECTRUM OF IdX₄



CHAPTER III : PHARMACOLOGY

a) Review of Biological Activities of Steroids

Estrogenic activity:

Estrogens are generally steroids, however many non steroidal compounds exhibit estrogenic activity. The physiological effects of the estrogens are observed in the target tissues, namely the uterus, vagina, anterior pituitary and mammary glands. Jensen and Jacobson, 1962 showed that, tritium labelled estrogen of high specific activity, gets bound to these target tissues and these tissues can selectively retain it for some time.

Allen and Doisy, 1923 were the first to make use of vaginal cornification in spayed female mice for estrogen assay. Later, spayed mice and rats were used by Kahnt and Doisy, 1928. Emmens, 1950 showed that the accuracy of this method depends on the completeness of ovariectomy and removal of the fallopian tubes. The sensitivity of the assay can be increased by direct instillation of the test compound into vagina (Sulman, 1952).

Martin and Claringbold, 1958 determined the increased mitotic activity in the vaginal epithelium as an index of estrogenic activity. The albino mice were ovariectomised and primed with 1 ug estrone subcutaneously, for 14 days. Single intravaginal administration of the test compound was done and the animals were killed 24 hour later. 7 hour before killing 0.1 mg cochlaine in 0.05 ml distilled water was injected subcutaneously to arrest mitosis. The vagina was removed, fixed and transverse sections of 6 μ thick were

stained with Heidenhain's haematoxylin and examined microscopically. Martin, 1960 reported the reduction of 2,3,5 - triphenyl tetrazolium chloride (TTC) in the vaginal epithelium in mice. Lawson et.al., 1939 reported that the time required for complete vaginal opening gives an accurate reliable index of estrogenic activity. Using this observation Hartman and Littrell, 1945 produced vaginal opening by injecting the compound locally around the vaginal opening in 21-day-old female rats.

Atwood, 1938 described an assay based on the observation that the uterus responds to estrogens by increased uptake and retention of water. The peak was observed 6 hour after administration. Rubin et.al., 1951 studied this method extensively and described the sensitivity of three naturally occurring estrogens. Jonsson and Terenius, 1965; and Terenius 1966, determined the affinity of the test compound for the estrogen sites in the uterus. They incubated the isolated uterus of rat with the labelled estrogen and the uptake of labelled estrogen was determined. The test compounds incubated with the labelled estrogen in assaying their effectiveness in competing for the receptors in the uterus.

Dorman et.al., 1940, demonstrated that the chick oviduct is suitable for assay of estrogen given subcutaneously or orally. Hertz, 1948 pointed out that pterylglutamic acid is essential for the responsiveness of the oviduct.

Progestational activity :

Since the discovery of progesterone (Corner, 1929) its isolation and synthesis and the establishment of its structure, several progestational steroids have been synthesized and parallel to this various methods have been devised for its quantitative study.

Endometrium :

Corner and Allen, 1929 described for the first time proliferation of the endometrium in the rabbit as an indication of progestational activity. McPhail, 1934 further graded this activity from 0 to +4 depending upon the formation of the glandular tissue. The improvement was made by Pincus and Vertheessen, 1937. They projected the collected sections of one uterine horn of each treated rabbit onto graph paper, and the areas of both the stromal and the glandular parts of the mucosa were determined by planimetry. Pincus *et.al.*, 1957 plotted the mean G/H ratio (where G is the area of glandular part and H is the area of total mucosa) against log dose. Mac Ginty *et.al.*, 1959 modified the McPhail method to determine the progestational activity of the substances applied locally to the endometrial surface. The rabbit was primed with oestrogen, 24 hour after the last dose the test compound was injected into the lumen of the uterus in 0.1 ml of sesame oil.

Following the observation of Lutwak - Mann, 1959 that

the level of carbonic anhydrase in the uterine endometrium depends upon the functioning of corpus luteum. Pincus *et al.*, 1957; and Ogawa and Pincus, 1961 derived a quantitative relationship between enzyme activity and the dose of progesterone administered to a estrogen primed rabbit. They primed the rabbits with estrogen. Progesterone was injected daily for 5 days. The rabbits were killed on 6th day, the endometrium was scraped, and weighed, and the enzyme activity was determined by colorimetric method of Philpot and Philpot, 1936, modified by Miyake and Pincus, 1950. The manometric method for carbonic anhydrase enzyme activity was suggested by Ogawa and Pincus, 1960. Ogawa and Pincus, 1961 described a simplified test system based on carbonic anhydrase enzyme levels in the endometrium, following a single injection of progesterone administered to nonprimed immature rabbit. The rabbits were killed 4 days after treatment and the endometrial weight and carbonic anhydrase activity were determined.

Leichert, 1928 reported the deciduoma reaction in rat and guinea pig. The estrogen primed and progesterone treated animals respond to traumatization of the uterus by formation of a maternal or placental tumor. Atwood, 1939 investigated this phenomenon in more detail and suggested this method for test purpose. Groop and Micas, 1958 employed pseudopregnant rats. The rats were made pseudopregnant by means of electrical stimulation of the cervix. On the fifth day animals were ovariectomized and one uterine horn was traumatized by inserting

27 gauge needle. Progesterone was injected subcutaneously daily for 5 days. Rats were killed on the fifth day. Valrado and Lissaw, 1951 measured the diameter of each horn, while Greenwald, 1961 suggested to weigh each horn. The percentage increase in weight of the traumatized horn over the nontraumatized horn was calculated and plotted against the dose of progesterone. Elton and Lågren, 1953 used rats one week after ovariectomy. The animals were primed with 1 ug of oestrogen daily subcutaneously for 4 days. 24 hour after the last dose, they were treated with progesterone for 9 days. On the fifth day the uterine horn was traumatized by injecting intraluminally, 1 mg of histamine dihydrochloride in 0.005 ml of saline (Barrow *et al.*, 1958). Madjerck, 1960 used ovariectomized virgin female mice. They were left for 3 days without any treatment. From day 4 to 7 the test compound was administered. On the 5th day, one uterine horn was traumatized by passing a thin nylon thread several times through the uterine wall. On the 6th day both uterine horns were cut off, weighed and examined histologically. The classification was made on Astwood's scale.

Effect upon ovulation :

Progesterone is known to have a biphasic effect on ovulation, mediated either by stimulatory or inhibitory effects on production and release of pituitary gonadotrophins. Progesterone injected less than 4 hour before the time of ovulation in rabbits facilitates ovulation, however Sawyer, 1952 has

shown that the ovulation is inhibited by injecting progesterone 4 hour or more, prior to the anticipated time of ovulation. Mao Gernack and Moyer, 1963 described a technique using 22-day-old immature female rats, maintained on a constant cycle of illumination, consisting of 14 hour of light and 10 hour of dark. They were injected subcutaneously with 15 international units (IU) of pregnant mare serum gonadotrophin (PMS), in 0.5 ml of saline. Two days later, progesterone was injected and on the following day rats were killed, the oviducts were dissected out and number of ova were counted.

Rabbit ovulates within a few hours after mating or after intravenous injection of copper acetate (0.5 mg/kg using 1% copper acetate in 0.9% saline). Progesterone injected 24 hour before induction of ovulation prevents ovulation. The rabbits are killed and ovaries are examined after 18-24 hour and the total number of ovulation points are recorded. Chipley, 1965 applied progesterone and 19-nor progesterone percutaneously.

Implantation of ova and maintenance of pregnancy :

Both oestrogens and progestogens show biphasic effects on implantation of fertilised ova and on the maintenance of pregnancy in laboratory animals. Dreisbach, 1953 and Edgren and Chipley, 1961 reported that small doses of oestrogen (10-20 ug oestrone/kg) caused a rapid expulsion of ova from the fallopian tubes of the rat. In contrast 100-160 ug/kg

of estrone did not affect the ova after they reached the uterus. Chambon, 1949 has shown that, small doses of progesterone alone delayed the implantation in the rat, and moderate to high doses are required to maintain pregnancy. Stucki and Forbes, 1960 demonstrated that many progestogens required the presence of estrogen to maintain the pregnancy in rats. Estrogen is not required with progesterone for the maintenance of pregnancy in hamster (Orsini and Meyer, 1962).

Androgenic activity :

Androgenic activity depends upon the sensitivity of the accessory sex organs of the male rodent (ventral prostate and seminal vesicles) and of secondary sex structures of birds and mammals (capon or chick comb, modified sebaceous glands of the hamster and rat and the exorbital lachrymal gland of rat).

Chick comb :

The ratio, of chick's comb in mg to body weight in gm, has been used successfully as a measure of response in androgen assays. The test compound may be administered by injection, orally or by direct application. Dorfman and Dorfman, 1948 studied extensively the sensitivity of different breeds of chicks and found that white leghorn was the most suitable for androgen assays. The injection method was devised by Frank et.al., 1942. Munson and Shaps, 1958 employed the method of

Frank et.al. but they changed the solvent. They used absolute alcohol instead of sesame oil and applied the daily dose in 0.01 ml.

Capon comb :

Gallagher and Koch, 1935 injected androgenic compound into 6-9 months old capons that were castrated at 9 weeks of age. The measurements of maximal length and height of the comb were taken before treatment and 24 hour after the last of the three or five daily injections or imunctions.

Pfeiffer et.al., 1944 suggested the use of bill pigmentation in the house sparrow as an indication of androgenic activity.

Accessory sex organs of the rodent :

Androgenic substances cause increase in weight of seminal vesicles and ventral prostate gland of castrated mice and rats. Dorfman, 1962 reported that the mouse test was more sensitive than the rat test. The literature contains many variations in the age, the time elapsed between castration and treatment, and duration of the treatment. Herzberger et.al., 1953 treated the animals on the day of castration. Arcoli et.al., 1960 treated the animals on the next day. Suchowsky and Junkmann, 1962; and Potts et.al., 1960 started the treatment after one week. Suchowsky and Junkmann, 1962 administered the test compound daily in 0.2 ml sesame oil orally and subcutaneously. The animals were killed on the day after the last administration. The fresh wet weight of preputial glands, ventral prostate.

tate, seminal vesicles were determined on a torsion balance.

Rat preputial gland :

The preputial glands are large, modified sebaceous glands, which do not open into a hair follicle. Jones and Goodbury, 1964 showed that these glands can be used as models for studying the effects of androgens. Castrated or spayed female rats were treated daily for 14 days, the increase in wet weight of the preputial glands were determined. Using this assay system de Groot *et al.*, 1965 demonstrated a parallelism between hypertrophy of the sebaceous glands and the preputial glands, with large doses of progesterone (10 mg/day for 14 days), without apparent stimulation of the seminal vesicles. Huggins *et al.*, 1955 reported that doses of androgens too low to stimulate the prostate, caused an increase in size of the preputial glands.

Costovertebral spot of hamster :

The costovertebral spots of the hamster lie on the dorsal side, approximately in the region above the kidneys. In response to androgenic stimulation in castrated male hamsters, the spots rapidly increase in size and in degree of pigmentation. Hamilton and Montagna, 1959 in histological examination showed marked deposition of melanin and great increase in the size and number of sebaceous gland cells.

Exorbital lachrymal gland of therat :

Cavallero, 1967 has described an assay system which depends upon the vesicular-mucous change in the glandular acini, which can be quantified histologically.

Female fetuses :

The effect of androgenic steroids on female fetuses have been described by Reeves et.al., 1960. They have discussed the determination of the anogenital distance and histological evaluation of the transverse sections of the pelvic region. Suchowsky and Neumann, 1962 described a method which deals with the longitudinal section. They administered the compound subcutaneously, on the 16th day of pregnancy, in 0.2-0.4 ml sesame oil, for 4 consecutive days. On the 21st day of pregnancy, the fetuses were removed from the uterus, cut at the umbilical level and fixed for 2-10 days in Bouin's solution. After fixation anogenital distance was measured with micrometer gauge. The criteria for androgenic action on development of vagina were:

- 1) reduction in length of the urogenital septum.
- 2) epithelial stimulation of the dorsal area of the ventral clitoral vestige.
- 3) length, course and opening of urethra.
- 4) shape of corpora cavernosa.
- 5) anogenital distance.

Anabolic activity :

Theoretically, it is possible that a compound may be found which stimulates growth of the levator ani muscle without stimulating growth of prostate or seminal vesicles. Such compound could be probably an anabolic agent devoid of androgenic activity.

Levator ani muscle of the rat :

Eisenberg and Gordon, 1950 reported an increase in weight of levator ani muscle of the rat due to testosterone. Horchberger *et.al.*, 1953 studied and improved the method of Eisenberg and Gordon, 1950. They weighed the ventral prostate, seminal vesicles and the levator ani. Dinni and Geiger, 1957 doubted the validity of the levator ani method. Hoteck and Drolich, 1961 reported a four fold increase in uptake of $1- (1-^{14}C)$ aminoisobutyric acid by the levator ani muscle with in 52 hours in castrated male rats. Saunders and Brill, 1957; Kochakian and Endahl, 1959; and Suchowsky and Junkmann, 1962 used the effect on body weight of castrated and of normal rats as a criterion for measuring the complex anabolic activity.

Nitrogen metabolism :

Arnol *et.al.*, 1959 described the nitrogen metabolism method, which more closely resembles clinical test methods for anabolic agents than weighing the levator ani. Rats were

placed on a restricted diet, till constant body weight was obtained.

Total urinary nitrogen excretion before drug administration was determined. The decrease in urinary nitrogen after drug administration indicates the anabolic or nitrogen retaining activity.

Anti-inflammatory activity :

Inflammation can be divided broadly into three separate tissue reactions:

- a) dilation and increased permeability of protein of small blood vessels.
- b) emigration of leucocytes from venules and capillaries.
- c) development of the cellular exudate in the injured tissue.

Rat hind-paw edema :

The edema producing substance is injected, usually in 0.1 ml volume, into the dorsum of the foot. At some time between 30 minutes and 3 hours later, the edema is measured. The volume of the edema can be measured volumetrically (if there are exudates in body cavities), gravimetrically by weighing a standard area of tissue or with the aid of plethysmography. Denenjoy, 1952 showed that the plethysmography gives the most reliable results. Vinter et al., 1962 studied the anti-inflammatory activity in rat foot paw by inducing the edema by calcium caragenin. The other edema inducing substances used

are formaldehyde, dextran, ovalbumin, 5-hydroxy tryptamine and histamine. Cortisone and phenylbutazone have been found to cause a modest diminution of acute inflammatory swelling induced by ovalbumin (Wilhelmsen, 1960). With dextran or 5-hydroxytryptamine, salicylate was found to give partial protection against edema as that offered by phenylbutazone, oxyphenylbutazone and aminopyrine (Domenjon, 1960).

Experimental pleurisy :

Spector and Willoughby, 1959a described a method based on measuring the volume of an acute inflammatory exudate by inducing the pleurisy in rat by intraperitoneal injection of turpentine. The exudate was collected during 24 hour period. Wilhelmsen, 1960 reported that phenylbutazone derivatives have some beneficial effects on peritonitis in the rat by intraperitoneal injection of formalin. The same worker has used chemosis after application of irritants, such as mustard oil, to the rabbit's eye as an index of the efficacy of anti-inflammatory drugs (Wilhelmsen, 1949, 1950).

Thermal injury :

Thermal injury to the skin can also be used as a guide to study the anti-inflammatory activity. Burns of standard duration and intensity are produced in an anaesthetized rat, and the resultant edema is measured by excising and weighing a standard area of skin. Large doses of sodium salicylate or quinine are

effective in this test (Spector and Willoughby, 1960). Thermal injury can be assessed by observing the degree of leakage into the damaged area by a circulating azo dye. The dye is previously injected intravenously. Trypan blue and potamine blue are the most popular stains. Wilhelm and Mason, 1960 demonstrated that histamine antagonists prevent the appearance of increased vascular permeability in thermal injury, by estimating the amount of dye leaking into the skin.

Leakage of dye stuff :

Leakage of circulating protein bound dye can also be used to measure, the ability of drugs to suppress the effect of particular endogenous mediators on vascular permeability. Spector, 1959 estimated the intensity or the area of the patch of dye staining by injecting intradermally, histamine or bradykinin into the abdomen or flank of the rat. Judah and Willoughby, 1962 determined dye leakage quantitatively, by excising the area of skin and extracting the dye. Willoughby, 1960 standardized an increase in vascular permeability in the rat intestine after abdominal irradiation. The increase measured by extracting and estimating the amount of protein bound Trypan blue leaking into the gut wall from the permeable vessels, the dye having been previously injected into the tail vein.

Arthus reaction :

An intradermal injection of antigen or antibody rapidly

results into developing inflammatory reaction. Salicylate and cortisone have been found effective in suppressing this reaction (Harko and Smith, 1960). Gell and Hinde, 1951 induced delayed hypersensitivity in guinea-pig and rabbits as tuberculin reaction and the effect of drugs on the reaction was measured by the area of erythema. Murray *et al.*, 1962 used homograft rejection as a test system.

Other hyperergic inflammatory reactions have been used to test the effect of anti-phlogistic drugs on the acute vascular response. Wilhelmi, 1949-1950 injected antigen into the joints of sensitized rabbits to produce an experimental hypersensitive arthritis.

Ultra violet erythema :

Adams, 1960; Wilhelmi and Demanjes, 1951 tested the effect of drugs on the acute vascular response to the injury caused by ultraviolet radiation. They found sodium salicylate, aspirin, phenylbutazone, amidopyrine and cortisone highly effective in this test. Adams, 1960 radiated the depilated skin for 20 seconds and the resultant erythema was graded by eye.

Leucocyte emigration :

Fox and Spector, 1962 showed the emigration of leucocytes after a single injurious stimulus. The irritant substance or an antigen is injected into a sensitized animal, intradermally or subcutaneously. The area excised after 6 hours. The tissue is examined histologically and the number of particular type

of leucocyte is counted. Dougherty and Schneehall, 1955 studied the inflammatory phenomena by means of mast-cell disruption and changes in fibroblasts. Allison *et.al.*, 1956 studied the effect of drugs on the adhesion of leucocytes to vascular epithelium and their sub-sequent emigration, with the aid of Clark-Sandison rabbit-ear chamber.

Granuloma pouch formation :

The granuloma pouch was introduced by Solyo, 1953a, and soon used as a means of assaying anti-inflammatory drugs by Tolksdorf, 1959; and Wilhelm, 1960. 25 ml. of air was injected into the dorsal subcutaneous tissue. Small volume of turpentine oil or croton oil was injected to the same site. The extent of inflammation was measured 4-14 days later by estimating the thickness of the wall of granulation (Solyo, 1953b), or the weight of excised pouch (Solyo, 1954), or the fluid exudate within the abscess (Robert and Resenick, 1957). Reiser *et.al.*, 1950 and Meyer *et.al.*, 1953 implanted the cotton-wool pellets of known weight, into subcutaneous tissues, and the granulation tissue was removed after 4-5 days and was weighed. Thomas *et.al.*, 1960 injected irritants or polysaccharides into joint cavities, and estimated the inflammatory infiltration in the synovial membrane.

b) Materials and Methods

Id₁, thus isolated was screened for the following biological activities possessed by steroids.

Estrogenic/antiestrogenic activity in immature ovariectomised rats :

Estrogenic and antiestrogenic activities were studied by uterine weight assay method as described by Evans *et al.*, 1941. Immature female rats weighing 50-60 gm were used for the evaluation. Animals were ovariectomised under light ether anaesthesia as described by Zarrow *et al.*, 1964.

Ovariectomy in rats:

Procedure: The rats were anaesthetised with ether. The animals were secured on a wooden board with its dorsal side up. The hair on lower back were clipped. Laparotomy was performed by two small incisions just above the pelvis across the midline of the back. After isolating the ovaries, they were carefully cleared from the surrounding fatty tissues. The blood supply to the ovary was completely ligated. The ovaries were then cut off and both horns of uterus pushed back in the peritoneal cavity and the muscular flap and the outer skin was sutured with the help of sterile thread.

All animals were allowed to rest under identical laboratory conditions for 2 weeks before use. Vaginal smears were examined daily to ascertain that vaginal epithelium was not showing any estrogenic influence. Animals which did not show any cornified

epithelial cells in their vaginal smears were selected for experiment and divided into four groups of 6 animals each.

The animals received the drugs dissolved in 0.1 ml of olive oil subcutaneously for 7 days as follows.

Group I : Olive oil 0.1 ml.

Group II : Ethinyl estradiol 0.1 ug

Group III : Ethinyl estradiol 0.1 ug
and Idx₁ 0.5 ug.

Group IV : Idx₁, 0.5 ug.

Ethinyl estradiol (John Wyeth Laboratories), and Idx₁ were dissolved in olive oil. The vaginal smears of all animals were examined daily during the treatment.

All animals were sacrificed on the eighth day i.e., 24 hour after the administration of last dose of drug. Uteri were removed, trimmed of fat and weighed immediately on a torsion balance (Technipor, Poland). The uterine weight in mg per 100 gm of body weight was calculated in each group and was compared with the control group.

In order to study the estrogenic activity in detail, log dose response of Idx₁ was obtained for the dose of the drug and uterine glycogen content and uterine water uptake.

Uterine glycogen :

The immature female rats were ovariectomised as described before and were allowed to rest for 2 weeks, under identical laboratory conditions before use. Vaginal smears were examined daily to ascertain that the vaginal epithelium was not showing any oestrogenic influence. Animals which did not show any cornified epithelial cells in their vaginal smears were selected for experiment and divided into 9 groups of 6 animals each. The groups received the drugs dissolved in 0.1 ml of olive oil subcutaneously for 7 days as follows.

- Group I : Olive oil
- Group II : Ethinyl estradiol 0.01 ug
- Group III : Ethinyl estradiol 0.025 ug
- Group IV : Ethinyl estradiol 0.05 ug
- Group V : Ethinyl estradiol 0.1 ug
- Group VI : Idx, 0.05 ug
- Group VII : Idx, 0.125 ug
- Group VIII : Idx, 0.250 ug
- Group IX : Idx, 0.5 ug

All animals were sacrificed on the 8th day i.e., 24 hour after the administration of last dose. Uteri were removed, trimmed of fat and weighed immediately on a torsion balance. The uterine weight in mg per 100 gm body weight was calculated in each group and was plotted against the log dose.

The uteri were then processed for the estimation of glycogen.

Estimation of glycogen by Anthrone method :

Principle : Sulphuric acid causes dehydration of sugar to form a furfural derivative which then condenses with anthrone to form a blue coloured compound, which can be read in a colorimeter at 660 nm.

Reagents :

- a) Anthrone reagent : 0.5 gm. of anthrone (BDH, LR) was dissolved in 250 ml. of 98% (v/v) sulphuric acid (AR). It was freshly prepared before each series of determination.
- b) Potassium hydroxide solution (30%) : 30 gm. of potassium hydroxide (BDH, LR) was dissolved in 100 ml. of distilled water.
- c) Alcohol : 95%.
- d) Stock glycogen standard solution : 100 mg. of glycogen (C grade, California Corporation for Biochemical Research), were dissolved in 100 ml. distilled water (1mg/ml).
- e) Working standard : The stock solution was diluted (1 in 10) with distilled water to give concentration of 100 µg/ml.

Procedure :

The uteri were weighed and homogenised in 1 ml. of 30% potassium hydroxide solution. The homogenate was kept on boiling water bath for 15 minutes. It was then cooled and

3.5 ml of 95% ethyl alcohol was added. The mixture was brought to boiling and then immediately cooled. It was centrifuged at 5,000 r.p.m. for 10 minutes. The supernatant was discarded and the precipitate washed with 3 ml. of 95% ethyl alcohol, then dissolved in 2 ml. of distilled water. The tubes were kept in an ice bath and 4 ml. of anthrone reagent was added dropwise to avoid heating of tubes, due to exothermic reaction. It was then kept on a boiling waterbath for 10 minutes, cooled and the blue green colour developed was measured at 660 nm on spectronic 20 (Bausch and Lomb), against 2 ml. of distilled water and anthrone reagent.

Standards containing 10-60 ug of glycogen were treated similarly and glycogen content of the uterus was calculated by extrapolation from the standard curve.

Uterine water retention :

The assay was carried out by the method described by Astwood, 1938. Immature female mice were divided into 9 groups of 6 animals each. The groups received the drugs, dissolved in 0.1 ml of olive oil, subcutaneously for 3 days as follows.

- Group I : Olive oil 0.1 ml.
- Group II : Ethinyl estradiol 0.001 ug.
- Group III : Ethinyl estradiol 0.01 ug.
- Group IV : Ethinyl estradiol 0.1 ug
- Group V : Ethinyl estradiol 1 ug
- Group VI : Idx, 0.005 ug.

Group VII : Idx, 0.05 ug.

Group VIII : Idx, 0.5 ug.

Group IX : Idx, 5.0 ug

On the 4th day i.e., 24 hour after the last dose, the animals were sacrificed, the uteri were exposed wet weights were determined on a Torsion balance (Technipart, Polant). The uteri were dehydrated in an oven at 100°C for 24 hour. The dry weights of the uteri were noted. The percentage of water content was calculated for each group and was plotted against the log dose.

Progestational/antiprogestational activity :

These activities were studied by the deciduoma formation described by Astwood, 1953 & modified by Zarrow et.al., 1959. The rats were ovariectomized as described earlier. Ovariectomized rats were primed with 17- α -oestradiol (1 ug/day), subcutaneously for 4 days. The animals were divided into 4 groups of 6 rats each. The groups received the drugs dissolved in 0.1 ml. of olive oil subcutaneously for 9 days as follows.

Group I : Olive oil 0.1 ml.

Group II : Progesterone 0.5 mg

Group III : Progesterone 0.5 mg and Idx, 2.5 mg.

Group IV : Idx, 2.5 mg.

On the day 5th of progesterone treatment a uterine horn was traumatized, by injecting histamine dihydrochloride (1 mg

in 0.05 ml. of saline) intraluminally. On the 10th day i.e. 24 hours after the last dose, the animals were sacrificed, and both the uterine horns were removed. The weight of each horn was determined (Grocenwald, 1961). The percentage increase in weight of the traumatized horn over the control horn was calculated in each group and was compared.

Androgenic/antiandrogenic activity :

These activities were studied in immature castrated rats by the method of Dorfman and Steven, 1960. Male albino rats weighing between 50-40 gm were selected for this experiment. These animals were castrated as described by Zarrow, 1964.

Castration in rats :

Procedure : The rats were anaesthetized with ether. The animal was secured on a wooden board with its ventral side up. The hind legs were tied and the hair outlying the scrotal area were clipped. An incision was made through the skin of the scrotum and the testis on one side was separated from the surrounding tissue by blunt dissection. A second incision was made through the transparent tunica vaginalis and the testes was exposed. A ligature was made around the tunica vaginalis and spermatic cord. The testes was removed by a cut close to the ligature. The second testes was then removed in the same manner and skin incision was closed with the help of sterile suture.

The animals were allowed to rest for one week under identical laboratory conditions before use. The animals were divided into 4 groups of 6 animals each. Each group received the drugs dissolved in 0.1 ml of olive oil subcutaneously for 7 days as follows.

- Group I : Olive oil 0.1 ml.
- Group II : Testosterone (Nutritional Biochemical Corp.)
50 ug.
- Group III : Testosterone 50 ug, and Idx, 250 ug.
- Group IV : Idx, 250 ug.

On the 8th day, the animals were weighed and change in body weight was recorded. All animals were sacrificed and the seminal vesicles, and ventral prostate were dissected out, blotted on the clean filter paper and weighed on Torsion balance. The weights of ventral prostate and seminal vesicles were compared among various groups.

Anabolic activity :

Anabolic activity was studied by the method of Horshberger et.al., 1953 in castrated rats. Male albino rats weighing between 50-60 gm were selected for this experiment. The animals were castrated as described earlier.

The castrated rats were divided into 3 groups of 6 rats each. Each group received the drugs dissolved in 0.1 ml of olive oil for 7 days subcutaneously as follows.

Group I : Olive oil 0.1 ml.

Group II : Testosterone 50 ug.

Group III : Idx, 250 ug.

On the 8th day i.e. 24 hour after the last dose the animals were sacrificed and levator ani muscle was dissected out by the following method.

The skin of the scrotum was cut along the midline from the base of penis to the anus. Fatty deposits were cleared away using forceps, and the small white Cowper's glands were located beneath the bulbocavernosus muscle and looping dorsally over the rectum. The muscle was freed by blunt dissection using forceps. The rectum was cut and was reflected toward. After freeing the levator ani from the rectum, it was cut at its insertion into the bulbocavernosus muscle (Turner and Hebborn, 1971).

The levator ani muscle was weighed. The weights of levator ani muscle of various groups were compared.

Anti-inflammatory activity:

The activity was screened by inducing the edema by calcium - casein in rat foot paw as described by Winter et al., 1962. Albino rats of either sex, weighing between 100-120 gm were divided into 3 groups of 6 rats each. Each group received atom.

the treatment as follows.

Group I : 5.0 ml of distilled water orally.

Group II : Phenylbutazone 100 mg/kg. orally.

Group III : Idx, 300 mg/kg orally.

The paw volumes of rats of all the groups were recorded plethymographically before calcium caragenin injection. One hour after feeding the drugs calcium caragenin was injected subcutaneously in each paw of the rat. Three hours after the calcium caragenin injection the paw volumes of rats were recorded. The paw volumes of control and other groups were compared.

Pharmacodynamic actions of Idx, :

In addition to the activities described above, Idx, was subjected to following pharmacodynamic actions.

Effect of Idx, on cardiovascular system :

Effect of Idx, on cat blood pressure : (Perry, 1968)

Cats of either sex, weighing between 1.5 to 2.5 kg were used. They were anaesthetised with sodium pentobarbitone (40 mg/kg). The trachea, femoral vein were cannulated. The carotid artery was cannulated by Sim's cannula connected through a mercury manometer to a pointer to record the blood pressure on smoked kymographic paper on a drum. The cats were then given heparin (100 I.U./kg) and allowed to stabilise for 30 minutes.

A dose response curve was obtained for adrenaline bitartrate (1 to 3 $\mu\text{g/kg}$, I.V., Haffline Biopharmaceutical Corporation Limited, Bombay) and acetyl choline bromide (0.5 to 2 $\mu\text{g/kg}$, I.V., BHI, England). Id_x (0.1 μg to 1 $\mu\text{g/kg}$) was administered intraperitoneally in suspension form. Responses were recorded upto 1 hour. The responses to the standard doses of adrenaline and acetylcholine were repeated in order to check for any potentiating or inhibiting effect of Id_x on the responses to these agonists.

Effect of Id_x on isolated guinea pig auricles :

A guinea pig (250 gm and above) was killed by stunning and bleeding. The thoracic cavity was opened at once and the intact heart was removed and put into Ringer-Locke solution.

(Ringer-Locke solution :- Na^+ , 155.8 mM; K^+ , 5.6 mM; Ca^{2+} , 4.3 mM; Cl^- , 163.0 mM; HCO_3^- , 1.8 mM; Glucose, 5.0 mM).

The pericardium was removed and the auricles gently cut away taking care not to damage the Sino-Atrial Node. They were mounted in an organ bath containing Ringer-Locke solution and through which oxygen was bubbled.

Responses were taken with a lightly sprung lever (magnification X 10) on a smoked kymographic paper. Responses to adrenaline (50 μg , 100 $\mu\text{g/ml}$), acetylcholine (50 μg , 100 $\mu\text{g/ml}$) and Id_x 100 $\mu\text{g/ml}$ were recorded.

Effect of Id_1 on smooth muscle :

Effect of Id_1 on the isolated guinea pig :

A guinea pig was killed by a blow on the head and cutting the throat. The abdomen was opened and the ileum was lifted out. A piece of the ileum, about 5 cm long was removed, about 5-6 cm from the caecal end and placed in a dish containing Tyrode solution previously warmed to 37°C. Tyrode solution (Na^+ , 149.2 mM; K^+ , 2.7 mM; Ca^{2+} , 3.6 mM; Mg^{2+} , 2.1 mM; Cl^- , 145.3 mM; H_2PO_4^- , 0.4 mM; HCO_3^- , 11.9 mM; Glucose, 5.0 mM).

The ileum was then gently flushed with warm Tyrode and the mesentery cut off. A piece about 3 cm long was then cut off and tied with thread anti-symmetrically and mounted in an organ bath containing tyrode solution through which air was bubbled. The preparation was allowed to equilibrate for 30 minutes, with changes in solution every 10 minutes, before the start of the experiment.

Responses were recorded to standard concentrations of acetylcholine (0.25 - 1.0 ng/ml) and histamine (0.25 - 1.0 ng/ml) using a frontal writing lever (Magnification $\times 10$, load = 0.5 gm) on a smoked kymographic paper. Responses to Id_1 (10-100 $\mu\text{g/ml}$) were also recorded similarly.

Atropine (20 ng/ml) and Noppyramine maleate (2 ng/ml) were used to antagonise the responses of the standard agonists as well as those due to Id_1 .

Effect of I_{dx_1} on isolated rat uterus :

Adult female albino rats weighing between 120-150 gm were used. The animals were pretreated by injecting intramuscularly 1 ug of ethinyl oestradiol 24 hours before the experiment. The rat was killed by a blow on the head abdomen was cut opened. The two horns of uterus were dissected out and they were transferred to a dish containing De-Jalen's solution. (De Jalen's solution: Na^+ , 163.4 ml; K^+ , 5.4 ml; Ca^{2+} , 0.27 ml; Cl^- , 163.17 ml; HCO_3^- , 5.9 ml; and Glucose, 2.8 ml).

The horns of the uterus were separated out and freed from adhering tissues. One horn was tied to the carbogen tube in an organ bath containing De Jalen's solution and bubbled with carbogen (Oxygen 95% and carbon dioxide 5%). The upper end of the tissue was connected to frontal writing lever (Magnification $\times 10$, load 0.25 gm). The responses of the tissue to the drugs were recorded on cracked kymographic paper.

The responses of oxytocin (5×10^{-4} I.U. to 1×10^{-5} I.U./ml; Parke and Davis, Bombay) and acetyl choline (0.5 ug to 2.0 ug/ml; BDH, England) and I_{dx_1} (10 ug to 100 ug/ml.) were recorded.

Effect of I_{dx_1} on skeletal muscle

Effect of I_{dx_1} on isolated rat phrenic nerve diaphragm :

Adult albino rat weighing about 150-200 gm was killed by a blow on head. The thorax was cut opened on left side of the sternum, and its frontal part was removed. The phrenic nerve

was separated from connective tissue to a length of about 3 cm from the diaphragm. The diaphragm was then cut in a fan like shape with phrenic nerve attached and then mounted on platinum electrode, placed in Krebs' solution, through which a mixture of 95% oxygen and 5% carbon dioxide was passed in a bath of about 50 ml., maintained at 37°C.

(Krebs' solution: Na^+ , 141 mM; K^+ , 5.9 mM; Ca^{2+} , 2.6 mM; Mg^{2+} , 1.2 mM; Cl⁻, 104.8 mM; H_2PO_4^- , 2.2 mM; HCO_3^- , 24.9 mM; SO_4^{2-} , 1.2 mM; Glucose, 10 mM).

The nerve was stimulated by rectangular pulses (Aplab, square wave stimulator) of width 0.1 sec., at 1-2 volts and at frequency of 0.2 Hz. The responses were recorded on a smoked paper by a lightly sprung lever (magnification x 10). Tetanic stimulation was given by increasing the frequency to 20 Hz. Idx₁ (100 µg/ml) was tested on this preparation.

Toxicity studies :

Acute toxicity of Idx₁ :

An acute toxicity was done in animals to find out LD₅₀ of Idx₁. Adult female mice weighing 25-30 gm and adult female rats weighing between 100-120 gm were used in a group of 6 animals each. The drug was administered orally and subcutaneously ranging from 1 gm/kg to 10 gm/kg body weight. All animals were observed over a period of 48 hours.

RESULTS

Table 1 indicates the effect of ethinyl estradiol and IdX_1 on the uterine weight in immature ovariectomised rats. All the rats treated with ethinyl estradiol and IdX_1 showed cornification of the vaginal epithelium throughout the treatment. Estradiol at a dose of 0.1 ug/rat has produced an increase in uterine weight by about 117.0%. Similarly IdX_1 at dose of 0.5 ug/rat has produced 156.0% increase in uterine weight. Further the combination of estradiol and IdX_1 at the same dose levels has produced 178.0% increase in uterine weight which is about 61.0% more than the uterine weight compared to the ethinyl estradiol reacted animals.

All the animals treated with estradiol and IdX_1 showed a cornification of vaginal epithelium throughout the treatment. Ethinyl estradiol and IdX_1 have produced a dose dependent increase in the uterine weight. Ethinyl estradiol has produced 63.67, 81.95, 129.65, 144.98% increase at the doses 0.01 ug, 0.025 ug, 0.05 ug, respectively. Similarly IdX_1 has produced 45.59, 61.99, 103.44, 121.00% increase at the doses 0.125 ug, 0.25 ug, 0.5 ug respectively. These increase in uterine weights are statistically significant (Fig. VII).

Further the uterine glucogen content of the above uteri was estimated as per the method described earlier. The results are shown in the table III. It clearly shows that the uterine glycogen content has simultaneously increased with respect to the uterine weight in all the animals treated with ethinyl estradiol and IdX_1 , which is statistically significant. The increase in the glycogen

content in dose dependent in the both cases. The increase being 12.36, 19.11, 30.97, 38.15% in case of ethinyl estradiol and 07.38, 16.10, 21.76, 33.10% in case of IdX_1 at the dose levels studied (Fig VIII).

The effect of ethinyl estradiol and IdX_1 on the uterine water retention was studied as described earlier. The results are given in the table IV. Both ethinyl estradiol and IdX_1 have produced a dose dependent increase in the uterine weight as well as increase in the water content of the uterus at the doses indicated in the table. The results are indicated graphically in the Fig.IX and X.

The anabolic effects of testosterone and IdX_1 were tested in castrated rats are shown in the table V. In all the four groups the increase in body weight was almost same indicating that no significant increase was seen in the treated animals as compared to the controls. However the weight of the levator ani muscle of the testosterone treated animals increased significantly as compared to that of control. IdX_1 failed to produce any increase in the weight of levator ani muscle neither it showed any effect in the testosterone treated animals.

Table VI indicates effect of testosterone and IdX_1 on the weight of seminal vesicles and of ventral prostate gland of castrated rats. Testosterone produced significant increase in the weights of both seminal vesicles and ventral prostate gland; but IdX_1 failed to produce any effect 'parse' or on the effect of testosterone.

of testosterone.

Progestational activity of IdX_1 was studied in rats using the method described earlier. Progesterone was taken as a standard. The results are indicated in table VII. Progesterone at the dose of 0.5 mg produced about 82% increase in the weight of uterine horn as compared with its control, which is highly significant, but IdX_1 at the dose of 2.5 mg failed to produce any significant increase in the weight of treated uterine horn, neither it had any effect on progesterone treated horn.

The antiinflammatory activity of IdX_1 was studied using the method of rat paw edema as described earlier and phenylbutazone was taken as a standard. Phenylbutazone at the dose of 100 mg/kg produced about 70% protection in paw edema but IdX_1 at dose of 300 mg/kg failed to produced any significant protection.

Other Pharmacological activities :

- a) Cat blood pressure: IdX_1 upto dose of 1 mg/kg did not produce any effect on cat blood pressure. It did not altered the responses induced by adrenaline and acetylcholine.
- b) Isolated guinea pig auricles: On this preparation IdX_1 did not produce any effect upto a dose of 100 ug/ml. It also failed to produce any effect on the responses induced by adrenaline and acetylcholine.
- c) Isolated guinea pig ileum: IdX_1 at dose of 100 ug/ml did not produce any effect on the responses induced by acetylcholine and histamine.

- d) Isolated rat uterus: On this preparation IdK_1 did not show any effect 'per se' or on the effects of oxytocine and acetylcholine upto the dose level of 100 ug/ml.

Table I: Estrogenic/Antiestrogenic activity in immature ovariectomized rats

Group	Drug Dose/(rat/day) S.C.	No. of animals	Uterine wet weight Mean $\pm$ S. E.	Vaginal smear	% increase in uterine weight	p value
I	Olive oil 0.1 ml	6	76.3 $\pm$ 2.7	Negative	-	-
II	Ethinyl estradiol 0.1 μ g	6	163.45 $\pm$ 4.55	Positive	116.84	< 0.001
III	Ethinyl- estradiol 0.1 μ g + IdX ₁ 0.5 μ g	6	211.8 $\pm$ 7.06	Positive	177.58	< 0.001
IV	IdX ₁ 0.5 μ g	6	172.1 $\pm$ 6.85	Positive	155.57	< 0.001

Table II : Estrogenic activity in immature ovariectomised rats

Group	Drug/Dose rat/day S.C.	No. of animals	Average uterine weight mg/100 gm body weight (Mean $\pm$ S.E.)	% increase	Vaginal smear	p value
I	Olive oil 0.1 ml	6	84.25 $\pm$ 1.53	-	Negative	-
II	Ethinyl estradiol 0.01 μ g	6	137.9 $\pm$ 2.45	63.67	Positive	<0.001
III	Ethinyl estradiol 0.025 μ g	6	153.3 $\pm$ 2.78	81.95	Positive	<0.001
IV	Ethinyl estradiol 0.05 μ g	6	193.46 $\pm$ 3.44	129.62	Positive	<0.001
V	Ethinyl estradiol 0.1 μ g	6	206.4 $\pm$ 1.74	144.98	Positive	<0.001
VI	IdX ₁ 0.05 μ g	6	122.66 $\pm$ 4.22	45.59	Positive	<0.001
VII	IdX ₁ 0.125 μ g	6	136.48 $\pm$ 2.51	61.99	Positive	<0.001
VIII	IdX ₁ 0.25 μ g	6	171.15 $\pm$ 2.94	103.14	Positive	<0.001
IX	IdX ₁ 0.5 μ g	6	186.2 $\pm$ 4.15	121.00	Positive	<0.001

Table III : Uterine glycogen content in ovariectomised rats

Group	Drug/Dose/rat/ day. S.C.	No. of animals	Average uterine glycogen ug/100 mg uterine weight	% increase	Vaginal smear	p value
I	Olive oil 0.1 ml	6	74.65 $\pm$ 1.46	-	Negative	-
II	Ethinyl estradiol 0.01 ug	6	83.88 $\pm$ 1.13	12.36	Positive	< 0.001
III	Ethinyl estradiol 0.025 ug	6	88.92 $\pm$ 0.76	19.11	Positive	< 0.001
IV	Ethinyl estradiol 0.05 ug	6	97.77 $\pm$ 1.18	30.97	Positive	< 0.001
V	Ethinyl estradiol 0.1 ug	6	103.13 $\pm$ 1.34	38.13	Positive	< 0.001
VI	IdX ₁ 0.05 ug	6	80.16 $\pm$ 1.87	07.38	Positive	< 0.05
VII	IdX ₁ 0.125 ug	6	86.67 $\pm$ 1.15	16.10	Positive	< 0.001
VIII	IdX ₁ 0.25 ug	6	90.9 $\pm$ 2.13	21.76	Positive	< 0.001
IX	IdX ₁ 0.5 ug	6	99.36 $\pm$ 3.27	33.10	Positive	< 0.001

Table IV : Uterine water retention in immature mice

Group	Drug/Dose mouse/day S.C.	No. of animals	Uterine wet weight (mg) Mean + S.E.	Uterine water content %
I	Olive oil 0.1 ml	6	10.5 $\pm$ 1.38	81.1
II	Ethinyl estradiol 0.001 μ g	6	29.5 $\pm$ 1.53	84.95
III	Ethinyl estradiol 0.01 μ g	6	38.5 $\pm$ 2.18	86.4
IV	Ethinyl estradiol 0.1 μ g	6	46.5 $\pm$ 2.77	87.65
V	Ethinyl estradiol 1 μ g	6	52.5 $\pm$ 2.35	88.7
VI	IdX ₁ 0.005 μ g	6	22.5 $\pm$ 1.14	84.15
VII	IdX ₁ 0.05 μ g	6	29.1 $\pm$ 1.83	85.45
VIII	IdX ₁ 0.5 μ g	6	36.2 $\pm$ 1.34	86.6
IX	IdX ₁ 5.0 μ g	6	44.6 $\pm$ 1.56	87.4

Table V : Anabolic activity in castrated rats

Group	Drug/Dose/rat/day S.C.	No. of animals	Body weight (g)		Weight of Levator ani muscle (mg) + S.E.	p value
			Before	After		
I	Olive oil 0.1 ml	6	55.2	92.8	11.6 ± 0.23	-
II	Testosterone 50 µg	6	53.3	95.09	22.1 ± 1.2	< 0.001
III	Testosterone 50 µg + IdX ₁ 250 µg	6	60.6	95.3	20.9 ± 1.3	< 0.001
IV	IdX ₁ 250 µg	6	62.3	98.2	10.8 ± 0.41	-

Table VI : Androgenic/antiandrogenic activity in castrated rats

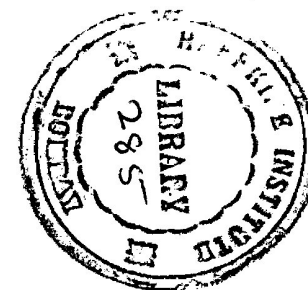
Group	Drug/Dose/rat/day S.C.	No. of animals	Weight of seminal vesicles (mg) Mean + S.E.	Weight of ventral pros- tate (mg) Mean + S.E.	p value
I	Olive oil 0.1 ml	6	9.4 ± 0.31	6.5 ± 0.17	-
II	Testosterone 50 µg	6	51.8 ± 2.87	49.7 ± 2.53	< 0.001
III	Testosterone 50 µg + IdX ₁ 250 µg	6	53.1 ± 4.38	47.8 ± 3.92	< 0.001
IV	IdX ₁ 250 µg	6	10.1 ± 0.47	5.9 ± 0.43	-

Table VII : Progestational/anti progestational activity in estrogen primed female rats

Group	Drug/Dose/rat/ day/ S.E.	Weight of uterine horn control (mg) Mean + S.E.	Weight of traumatized uterine horn (mg) Mean + S.E.	% increase over control horn	p value
I	Olive oil 0.1 ml	87.18 ± 2.7	90.30 ± 2.78	03.57 ± 3.9	-
II	Progesterone 0.5 mg	81.98 ± 3.12	149.5 ± 5.57	82.36 ± 6.38	< 0.001
III	Progesterone 0.5 mg + IdX ₁ 2.5 mg	86.5 ± 3.81	161.25 ± 6.73	86.41 ± 7.84	< 0.001
IV	IdX ₁ 2.5 mg	83.66 ± 2.75	87.33 ± 2.54	04.38 ± 2.58	> 0.05

Table VIII : Antiinflammatory activity

Group	Drug/Dose/ rat/orally	No. of animals	Initial paw volume	Paw volume after caragenin infec- tion.	% protec- tion
I	Distilled water 5.0 ml (control)	6	58.2 $\pm$ 1.45	105.7 $\pm$ 1.75	-
II	Phenyl-butozone 100 mg/kg.	6	57.5 $\pm$ 3.4	71.4 $\pm$ 3.5	70.0
III	IdX ₁ 500 mg/kg	6	60.0 $\pm$ 3.9	105.54 $\pm$ 4.15	3.79



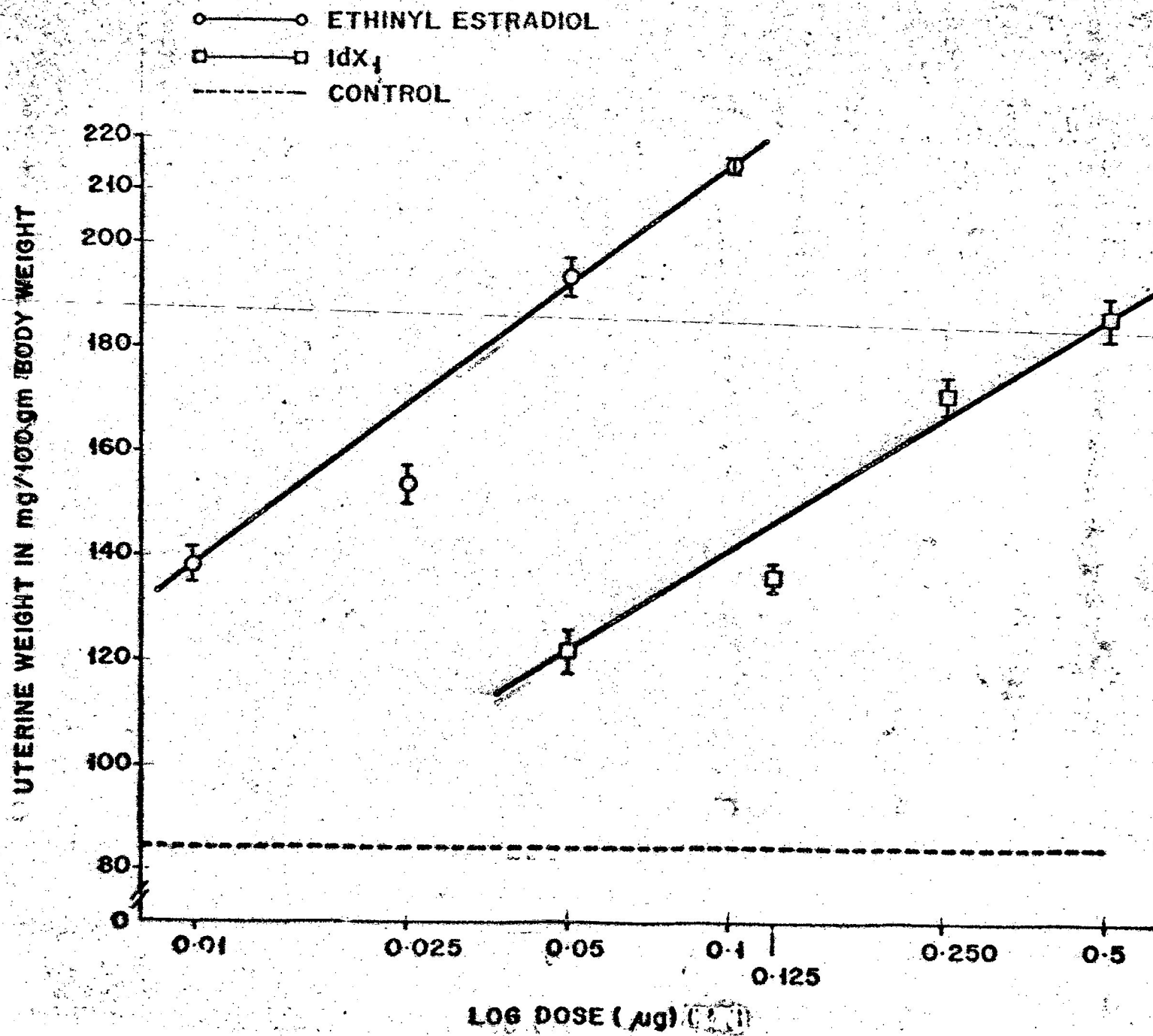


Fig. Uterine Glycogen content in Immature Ovariectomised Rats.

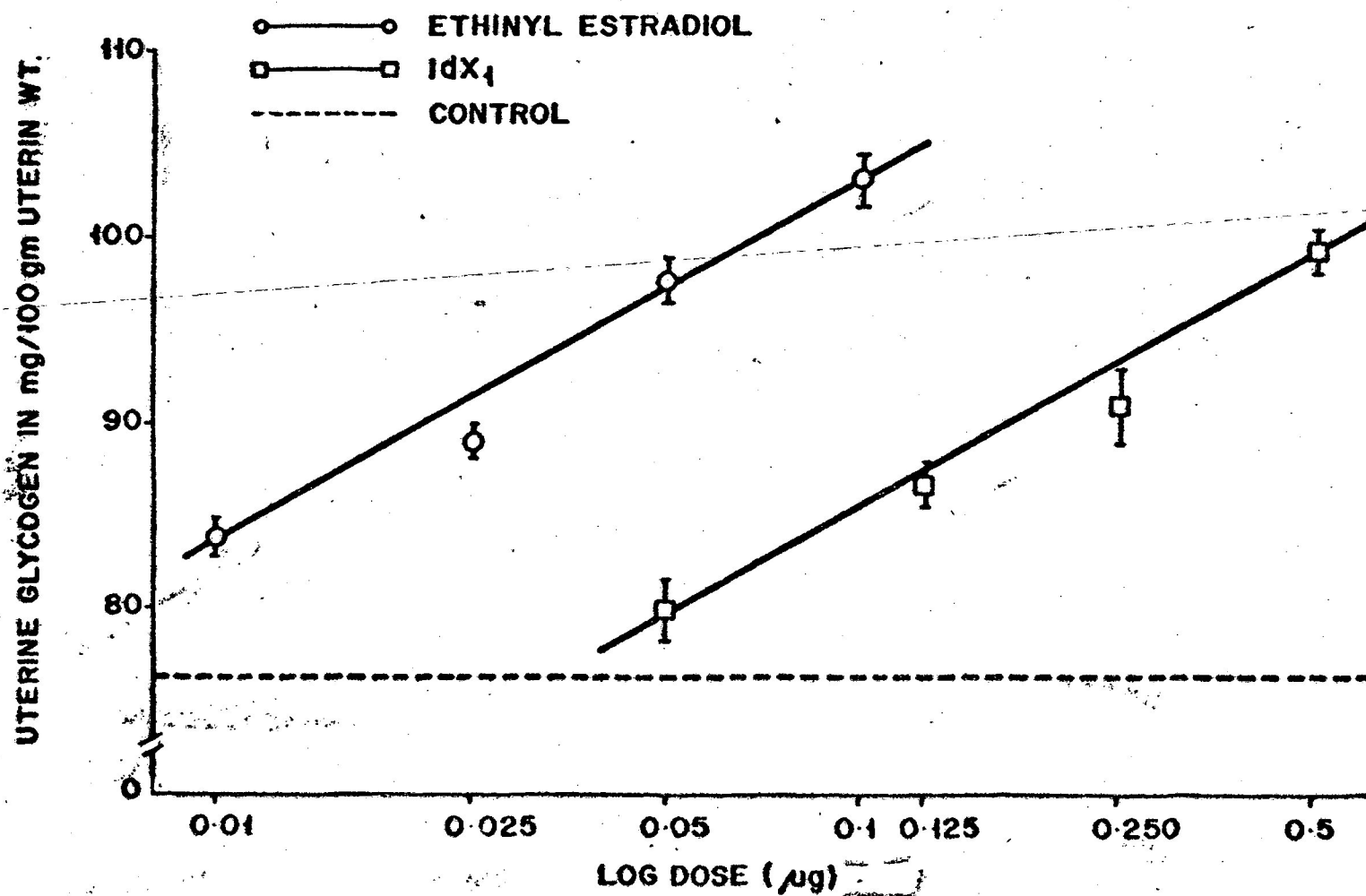


Fig. 1—Uterine weight assay in immature mice.

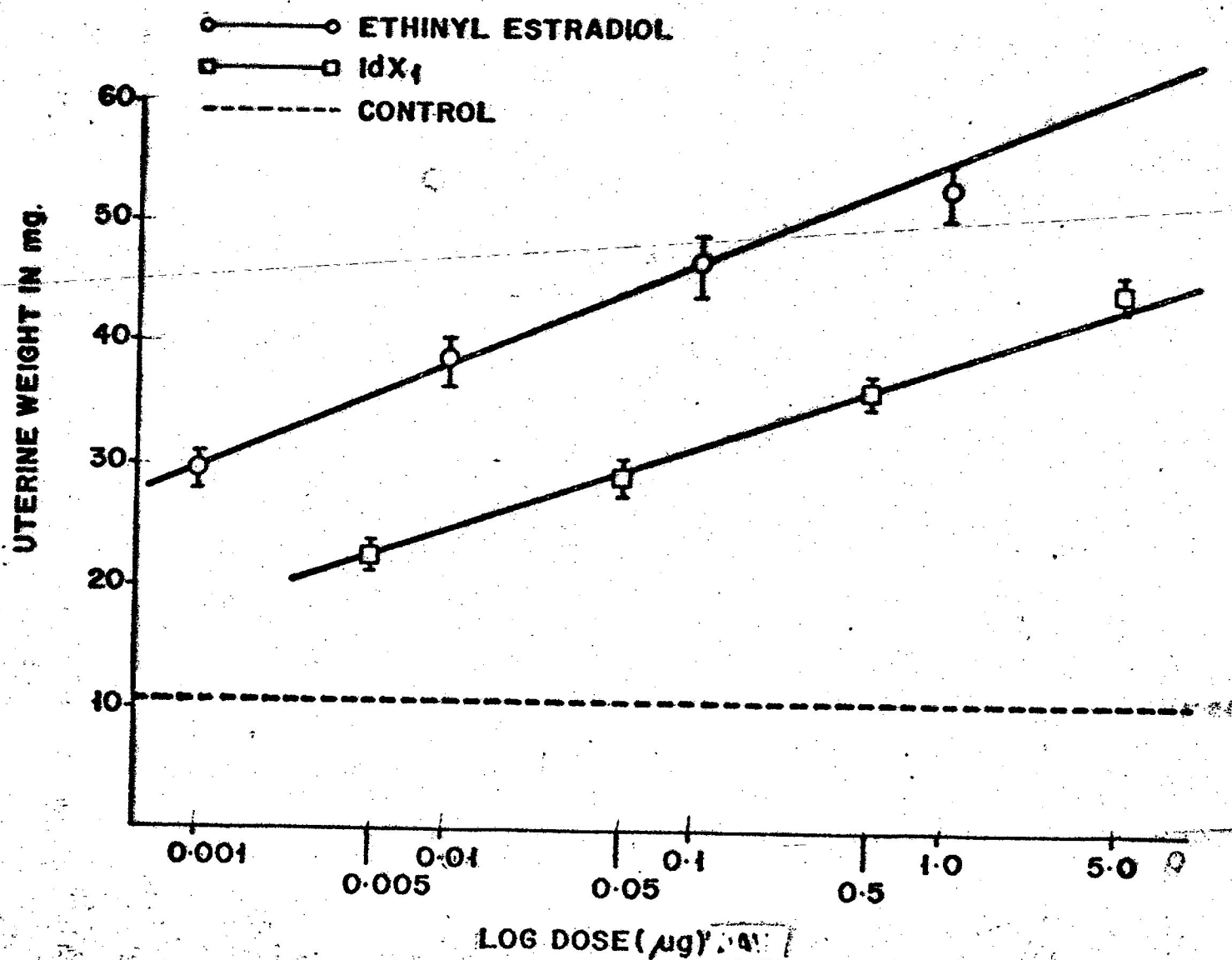
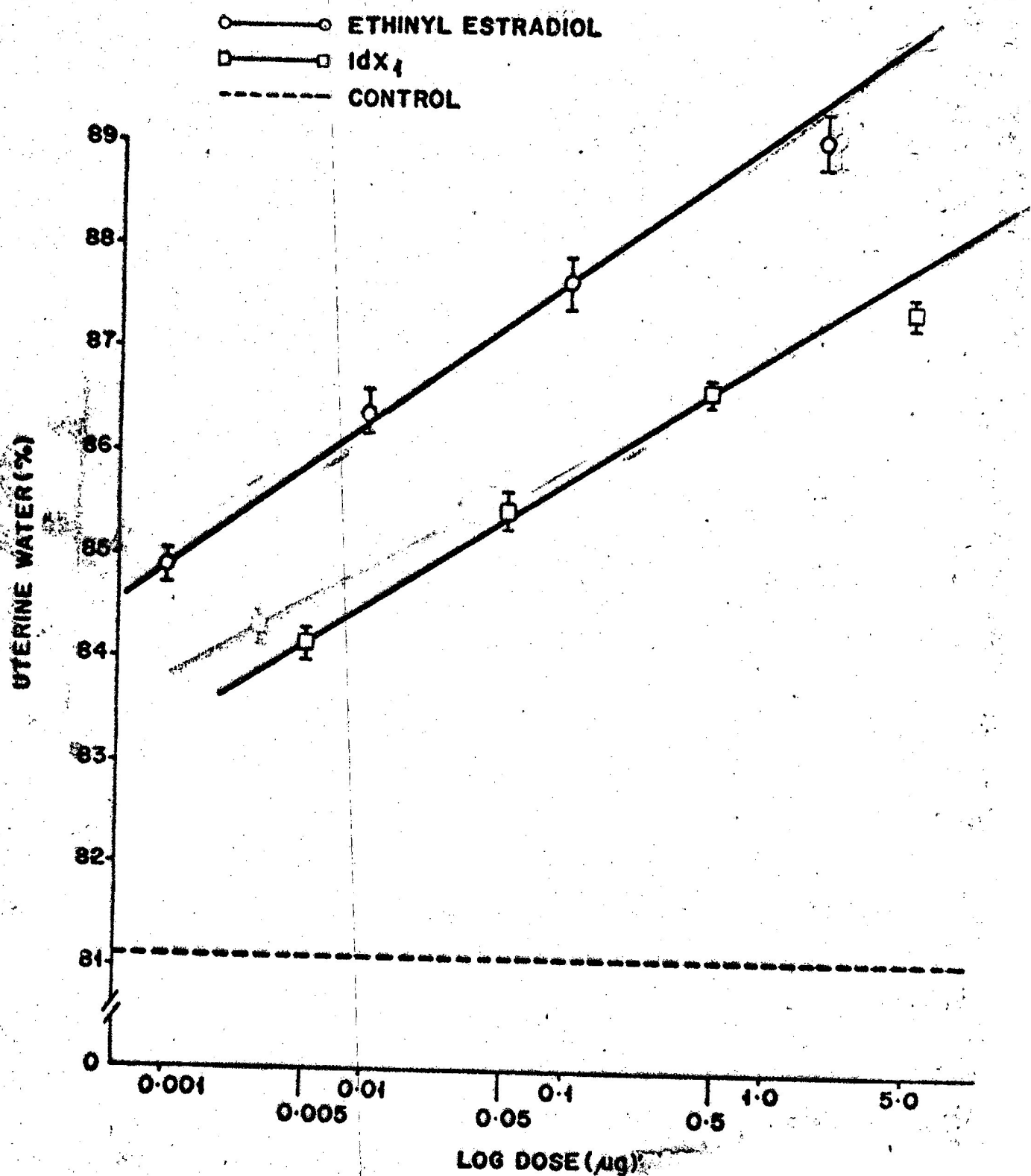


Fig - Uterine water retention assay in immature mice



DISCUSSION

The plant *Ipomoea digitata* has not been studied much, in the past for its pharmacological activity. Even in Indian system, of medicine the plant has a very limited utility. One of its use in ayurveda is for its lactogenic effect. The present work was undertaken to study the pharmacological actions in general. Since our chemical studies have revealed that the fraction isolated in the pure form i.e. IdX₁, is steroid more attention was paid to its steroidal activities viz. estrogenic, progestational, androgenic, anabolic and antiinflammatory effects.

Its estrogenic activity was studied in immature mice and immature ovariectomised rats. Ethinyl estradiol was taken as a reference standard. In uterine weight assay in immature ovariectomised rats both ethinyl estradiol and IdX₁ have produced an increase in the uterine weight at dose of 0.1 ug and 0.5 ug/rat/day respectively. The increase in uterine weight as compared to its control group is statistically significant (table I). All the animals in the groups treated with ethinyl estradiol and IdX₁ have shown cornification of the vaginal epithelium. This is a characteristic property of estrogens (Allen and Doisy, 1923).

The effects of both these compounds are dose dependent in ovariectomised rats. The increase in uterine weight is more with the increase in the dose of each compound. Fig.VII shows clearly a parallelism in the effects between these two compounds, wherein ethinyl estradiol is about 10 times more potent than IdX₁. These experiments indicate that this steroid IdX₁, has got effects on the uterine weight similar to estrogens. Estrogens are known to

produce increase in the uterine weight (Evans et.al. 1941).

In the same animals the uterine glucogen content was estimated. Table III clearly indicates that ethinyl estradiol has produced a significant increase in the uterine glycogen content and this increase is dose dependent. Van,der Vico (1942), has shown that estrogens increase the uterine glycogen. IdX₁ also has shown a dose dependent increase in the uterine glycogen content. Fig. VIII indicates a parallelism between the two compounds. These results indicate that IdX₁ has similar effects as that of ethinyl estradiol on immature ovariectomised rats.

In immature female mice the similar studies were made viz. the effect on uterine weight and on uterine water retention. Astwood (1938), has described an assay based on the observation that uterus responds to estrogens by increased uptake of water. Ethinyl estradiol has produced a significant increase in the uterine weight and also uterine water retention which is dose dependent similar effects were observed with IdX₁ (Table IV) Figures IX and X exhibit similarity and parallelism between the effects of these two compounds.

It appears from these results that IdX₁ is about 10 times less potent in immature ovariectomised rats and about 50 times less potent in immature mice than estrogen. It shows that immature ovariectomised rats are more sensitive to IdX₁ than immature mice. Emmens (1950) has shown that immature ovariectomised rats are more sensitive to estrogens than immature rats/mice.

The compound IdX₁ does not seem to possess either progestational activity in the estrogen primed rats because it has failed to produce any increase in the treated uterine horn over the untreated uterine horn either alone or in combination with progesterone. However, progesterone has produced a significant increase in the weight of treated uterine horn over the untreated uterine horn as compared to control. Progesterone is known to produce increase in weight of the traumatized uterine horn primed with estrogen (Greenwald, 1961).

It is well known that testosterone has got both androgenic and anabolic activities. In our experiments also it was observed that testosterone produces increase in the weight of seminal vesicles and of ventral prostate gland, which shows the androgenic activity. It also produced increase in the weight of levator ani muscle in castrated rats, which is the indication of anabolic activity (Eisenberg and Gordon, 1950). However, IdX₁ has failed to show any of these effects in castrated rats. It also failed to produce any effect on testosterone treated castrated rats.

Further IdX₁ was tested for its anti inflammatory activity using rat paw edema method (Winter et.al., 1952). Phenyl butazone at a dose of 100 mg/kg has produced about 70% protection, whereas IdX₁ even upto a dose of 500 mg/kg did not produce any protection. This clearly indicates that the compound under study does not possess anti inflammatory activity.

The general pharmacological screening of IdX₁ indicates that it does not possess any effect on other systems. On cat blood pressure it has failed to produce any effect 'persay' or on the

responses induced by standard agonists. However, Tiwari et al., (1965), have reported that ether soluble fraction of *Ipomoea digitata* has got hypotensive effect. They have also shown that the slowing of the frog heart in situ.

Similar observations were reported by Matin et al., (1969). However, they have isolated a glycoside known as paniculatin which produces rise in blood pressure and stimulates the heart. Since IdX₁ does not have either of these, it differs from the above two compounds.

On smooth muscle preparations like rat uterus and guinea pig ileum IdX₁ has failed to show any effect per se or on the responses induced by acetylcholin, histamine. The ether soluble fraction of *Ipomoea digitata* have been shown to have relaxant effect on smooth muscle preparations as reported by Tiwari et al., (1965) and Matin et al., (1969). However, the glycoside, paniculatin, has been shown to possess spasmogenic effect on smooth muscles. It is shown to produce va so constriction, broncho constriction and it is also shown to be oxytocic (Matin et al., 1969). This indicates that IdX₁ is steroid which is devoid of any effect on smooth muscles.

CONCLUSION

The tubers of the plant *Ipomoea digitata* contains a number of steroids. One of them is found to be β -sistosterol. The top fraction of the TLC, which has been studied in detail for its biological activity is also a steroid and the structure of it is yet to be elucidated. This steroid, coded as IdX₁, possesses a strong estrogenic activity as evidenced by its effects on the uterus of the immature ovariectomised rats and immature mice. It is about 10 times less potent than ethinyl estradiol. The estrogenic activity seems to be specific because it has failed to exhibit other steroidal activities like progestational, androgenic, anabolic and antiinflammatory activities. It does not possess any other biological activities.

Since the compound is having estrogenic activity, it may possess antifertility activity too. And if proved, the drug will be of a great help in the population control.

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PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES
ON XIPHOEA DIGITATA

A
SYNOPSIS
SUBMITTED TO THE
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SYNOPSIS

Name of the candidate	K.S.ATHAWALKAR
Degree	M.Pharm(Tech.)
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Subject	Phytochemical and pharmacological studies on <i>Ipomoea digitata</i>
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Steroidal drugs introduced in therapeutics have played a very important role in reducing human suffering in diseases. The demand for steroidal drugs, particularly the antifertility agents, have risen to a very high level. Consequently, easily available natural sources have been searched for intermediates for steroid synthesis. This has given a big boost to phytochemistry. The search for antimalignant drugs has also caused the chemist to look forward to the plant kingdom for the newer groups or nuclei active in cancer. This search has also been fruitful and a large number of organic molecules which even the modern synthetic chemist would not have dreamt of have shown promising anticancer activity.

A tuber of plant (*Ipomoea digitata*) was chosen for detail investigation so as to isolate pure steroids. Our literature survey did not reveal any work done on the presence of steroids except B-sitosterol reported by

Tewari et al (1964) in this plant. However our preliminary work on petroleum ether extract exhibited the presence of steroid nucleus by Libermann - Burchard reaction.

It was, therefore, decided to isolate the steroid from petroleum ether extract of the tuber of this plant, which is different from D-sitosterol, which was confirmed by testing it, on thin layer chromatography, using solvent system chloroform:pet.ether (90:10).

PHYTOCHEMICAL STUDIES

An attempt was made to isolate and purify the compound giving positive Libermann-Burchard reaction. The following flow chart shows the procedure adopted by us for the isolation of the steroid:

Ipomoea digitata tubers
dried, powdered and
extracted with Pet.ether
(40-60 °C)

Adsorption column chromatography
on activated silicagel
Eluted successively with pet.ether-
chloroform mixtures

Compound Idx₁

PHYSICO-CHEMICAL PROPERTIES

The compound isolated by column chromatography on activated silicagel as mentioned above was waxy in nature. It was highly soluble in chloroform, pet. ether and partially soluble in acetone, insoluble in methanol. Qualitative elemental analysis showed that the absence of nitrogen, sulphur and halogens. Quantitative analysis showed that carbon is 75.76%, hydrogen 11.81% and oxygen 12.43% giving a probable empirical formula $C_{10}H_{18}O$.

The R_f value of the compound (IdX_1) as tested on silicagel TLO with chloroform:pet. ether (90:10) was 0.76. The compound was tested for the presence of steroid. It gave appositive Libermann-Burchard reaction indicating the presence of steroid. Tests for other classes of compounds (such as alkaloids, glycosides, salponins etc.) were negative.

The U.V. spectrum of the compound shows absorption maxima at 250 nm. The I.R. spectrum shows peaks at 1220 cm^{-1} , 1420 cm^{-1} , 1560 cm^{-1} , 1750 cm^{-1} , 2880 cm^{-1} , 2960 cm^{-1} and 3040 cm^{-1} . NMR and mass spectra analysis is in progress which will assist in elucidation of the possible structure of the compound.

BIOLOGICAL ACTIVITIES

The IdX_1 was further screened for the following biological activities.

(A) Anabolic activity

Anabolic was studied by the method of Herzhberger et al, 1953, in castrated rats using testosterone (50 µg/rat/day) s.c. as a standard anabolic agent. The castrated rats were divided into three groups of 6 rats each. The groups received separately (i) vehicle only (olive oil), (ii) Testosterone (50 µg/rat/day) s.c.; (iii) IAX₁ (250 µg/rat/day) s.c. daily for seven days. Twenty four hours after the last dose the animals were sacrificed and the levator ani muscle was dissected out and weighed. The weights of the levator ani and from animals of different group were compared. No significant change was observed in the weight of levator ani muscle in the drug treated group.

(B) Androgenic/antiandrogenic activity

These activities were studied in the method of Dorfman and Steven (1960) in castrated rats using testosterone (50 µg/rat/day) s.c. as a standard androgenic agent. The castrated rats were divided into four groups of 6 rats each. The groups received separately (i) vehicle only; (ii) testosterone (50 µg/rat/day) s.c. (iii) testosterone + IAX₁ (250 µg/rat/day) s.c. and group (iv) IAX₁ (250 µg/rat/day) s.c. The seminal vesicles and ventral prostate gland weights were compared. No significant androgenic or anti-androgenic activity was observed.

(C) Estrogenic/antiestrogenic activity

These activities were studied by uterine weight assay method, as described by Evans et al (1941). The ovariectomised rats were divided into four groups of 6 rats each. The groups received separately (i) vehicle only (ii) ethinyl estradiol (0.1 mg/rat/day) s.c. (iii) ethinyl estradiol + Idx₁ (0.5 mg/rat/day) s.c. in vehicle and group (iv) Idx₁ (0.5 mg/rat/day) s.c. in vehicle. The uterine weights of various groups were compared. The compound Idx₁ showed significant estrogenic activity in the drug treated group. It also showed additive estrogenic effect in the group (iii) which had received a combination of ethinyl estradiol and Idx₁.

(D) Progestational/antiprogestational activity

These activities were studied by the deciduoma formation described by Astwood (1939) modified by Zarrow et al 1958. Ovariectomised rats were primed with 17- β -estradiol (1 mg/day) s.c. for four days. The rats were divided into four groups of six rats each. The groups received separately (i) vehicle only (ii) progesterone (0.5 mg/day) s.c. group (iii) received progesterone (0.5 mg/day) s.c. + Idx₁ (2.5 mg/day) s.c. and group (iv) received Idx₁ (2.5 mg/day) s.c. only for nine days. On day 5th one of the uterine horns was traumatized whereas the other acted as control. Twenty hours after the last dose animals were sacrificed and both the uterine horns were removed and weighed.

separately in the individual animal. The percentage increase in the weight of the horn due to traumatization was compared between the various groups of animals.

No significant change in the uterine horn weights of different group was noted.

(E) Anti-inflammatory activity

This activity was studied by inducing the edema by Casearagenin in rat foot paw described by Winter et al (1962).

The rats were divided into three groups of 6 rats each. The groups (i) control (ii) standard phenylbutazone (100 mg/kg, orally) (iii) IdX, 300 mg/kg orally. The paw volumes of coragenin treated control phenylbutazone treated and IdX, treated were measured. No significant anti-inflammatory activity was found for the compound IdX, thus indicating that the drug has no anti-inflammatory activity.

OTHER PHARMACOLOGICAL ACTIONS

(a) Effect of IdX, on rat uterus: (De Jalon et al, 1945)

IdX, (100 µg/ml) caused contraction of the rat uterus (isolated) primed with estradiol. The drug has a slow onset of action.

(b) Effect of IdX, on isolated auricles

IdX, did not show any activity on isolated auricles

auricles of G.pig upto the dose range 100 μ g/ml.

(c) Effect of IdX₁ on isolated rat phrenic nerve diaphragm
(Dulbring, 1945)

IdX₁ upto the dose 100 μ g/ml did not in any way modify either the indirectly or directly evoked responses.

(d) Effect of IdX₁ on cat B.P.

The IdX₁ did not show any effect on cat blood pressure in doses upto 1 mg/kg.

Summary

From the experimental data it is seen that, the petroleum ether extract of the tubers of the plant possesses probably a steroid, which has not been described. The steroid exhibits a very promising estrogenic activity.

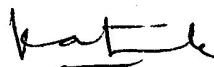
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Statement Under University Rule No. 0.412 and 0.413

As required by the University Rule No. 0.412 and 0.413,
I state that the work presented in this thesis is of
original in nature and so far to the best of my knowledge
has not been submitted for any degree of this or any
other University.

Whenever reference has been made to the work of
others, it has been clearly indicated along with the
source of information in the 'Bibliography'.



(K. S. Atrawalker)

Signature of the Candidate

Forwarded through the Research Guide



(Dr. M.B. Bhide)

Signature of the Research Guide.

