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Assessment of lactogenic potential of some traditional herbs

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SUMMARY

The entire plant of *Leptadenia reticulata* (Asclepidaceae) is extensively used as lactogen, traditionally, in veterinary practice. The plants of *Dregea volubilis* and *Pentatropis microphylla* (Asclepidaceae) are now used as its substitute and sometimes replace the original drug as traditional lactogen. The lactogenic potential of these drugs was studied in rats using, pup weight, weight of mother, parenchyma percentage, secretary rating, estimation of total protein content and glycogen content of mammary glands tissues as assessment parameters. HPTLC profiles of bioactive extracts were also generated to serve the authentification needs. The results of present studies show that *P.microphylla* forms a better substitute over *D. volubilis.*

Key words: *Leptadenia reticulate; Dregea volubilis; Pentatropis microphylla*; Lactogenic activity

INTRODUCTION

Leptadenia reticulata (L.reticulata) Wight & Arn. (Asclepidaceae) is termed as *Jivanti* in central and western part of India. Tribes utilize this plant as stimulant, tonic and in treatment of diseases related to reproductive system (Basu and Kirtikar, 1964). Traditionally the plant is reputed as lactogenic agent (Williamson, 2002) and studies have showed an enhanced development of mammary glands in lactating rats (Anjaria *et al.*, 1975). Clinical studies showed lactogenic properties of *L.reticulata* and one of its herbal formulations (Bhandari and Soni, 1979). Stigmasterol, tocopherol (Anjaria *et al.*, 1974), triterpene alcohol (Laxmanan and Subramanian, 1977), pregnane glycosides (Shrivastava *et al.*, 1994), flavonoidal aglycones and flavonoidal glycosides (Nair and Subramanian, 1968) were isolated from the aerial parts of *L.reticulata.*

Other two plants, *Dregea volubilis* Benth. ex Hook.f. (Gupta, 1985) and *Pentatropis microphylla* Wight & Arn are also described traditionally having similar therapeutic properties like that of *L. reticulata* (Vaidya, 1982).

Since these substitute plants have never been screened for their lactogenic potential, the present studies were planned to assess lactogenic potential and compare the results with that of *L.reticulata,* being a lactogenic drug in veterinary practice in India.

D.volubilis (Asclepiadaceae) is also described as *Jivanti* in *Ayurveda* sometimes supplied as substitute for *L.reticulata.* The plant is utilized as tonic, aphrodisiac and as a cure for burning sensation (Basu and Kirtikar, 1964). The seeds of *D.volubilis* possess ester glycosides with sterol genins (Venkta Rao *et al.*, 1967), while stems, leaves and bark showed presence of taraxerol and taraxerol benzoate, two

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steroidal compounds, kaempferol along with other unknown phytoconstituents is also reported with pregnane aglycones (Venkta Rao *et al.*, 1969, 1971). Different extracts of flowers of *D.volubilis* showed presence of volubiloside A, B, C and new polyhydroxy pregnane glycosides dregelol, volubilogenone and volubilol along with pregnane derivatives; drevogenin, isodrevogenin and 17- alpha-marsdenin (Sahu *et al.*, 2002; Panda *et al.*, 2003). The juice of the leaves was found effective in treatment of experimentally induced diabetes in rats (Shetty *et al.*, 1997). The plant showed anti-fibritic potential due to presence of pregnane and steroidal derivatives (Yoshimura *et al.*, 1983). The aqueous extract of the leaves were found to be hepatotoxic in rats (Tennekoon *et al.*, 1991).

P.microphylla (*Asclepidaceae*) is known as *Kakanasa* in *Ayurveda* and is also used as substitute of *Jivanti* in the treatment of diseases of reproductive system (Basu and Kirtikar, 1964). Traditionally aqueous extract of roots is used in treatment of gonorrhea. Different extracts of the aerial parts of the plant showed presence of octacosenol, alpha-amyrin, friedelin, beta- sitosterol, salicylic acid and some unidentified triterpenoids (Saraswathy *et al.*, 1990). Petroleum ether (pet.ether) and methanol extracts of all the three plants were selected for present studies. The basis of selection was the probable presence of phytosterols in pet.ether extracts and triterpenoidal saponins in methanol extract and their structural similarities with stigmasterol; a known lactogenic compound (Anjaria *et al.*, 1975).

MATERIALS AND METHODS

The plants were collected from near by tribal areas of Junagadh District, Gujarat state. The samples were authenticated by Dr. U.A.Baxi, Head, Botany Department, M.D. Science College, Porbandar, Dist. Junagadh. Gujarat. India. The voucher specimens of *L. reticulata* (HDT/MR/20041801), *D. volubilis* (HDT/MR/20042503) and *P. microphylla* (HDT/MR/20041101) were deposited at Pharmacy Department, Herbal Drugs Technology Laboratory, The M. S. University of Baroda, Vadodara. The collected plant materials were dried in sun and then under the shade for 30 days. The aerial parts were then powdered and used for further studies. Standard sample of stigmasterol was procured from M/s. Hi Media Lab. Pvt. Ltd.

Preparations of extracts

The dried aerial parts of the plants were powdered to a coarse powder and then successively extracted with petroleum ether (60°C - 80°C) (Pet ether) and methanol using Soxhlet extractor. The extracts were evaporated in vacuum evaporator at 40°C and the dried extracts were then stored in vacuum desiccators. Extractive values of the extracts are noted in Table 1. Stigmasterol and domperidone were used as positive control.

Phytochemical studies of extracts

The extracts were subjected to preliminary phyto-chemical analysis. Percentage extractive value was determined for each extract on dried weight basis of plant materials and qualitative tests were performed to ascertain presence of various types of phytoconstituents. Comparative TLC (Co-TLC) fingerprinting profile of each extract was also developed (Bladt and Wagner, 1996).

Table 1. Extractive values of *L.reticulata, D.volubilis and P.microphylla*

Sr No.	Extract	Extractive value avergae ± S.D.
1	Pet.ether extract of *L.reticulata*	0.60 ± 0.03
2	Pet.ether extract of *D.volubilis*	2.68 ± 0.25
3	Pet.ether extract of *P.microphylla*	2.20 ± 0.41
4	Methanolic extract of *P.microphylla*	13.32 ± 0.78

The results are expressed as average of three experiments along with standard deviation (S.D.).

Detection of stigmasterol

Presence of stigmasterol in pet.ether extract of *L.reticulata*, *P.microphylla* and *D.volubilis* was ascertained by comparing R_f and Ultraviolet-visible spectra (UV spectra) of the corresponding spots obtained in tracks of extracts and standard stigmasterol using co-TLC.

Assessment of lactogenic activity

The assessment of lactogenic potential was determined by adopting reported methodologies with necessary modifications (Anjaria *et al.*, 1975).

Animals

Wistar female rats weighing about 200 - 250 g were utilized as experimental animals. The animals were housed in standard conditions of temperature, humidity and light. They were fed with standard rodent diet and water *ad libitum*. Animal experiments were performed after obtaining necessary approval of Institutional Animal Ethics Committee.

Preparation of dose

The extracts were suspended in arachis oil as vehicle and used in the treatment. The quantity of extracts was incorporated in such a manner so that 0.2 ml of the suspension represents the daily dose in mg/kg, calculated on the basis of weight of each animal. The route of administration was per oral. (p.o.)

The colony of Wistar female rats was set up for breeding. Six female pregnant rats were randomly allotted to each group. The litter size was reduced to 6 pups with each mother rat on the second day of the delivery of pups. Treatments were administered from the 3rd to 23rd day, where the day of the delivery was counted as day one. Weight of mother rat and pups were recorded daily from 1st day to 23rd day. Mamactomy of two pectoral and two abdominal mammary glands of mother rats was performed 23rd day. One abdominal mammary gland was preserved in 10% w/v formosaline solution and utilized for histological studies. The slides were stained with hematoxylline stain (Galighe and Kozloff, 1971) and the histology slides were observed for the alterations in the microstructure of the mammary glands. The other set of mammary glands tissues were preserved in deep freeze at -20°C for the estimation of total glycogen content, while pectoral glands isolated and preserved in the same manner were used to estimate total protein content. The observations on the following parameters were recorded and compared with the results of control obtained in similar manner to evaluate the lactogenic potential of selected extracts during lactation.

Histological studies

The studies include observation of intensity of lactation changes in cellular architect of mammary glands (Eroschenko, 1978) quantified by determining the secretary ratings and percentage of parenchyma tissues (Anjaria *et al.*, 1975).

Weight of pups and mother rats

The weight of both mother rats and pups on 3rd day and 13th day was determined and the difference was recorded.

Biochemical parameters

The stored mammary glands tissues were subjected to the estimation of total protein and glycogen content. The total protein content of mammary glands was determined using Lowry-Hartree method, which is Hartree version of the Lowry assay, improves the sensitivity, less likely to be incompatible with some salt solutions, provides a more linear response. Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reacts with molybdenum/ tungsten blue reagent (Hartree, 1972). Glycogen content of the mammary glands tissues was estimated using modified phenol-sulphuric acid. The dissected

mammary glands of the lactating rats were digested with potassium hydroxide to convert the glycogen in to basic sugar units. The liberated sugars present in the digest were then precipitated by addition of ethanol and quantitatively estimated using the phenol-sulphuric acid reagent (Lo *et al*., 1970).

Toxicity studies

Acute toxicity studies were carried out according to OECD guidelines (OECD guideline for testing of acute toxicity of chemicals). Female rats were selected as experimental animals. As aqueous extract of leaves of *D.volubilis* was hepatotoxic (Tennekoon *et al*., 1991) and the phytoconstituents of other test extracts are similar in nature, detailed hepatotoxicity studies were planned. Various biochemical parameters selected to assess the hepatotoxicity were alkaline phosphatase (Kind and King, 1954), serum GOT (Frankel and Reitman, 1957), serum GPT (Frankel and Reitman, 1957). The histological studies of the liver were performed to detect any disturbances in the integrity of the microstructures (Vinay Kumar *et al*., 2004). The text extracts were administered at 200 mg/kg body wt for 23 days. Studies on isolated hepatocytes were also performed to confirm the probable role of the extract in proposed liver injury (Sarkar and Sil, 2006). The viability of the cells was selected as the parameter of the assessment of toxic effects. Trypan blue cell exclusion assay was performed to assess the viability of hepatocytes culture (Visen *et al*., 1998).

Statistical analysis

Results were subjected to statistical analysis using one way test for analysis of variance (Anova) and dunnet's test. $P < 0.05$ was selected as the level of significance.

RESULTS AND DISCUSSION

Phytochemical studies

The results of phytochemical analysis of both the extracts revealed the presence of phytosterols, phenolic compounds, saponins and triterpenoids. HPTLC fingerprinting profiles also confirmed presence of these components (Figs. 1, 2). The presence of stigmasterol was confirmed by comparing the R_f and the UVspectra (Fig. 3) of the spots obtained in tracks of standard stigmasterol (Fig. 1(d)) and test extracts (Figs. 1(a), (b), (c)).

Toxicity studies

All the test extracts were found to be safe up to 990 mg/kg in toxicity studies performed as per OECD

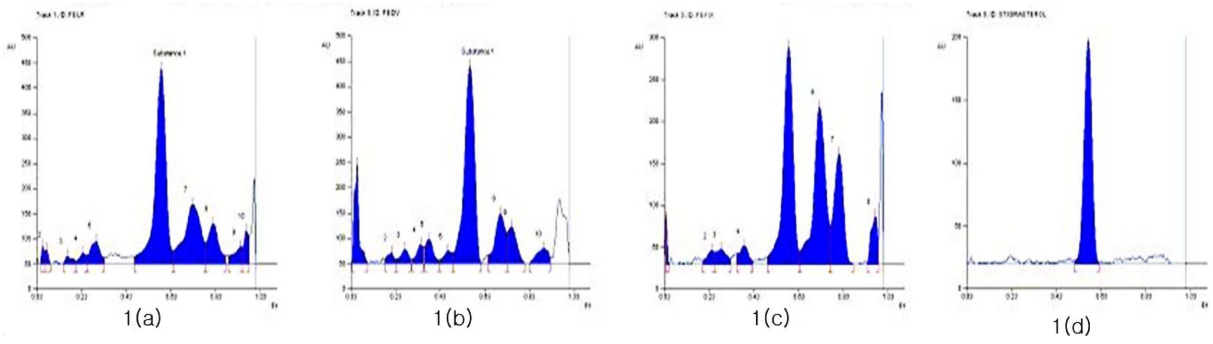

Fig. 1. HPTLC fingerprint of pet. ether extract of the selected plants along with the peak of stigmasterol standard. 1(a) PELR, 1(b) PEDV, 1(c) PEPM, 1(d) Stigmasterol. PELR: Pet.ether extract of *L.reticulata*, PEDV: Pet.ether extract of *D.volubilis,* PEPM: Pet.ether extract of *P.microphylla.* Plate 1(a) to 1(d): Toluene:Ethyl Acetate: Formic acid (9.0:4.5:0.05, v/v/v) was the solvent system and the plates were postchromatographed with 10% w/w sulphuric acid in methanol, heated at 110°C for 10 min and scanned at 366 nm using mercury lamp.

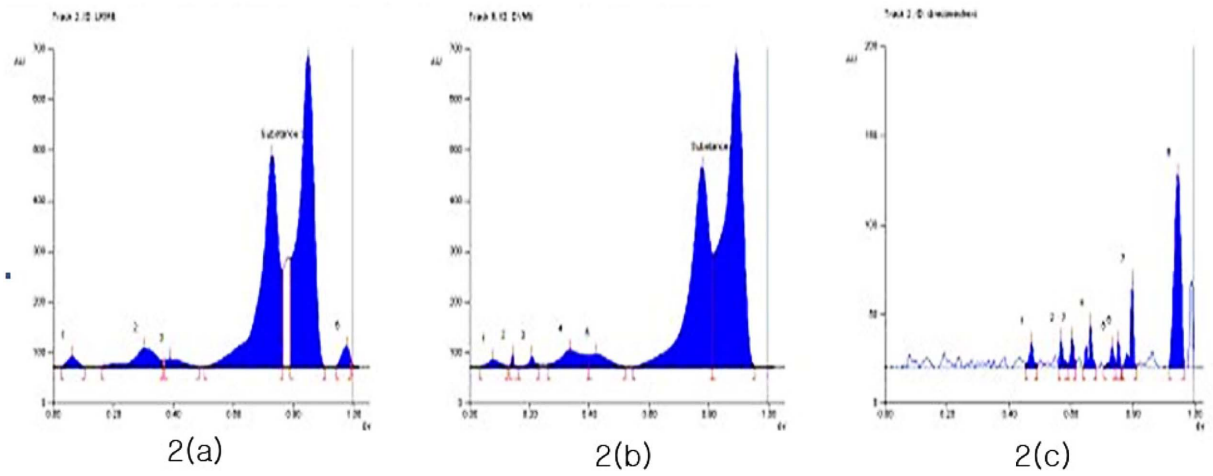

Fig. 2. HPTLC fingerprint of methanolic extract of the selected plants.
2(a) MELR, 2(b) MEDV, 2(c) MEPM. MELR: Methanolic extract of *L.reticulata,* MEDV : Methanolic extract of *D.volubilis,* MEPM: Methanolic extract of *P.microphylla.* Plate 2(a) and 2(b): Ethylacetate : Methanol : Water (2.5:0.65:0.5, v/v/v) was the solvent system utilized. Plate 2(c): Ethylacetate:Methanol:Water (10.0:1.35:0.1, v/v/v) was the solvent system for development of chromatogram. The plates were derivatized using Natural product polyethylene glycole reagent and after drying at room temperature they were scanned at 366 nm in fluorescence mode.

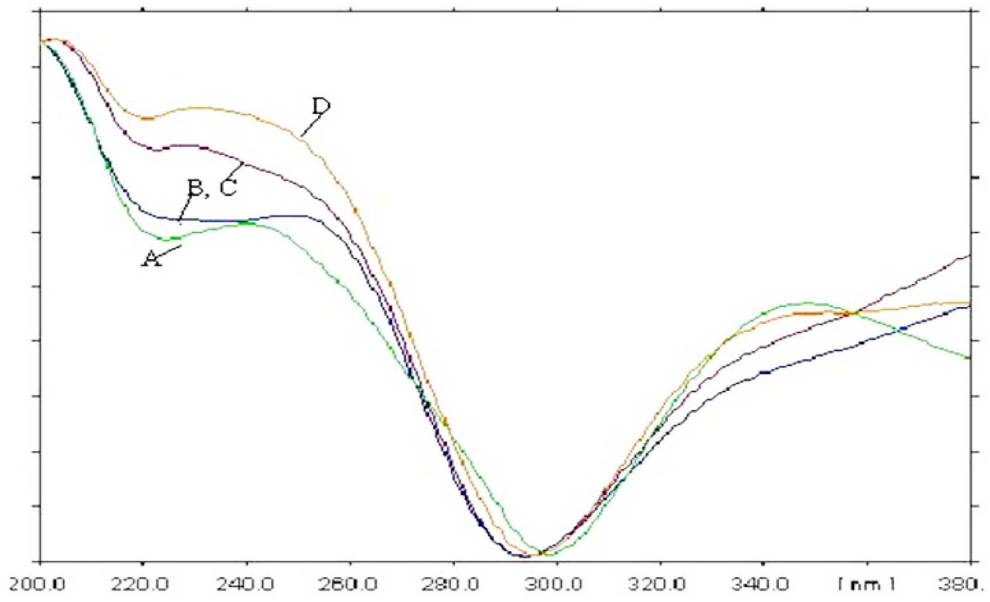

Fig. 3. Comparative UV spectra of stigmasterol standard and comparative spot in sample tracks.
Toluene: Ethyl Acetate: Formic acid (9.0:4.5:0.05, v/v/v) was the solvent system, the plates were scanned at 207 nm using Dueterium lamp and the UV spectra of the corresponding spots were taken. A: UVspectra of spot of stigmasterol standard spot. B: UVspectra of corresponding spot of stigmasterol in track of pet.ether extract spot of *D.Volubilis.* C: UVspectra of corresponding spot of stigmasterol in track of pet.ether extract spot of *L.reticulata.* D: UVspectra of corresponding spot of stigmasterol in track of pet.ether extract spot of *P.microphylla.*

guidelines. The results of the *in vivo* studies revealed no statistically significant alteration in the selected biochemical and histological parameters. *In-vitro* hepatotoxicity studies performed on cultured hepatocytes, revealed inability of the plant extracts to affect the viability of the culture at moderately high concentration (100 µg/ml of each extract) level, as compare to the negative control- consisted hepatocytes cultured in presence of vehicle only.

The animals of each group were subjected to assessment of mortality and behavioral changes daily while conducting the efficacy studies. There has been no mortality reported in test groups and in control groups at the reported dose levels in case of pet. ether extracts. In the case of methanol extract of *L.reticulata* and *D.volubiolis* the extracts did not elicit any physiological response at dose level 50 mg/kg body wt. whereas at 100 mg/kg body wt. two of the test animals became overexcited and killed all the pups, however, no mortality was observed at in case of mothers. In the case of methanol extract of *P.microphylla*, similar behavioral response was observed when the dose was increased to 200 mg/kg body wt. These extracts were therefore eliminated from further study. Since, pet. ether extract of the drugs possessing steroidal compounds did not show any such behavioral changes, may be due to the absence of methanol soluble phenolic compounds which in turn may not be responsible for lactogenic property.

Biochemical and histological studies

The results of animal experiments are tabulated in Table 2; indicate dose dependant increment in total protein content and glycogen content of the biopsiesd mammary glands in all treated groups. The increase in formation of milk requires more amount of normal sugar and proteins, therefore proportionate increase in reserves of tissue glycogen and proteins are necessary. The arithmetic mean amount of glycogen and total protein content in mammary tissues of treatment groups were found higher than those of the control groups, showing higher metabolic state of the tissues analyzed.

The lactation stage in mammary glands is characterized by enlargement of the alveoli and thus increment in parenchyma area with subsequent decrease in stroma is observed. The appearance of epithelial cells is cuboidal while the myoepithelial cells are flattened in shape (Mepham, 1983). The histological observations, from photomicrographs of the slides of each group shown as in Fig. 4 to 5 were recorded and quantitative determinations were made. The intensity of observed alterations is expressed in the form of secretory ratings. In case of all the treated groups the secretory ratings of the tissues increased significantly in a dose dependant manner, when compared to the respective control groups.

The yield of milk in any animal is the product of the output per active secretory cell and to the total number of such cells (Mepham, 1983). The parenchyma percentage represents the relative amount of the cells concerned with the lactation to the stroma. The parenchyma percentage is calculated as the ratio of area of parenchyma cell to the ground tissues in mammary gland stated in percentage. In present studies there has also been an increase observed in all test groups.

At the end of the 13th day, increment in body weight of pups and decrease in mothers' weight were observed and reported in percentage. According to the principles of bioenergetic, with the increase in the output of milk, the lactating mother rats lose body weights if increased energy output is not compensated by extra ration. The decreasing trend towards loss in mother rats body weights leads towards a conclusion that the effect may be secondary to lacto genesis in mothers (Anjaria *et al.*, 1975).

Prolactin is the hormone which ultimately controls the process of lactation (Guyton and Hall, 2006). The experiments are under progress involves role of the extracts assessed on serum level of prolactin, which will provide partly the information about mechanism through this extracts exerts lactogenic effect.

Table 2. Results of biochemical and histological studies

Sr. No	Description	Dose mg/kg body wt of animal per day p.o using pea nut oil as vehicle	Protein content mg/100 mg of wet mammary glands tissues	Glycogen content mg/100 g of wet mammary glands tissues	% parenchyma	Secretary rating	% alteration in mother's weight.	% alteration in pups' weight
1	Control	0.2 ml pea nut oil	9.261 ± 0.206	35.323 ± 1.083	57.771 ± 2.151	1.167 ± 0.105	-3.332 ± 1.467	110.707 ± 7.203
2	Positive control	4.5	12.014 ± 0.778***	47.438 ± 2.109*	67.563 ± 2.273***	2.666 ± 0.247***	-2.983 ± 0.436	159.760 ± 0.430
3	Stigmasterol	1.0	14.260 ± 0.300***	40.673 ± 0.5	65.989 ± 5.940*	2.166 ± 0.166***	-1.630 ± 0.220	140.467 ± 31.635
		2.00	16.988 ± 1.153***	53.564 ± 3.701***	77.891 ± 4.159***	2.750 ± 0.214***	-1.311 ± 1.109	178.750 ± 9.757
4	*L.reticulata* pet.ether extract	100	11.721 ± 0.579	47.732 ± 1.459	64.624 ± 2.275*	2.333 ± 0.166***	-3.385 ± 1.114	136.210 ± 11.147
		200	12.360 ± 0.835	54.458 ± 3.586***	69.495 ± 1.669***	2.416 ± 0.200***	-2.912 ± 0.970	158.460 ± 32.272
5	*D.volubilis* pet.ether extract	100	11.796 ± 0.224	48.450 ± 2.489*	63.998 ± 1.277*	2.333 ± 0.210***	-1.732 ± 0.907	100.030 ± 7.536
		200	12.689 ± 0.725***	52.239 ± 4.110***	73.408 ± 1.720***	2.666 ± 0.105***	-0.797 ± 0.158	107.653 ± 5.270
6	*P. microphylla* pet.ether extract	100	11.299 ± 0.730	37.430± 3.761	60.505 ± 2.361	2.083 ± 0.153*	-0.947 ± 0.203	149.13 ± 11.726
		200	12.491 ± 0.609***	55.331 ± 2.765***	65.358 ± 1.709*	2.333 ± 0.166***	-0.454 ± 0.311	152.307 ± 16.091
		400	12.454 ± 0.756*	67.907 ± 3.092***	72.653 ± 3.143***	2.583 ± 0.153***	0.210 ± 0.290***	154.693 ± 7.675
7	*P. microphylla* Methanolic extract	50	10.781 ± 0.379	38.867 ± 3.378	56.055 ± 3.698	2.416 ± 0.238***	-0.676 ± 0.163	165.368 ± 27.783
		100	11.934 ± 0.370	47.572 ± 3.894*	65.637 ± 2.546*	2.250 ± 0.281***	0.146 ± 0.348**	175.770 ± 14.121
		150	12.704 ± 0.531***	69.400 ± 2.641***	75.512 ± 2.636***	2.916 ± 0.238***	0.880 ± 0.258***	198.725 ± 9.643***
8	One way ANOVA	F	7.132	12.040	6.087	5.255	6.777	2.845
		df	13	13	13	13	13	13
		P	P< 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.005

N = 6, Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to respective controls. (-) sign indicates decrease. The test groups were compared to control group using ANOWA followed by dunnett's test.

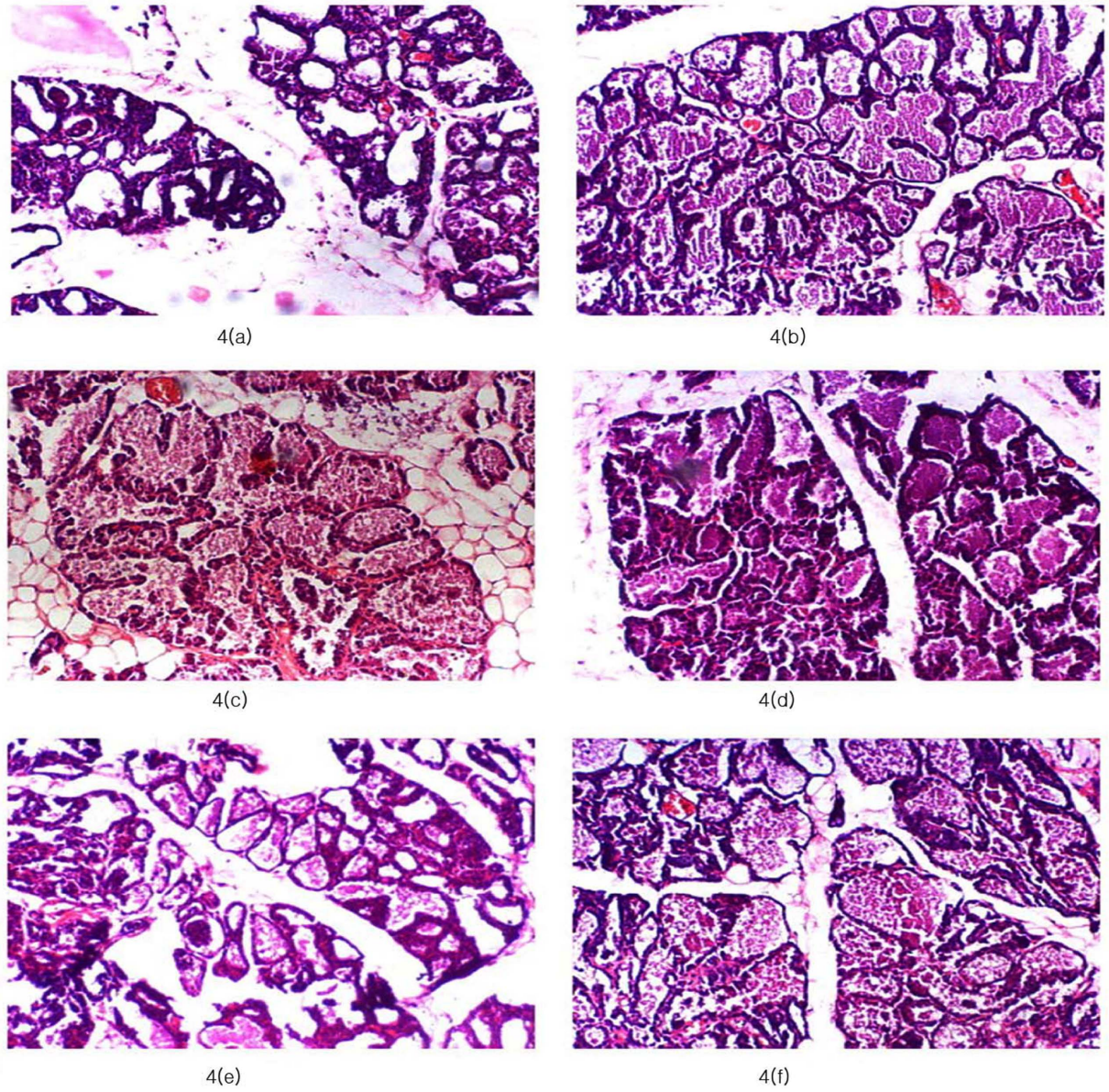

Fig. 4. Photomicrograph of Histology of Mammary glands.
4(a) control 0.2 ml Pea nut oil, 4(b) STG 2.0, 4(c) STG 1.0, 4(d) DMP 4.5, 4(e) LRPE 100, 4(f) LRPE 200. STG 2.0 = 2 mg/kg stigmasterol, STG 1.0 = 1 mg/kg stigmasterol,DMP. 4.5 = 4.5 mg/kg domperidone, LRPE 100 = 100 mg/kg pet.ether extract of *L.reticulata,* LRPE 200 = 200 mg/kg pet.ether extract of *L.reticulata.*

The results indicated *P.microphylla* is *better* substitute of *L.reticulata* when compared to *D.volubilis*. The studies also justify the selection of stigmasterol as marker for lactogenic potential of the plants and plant extracts.

The studies supports the traditional claims of *D.volubilis* and *P.microphylla* as therapeutic substitutes of *L.reticulata*, both of them can be utilized in place of *L.reticulata* as regular veterinary lactogens after through efficacy trials.

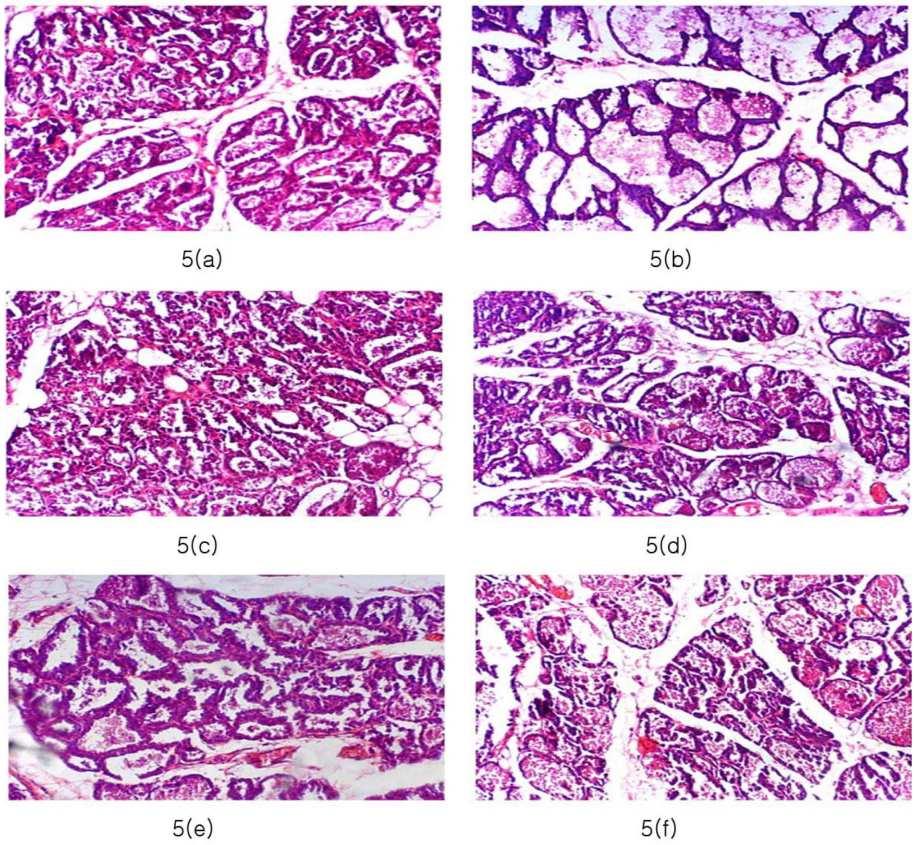

Fig. 5. Photomicrograph of Histology of Mammary glands.
5 (a) DVPE 100, 5 (b) DVPE 200, 5 (c)PMPE 100, 5 (d) PMPE 200, 5 (e) PMPE 400, 5 (f) PMPE 100. DVPE 100= 100 mg/kg pet.ether extract of *D.volubilis,* DVPE 200 = 200 mg/kg pet.ether extract of *D.volubilis,* PMPE 100 = 100 mg/kg pet.ether extract of *P.microphylla*, PMPE 200 = 200 mg/kg pet.ether extract of *P.microphylla*, PMPE 400 = 400 mg/kg pet. ther extract of *P.microphylla*, PMME 50= 50 mg/kg methanol extract of *P.microphylla.*

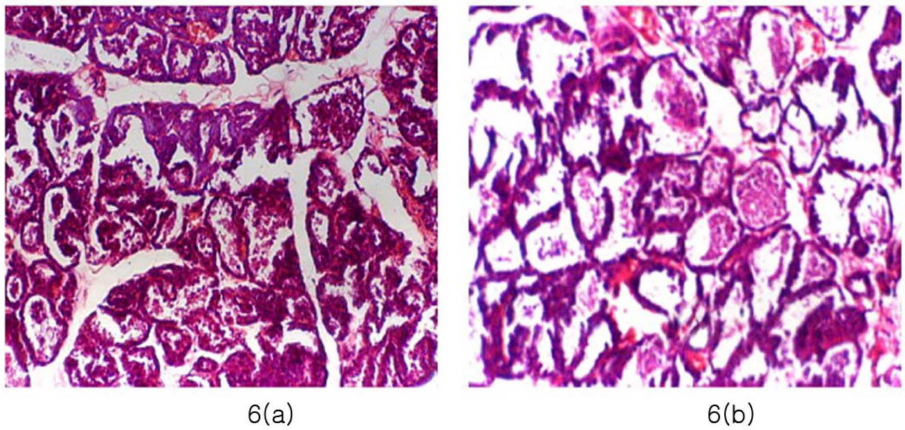

Fig. 6. Photomicrograph of Histology of Mammary glands.
6(a) PMME 100, 6(b) PMME 150. PMME 100 = 100 mg/kg methanol extract of *P.microphylla*, PMME 150 = 150 mg/kg Methanol extract of *P.microphylla.*

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