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A preliminary study on evaluating the galactagogic and antioxidant properties of *Bauhinia variegata* leaf extract on lactating mice

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Abstract
Mother's milk is important for survival, proper development and growth of the neonate for which continuous milk supply is essential. *Bauhinia variegata* is used as a galactagogue by various tribals of West Midnapore district, West Bengal, India but it lacks scientific basis.
Objectives: The present investigation was undertaken to investigate the potential of aqueous leaf extract of *Bauhinia variegata* in promoting milk production in lactating mice (*Mus musculus*) and its effect on growth of suckling pups and also to find its antioxidant status.
Methods: Galactagogue activity was evaluated in terms of quantity of milk produced from the mice treated with leaves extracts. Lactating mice (n=6 in each group) with at least 5 pups each were treated with the extract at 250 mg/kg and 350 mg/kg body weight while the control was administered with distilled water. A total of 18 lactating mother mice were used in the study. The mice were daily administered with the aqueous leaf extract via oral feeding with gavage which started from day 1 to day 15 and the performance of milk production were measured along the experimental duration by weight-suckle-weight method. Correspondingly the leaf extract was also subjected to qualitative, UV-Vis and GC-MS analysis for the presence of any suitable compound which enhances the milk production. Results were statistically analyzed by means of ANOVA and expressed as mean ± S.E., the growth rate of pups were measured along the experimental period.
Results: Aqueous leaf extract of *Bauhinia variegata* at both the doses significantly increased milk production (23.2%) than the control mice. The pups also gained weight significantly ($p<0.05$) with regard to control. Serum prolactin levels steadily increased from day 1 to day 8. However, the milk production was more in the mice treated with 250 mg/kg of leaves extract when compared to 350 mg/kg body weight. GS-MS chromatogram of the aqueous extract showed 20 major peaks besides a number of peaks with very narrow retention time. The fragmentation patterns for some of the peaks were compared with the library of NIST which revealed presence of several phytoestrogenic compounds. The information summarized here is intended to serve as a reference tool for ethno-pharmacologists and its scientificity.

Keywords: antioxidant, galactagogue, *Bauhinia variegata*, hormones

Introduction
Bauhinia variegata Linn (Caesalpiniaceae) commonly known as 'camel foot tree' is widely grown in India as an ornamental plant. It is traditionally used for the treatment of several diseases such as leprosy, bronchitis, tumors [1, 2]. Further it has been reported by several workers that it has chemo preventive properties [3]. The stem of the plant displays antibacterial and antiviral properties while the root shows anti-inflammatory activity [4, 5]. Phytochemical studies revealed presence of several flavonoids that have been isolated from seeds, stems and flowers [6-8]. After parturition continuously milk supply is essential for the new born baby and depends on maternal hormone secretion and these hormones include oxytocin which initiates and stops milk supply. Prolactin is another important hormone in this regard which also initiates and stimulates milk secretion [9]. Milk secretion is a complicated process because it depends on many factors viz. periodic removal of milk, emotion and stress. Removal of milk from the breast is also effected by MER (Milk Ejection Reflex) which in turn helps in the release of the hormone oxytocin. It is well known that lactogenesis is inhibited during pregnancy due to presence of placental steroids despite high levels of prolactin in blood, also during pregnancy alveoli and secretory ducts develop due to presence of oestrogen and progesterone [10, 11]. Breast feeding is quite problematic in urban areas because either the baby or new born cannot suckle properly or there may be some hormonal imbalance in the mother or if there is a problem in the breast tissue, also many females in the urban areas have the belief that suckling may alter shape of their breast.

As lactation primarily depends on hormone action which in turn also depends on emotion and stress so control of stress can effectively help in lactogenesis in an urban setting [12-14]. On the other hand, people residing in the rural areas often suffer from inhibition of lactation and they often depend on herbal galactagogues which are commonly available and less expensive. Further they often treat their farm animals with those herbs to increase their lactation. As far as our knowledge is concerned and after literature review there were less works where BV was used as herbal galactagogue. Hence the present investigation was conducted to evaluate whether *Bauhinia variegata* aqueous leaf extract can be used as herbal galactagogue in suitable mice model and if they do, what phytoconstituents are responsible for it?

Materials and Methods

Preparation of the leaf extract

The plant was collected from nearby villages of West Midnapore district, West Bengal, India during the month of March 2014 and identified. A voucher specimen has been deposited to Botany department (V-1244 MDC/2015) for record. Sundried leaves of Bauhinia variegate (henceforth BV) (200 g) were extracted in double distilled water (the ratio of plant material to solvent was 1:10m/v). The extraction was carried out at 50°C with constant stirring for 24 hours. The extract obtained was evaporated to dryness and stored at 4°C until required. The yield of the aerial part was 12.04% which was calculated by the following equation: Yield (g/100g of dry (plant material) =W1x100/W2, where W1 and W2 represented the weight of the extract after evaporation of solvent and the weight of the dry plant material, respectively.

Preliminary phytochemical screening

The presence or absence of phytochemical constituents was analyzed by routine procedures.

Shinodas test for Flavonoids: 100g of plant material was extracted with 5 mL ethanol and filtered. To 1 mL of the filtrate, magnesium ribbon and few drops of concentrated HCl were added. Pinkish red colour indicates presence of flavonoids.

Alkaloids: Plant material (25g) was boiled in 15 mL of 1% concentrated H_2SO_4 in 50% ethanol and filtered. To the filtrate 5 drops of NH_4OH was added followed by 15 mL chloroform and two layers were separated. The chloroform layer was extracted with 15 mL dilute H_2SO_4. On addition of 5 drops of Mayers reagent to the extract, a creamy red orange, brownish precipitate would indicate presence of alkaloids.

Tannins: To 2 mL of the filtrate, 1mL of ferric chloride was added, a blue to greenish black precipitate indicates presence of tannins.

Carbohydrates: 100 g of powdered leaves were boiled in 25 mL distilled water and filtered. To 1mL filtrate, 1mL of Molisch reagent was added followed by 1mL of concentrated H_2SO_4. Formation of reddish ring indicates presence of carbohydrates.

Reducing sugars: 1 mL of above filtrate was boiled with 2 mL of Fehlings reagent for 3-4 minutes. A brick red precipitate indicates presence of reducing sugars.

Glycosides: To 2 mL of the filtrate, 1mL of glacial acetic acid was added followed by 1 mL of ferric chloride solution. To this solution mixture 1 mL of conc. sulfuric acid was added, formation of green blue colour indicates presence of glycosides.

Steroids: 100 mg of powdered leaves was dissolved in 15 mL chloroform and filtered carefully. To 1mL of the filtrate obtained, 1mL of acetic anhydride was added followed by 500 µL of concentrated sulfuric acid, formation of deep blue green ring reveals presence of glycosides.

Total Phenolic content

The total phenolic content was determined spectrophotometrically following the method of Kim *et al* 2003 with minor modifications [15]. Briefly 1ml of the sample was mixed with 1 ml of Folin-Ciocalteau. After 5 minutes, 10 ml of 7% Na_2CO_3 was added followed by 10 ml of deionized water. They were mixed thoroughly and kept in dark for 90 minutes at room temperature. Then the absorbance was taken at 750nm against a suitable blank. The calibration curve was made by gallic acid solution and the total phenolic content was expressed as mg of gallic acid equivalents per gram of dried sample.

Antioxidant activity (DPPH free radical scavenging activity) of ethanolic extract

The antioxidant activity of the leaf extract of BV was conducted on the basis of the radical scavenging effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity [16]. Briefly diluted working solutions of the test extracts were prepared in ethanol. Ascorbic acid was used as standard in 50, 100, 150, 200, 400, 600, 800µg/ml solution. 0.002% of DPPH was prepared in ethanol and 1 ml of this solution was mixed with 1 ml of sample solution and standard solution (ascorbic acid) separately. These solution mixtures incubated in dark for 45 min and optical density was measured at 517 nm (Shimadzu UV-1800). Ethanol (1 ml) with DPPH solution (0.002%, 1 ml) was used as blank. The optical density was recorded and % inhibition was calculated.

Experimental animals

The study was conducted on random bred Swiss albino mice (*Mus musculus*) weighing about 23-28 g body weight under the supervision of Institutional Animal Ethical Committee (Midnapore College, West Bengal, India, Registration No: 1617/GO/a/12/CPCSEA, Ministry of Environment and Forest, Government of India). A total of 18 lactating dams with at least 5 pups each were used for the test.

They were divided into 3 groups, Group I served as normal control fed with distilled water, Group II, and III mice received orally aqueous leaf extract of BV at 250mg/kg b. wt., and 350 mg/kg b. wt. These two doses were selected based on acute and sub-acute studies. The milk productions of the dams were measured by the method of Lompo-Ouedraogo *et al* 2004 [17]. Briefly the pups were weighed at 8.00 A.M. which was considered (W1), then the pups were isolated from the mother for 6 hours and weighed again (W2), then the pups were again returned to their mother to feed for 1 hour and subsequently they were weighed again (W3). The daily milk yield was corrected for the loss of weight caused by the various metabolic processes in the pups during the suckling period (6 times). The value used was W2-W1/6. This value was multiplied by the amount of suckling hours per day and added to the daily suckling gain. Everyday gain in pup weight was measured from W2.

Prolactin levels: Serum prolactin levels were analyzed by the method of Tietz 1995 following manufacturers protocol using Elisa kits (Kruise Pathline, Ahmedabad, India) [18]

Ultraviolet visible absorption analysis (UV)
One g of BV leaf powder was kept overnight with 25 ml of distilled water with constant stirring and then filtered. An aliquot of the filtered sample was scanned using UV-visible Spectrophotometer (Shimadzu, UV-1800), at a range of 190-450 nm (scanning speed-medium, and slit width 1), to detect the characteristic wavelength of the leaf extract.

GC/MS analysis of aerial parts
GC/MS analysis of aqueous leaf extract of BV was conducted by Gas chromatograph coupled to a mass spectrophotometer equipped with a fused capillary column, Model No: Agilent 190915-433 (HP-5MS, 0.25mm×30m ×0.25µm). For GC/MS detection on electron ionization system with ionization energy of 69.9eV was utilized. The carrier gas was Helium (99%) with a constant flow rate of 1mL/min. The volume of the sample injected was 5µl in GC grade ethanol with an average velocity of 37cm/sec. The initial temperature of column was 50°C for 5 min and then it was programmed to 280°C. The total GC running time was 28 minutes.

Statistical analysis
Data on several biochemical estimations were analyzed by one-way ANOVA and expressed as mean ± SD from at least 3 separate observations. A probability of $p<0.05$ was considered statistically significant.

Results
The yield of the aqueous leaf extract of BV was 11.26% and table 1 represents qualitative estimation of various phytochemicals present in the extracts. The antioxidative property of this extract may be due to the presence of phenolic content which was 32.88±0.27 mg/ml, (gallic acid equivalent per 100 mg leaf extract). The aqueous leaf extract showed a dose dependent inhibition of DPPH activity with about 50% inhibition at concentration of 400 µg/ml compared to the standard ascorbic acid. Acute toxicity studies for 96 hours and subacute studies for 30 days with the aqueous leaf extracts reveal that even at higher concentrations the aqueous extract was not toxic to the animal's, occasional salivation and corner sitting was noted on cage side observation at 1900mg/kg b. wt. administration (table 2). The total phenolic content in the fraction of aqueous extract was 79.26 which was statistically more than that of control. Figure 1 showed percentage weight gain in pups which were based on n=5, with minimum 5 pups per litter. Weight of all pups increased at a steady state from day 1 to day 15 in all the groups, however the weight of pups increased considerably in pups fed with 250mg/kg b.wt when compared to 350 mg/kg wt. and normal controls which were statistically significant ($p<0.05$). UV-vis analysis of aqueous extract of BV revealed that it has two major peaks when scanned from 190 to 450 nm, the peaks were at 333.5nm and 271.5 nm with absorbance of 0.579 and 0.728 respectively (Figure 1a). It was also observed that that daily milk yield increased by about 23.2% in mice which were fed with 250 mg/kg b.wt of BV when compared to 350 mg/kg b.wt and normal controls and which were statistically significant (figure 2). When the variation of mean serum prolactin levels was analyzed from the serum of mice (mothers) it was observed that the mothers fed with BV 250mg/kg b.wt. were more at day 1 to day 8 when compared to normal and mice fed with aqueous BV 350 mg/kg b.wt. But it dropped significantly in mice fed with 350 mg/kg b.wt at day 3 when compared to distilled water controls and mice fed with 250 mg/kg b.wt. which we could not ascertain as to why this occurred. Further, there was a lacuna in the study of serum prolactin levels because we could not carry on till day 15, due to low serum levels and were also concerned that regular drawing of blood might harm the mother and subsequently the pups.
GC MS study reveals presence of about 20 different compounds with various retention time, molecular weight and peak area which have been presented in table 3. The prevailing compounds were 1 dodecanol, 3,7,11 trimethyl (6.05), Hexadecanoic acid ethyl ester (9.89), phytol (12.3), Squalene (9.8) and β-sitosterol (5.15).

Table 1: Preliminary phytochemical screening of BV leaf extracts.

Chemical compounds	Results
Flavonoids	++
Alkaloids	+++
Tannin	+
Carbohydrate	++
Glycosides	++
Reducing sugars	+
Steroids	++

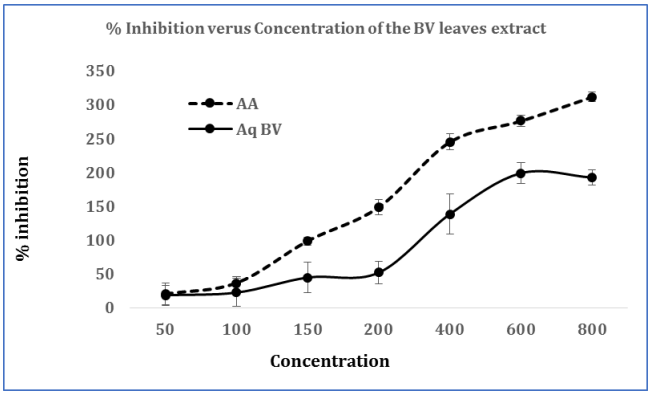

Fig 1: Percentage inhibition of aqueous leaf extract of BV against standard (Ascorbic acid AA)

Table 2: Showing acute and sub-acute toxicity studies, mortality and symptoms of mice treated with various concentration of ethanolic extract of *Bauhinia variegata.*

S. No	Dose/day	Mortality	Symptoms within 1 to 2 hours after administration of BV
Acute toxicity study [96 hours]			
1.	100 mg/kg	0/6	Nil
2.	200 mg/kg	0/6	Nil
3.	500 mg/kg	0/6	Nil
4.	900 mg/kg	0/6	Nil
Sub-acute toxicity study [30 days]			
1.	1200 mg/kg	0/6	Corner sitting, Sniffing
2.	1500 mg/kg	0/6	Salivation and corner sitting
3.	1900 mg/kg	0/6	Salivation, Corner sitting, Drowsiness, Sniffing each other

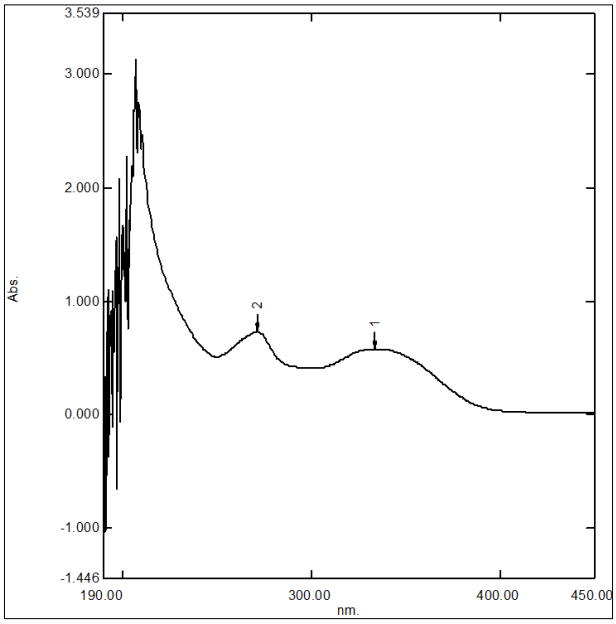

Fig 1a: The UV-vis scanning of aqueous extract of BV.

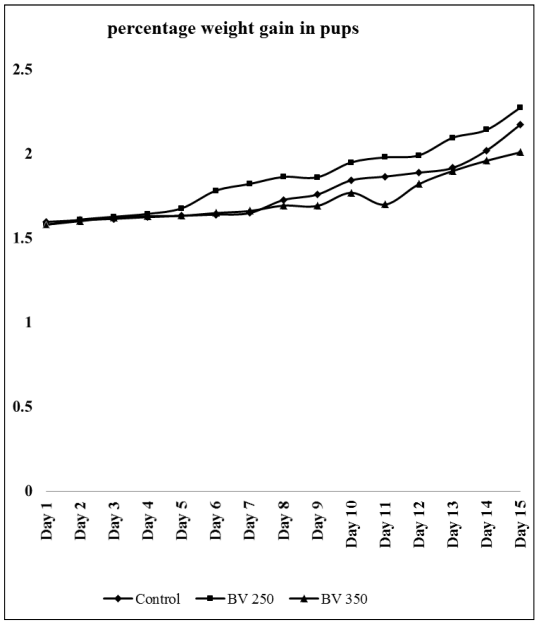

Fig 2: Graph showing percentage weight gain in pups from day 1 to 15.

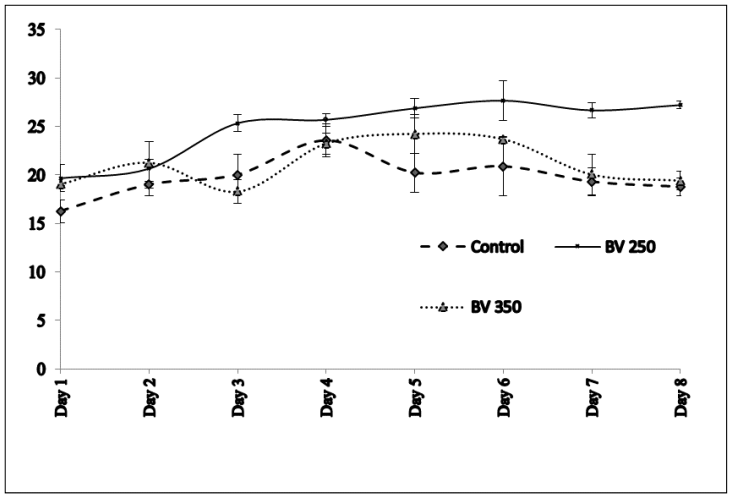

Fig 3: Variation in serum prolactin levels in pg/ml during 8day study period

Table 3: List of compounds identified by GC-MS from aqueous leaf extract of *Bauhinia variegate.*

S. No.	RT	Name of the compound	Molecular Formulae	Molecular Weight	Peak Area %
1.	8.03	2-Butyl-2,7-octadien-1-ol	$C_{12}H_{22}O$	182	3.01
2.	8.37	1-Dodecanol,3,7,11-trimethyl-	$C_{15}H_{32}O$	228	6.05
3.	8.94	2H-3, 9a-Methano-1-benzoepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3α, 5aα, 9α, 9aα)]	$C_{15}H_{26}O$	222	0.94
4.	13.44	Tert-Hexadecanethiol	$C_{16}H_{34}S$	258	3.56
5.	13.95	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	9.56
6.	14.27	13-Heptadecyn-1-ol	$C_{17}H_{32}O$	252	2.25
7.	14.50	9,12-Octadecadien-1-ol, (Z,Z)-	$C_{18}H_{34}O$	266	2.67
8.	14.90	1,2-Benzenedicarboxylic acid, butyl cyclohexyl ester	$C_{18}H_{24}O_4$	304	1.54
9.	15.10	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	3.47
10.	15.5	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	19.02
11.	16.00	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	9.89
12.	17.38	[1,1'-Bicyclopropyl]-2-octanoic acid, 2'-hexyl-,methyl ester	$C_{21}H_{38}O_2$	322	2.27
13.	17.48	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	4.30
14.	17.64	Phytol	$C_{20}H_{40}O$	296	12.30
15.	18.43	9,12-Octadecadienoic acid, methyl ester, (E, E)-	$C_{19}H_{34}O_2$	294	0.23
16.	18.44	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296	0.12

17.	18.94	Ethyl iso-allocholate	$C_{26}H_{44}O_5$	436	0.71
18.	23.77	Cyclopropaneoctanoic acid, 2-[2-pentylcyclopropyl)methyl]-, methyl ester, trans, trans-	$C_{21}H_{38}O_2$	322	3.17
19.	28.04	Squalene	$C_{30}H_{50}$	410	9.80
20.	37.28	β-sitosterol	$C_{29}H_{50}O$	414	5.15

Discussion

Food intake quality of the mother during pregnancy is important for the growth and development of pups. Traditionally people of several countries uses herbal galactagogues for increasing milk production sometime they use extracts of herbs or apply them as paste [19, 20]. They also use the same for their farm animals. Investigation done by various scientist showed that various plants have the ability to increase milk production such as *Trigonella foenum graecum*, milk thistle, asparagus, *Coleus amboinicus and Musa paradisiaca flower* [19-24]. The results of the present investigation suggest that the aqueous extract of BV contains numerous constituents that are capable of donating their hydrogen to a free radical thereby scavenging them. GC-MS analysis reveals a phytoestrogenic compound such as β-sitosterol which might be responsible for increasing milk production. Further research in this direction are required to validate the above findings. Further, phytoestrogenic compounds such as β-sitosterol may also be responsible for the formation of the estrogen hormone which helps in the development of milk ducts, alveoli of mammary glands and also increases concentration of the estrogen receptors [24]. On the other hand, several compounds such as phytol, squalene, hexadecenoic acid, octadecanoic acid and flavonoids present may be responsible for the antioxidant properties which might be due to action of a single compound or conjoint action of all the compounds. The donation of the electron can be measured by bleaching of DPPH (2, 2'-diphenyl-1-picrylhydrazyl) radical, where de-colourization of DPPH occurs due to addition of radical species or antioxidants and which is proportional to the concentration of the antioxidants. In the present investigation, we found that decrease in the absorbance of the reaction mixture which indicated significant free radical scavenging activity of the aqueous leaf extract of BV. The results of the present investigation suggest that aqueous extract has potential to be used as a galactagogue and a potent natural source of antioxidants and may be useful. Our findings also support the ethnomedicinal use of the leaf extracts for galactagogic purpose and would raise confidence among consumers towards its safety and effectiveness.

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