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EXPERIMENTAL PAPER

Phytochemical screening and galactagogic attributes of *Pergularia daemia* (Forssk.) Chiov. leaf extract in lactating mice (*Mus musculus*)

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Summary

Introduction: *Pergularia daemia* (Forssk.) Chiov. [PD] is used as a galactagogue by various tribes in Purulia district, India, lacks a scientific basis.

Objectives: The purpose of the study was to determine whether administration of aqueous leaf extract of *P. daemia* can increase milk production in lactating mice and encourages development in suckling pups.

Methods: 30 lactating mice (n=6 per group) with a minimum of 5 pups each were treated with the aqueous PD extract at 50, 100, 150 and 200 mg/kg body weight while the control group was administered with only distilled water. The mice were daily fed with aqueous leaf extract *via* gavage, which started at day 3 and continued to day 15 post-partum and milk secretion was assessed by the weight-suckle-weight method. The qualitative estimation, UV-vis analysis and GC-MS study of the leaf extract was also done for the presence of phytoconstituents.

Results: Administration of aqueous leaf extract led to a considerable increase in milk production (28.1%) and the pups gained weight substantially ($p < 0.05$) when compared to control. Milk production was enhanced in mice treated with 150 mg/kg b.wt. of leaf extract compared to other doses ($p < 0.05$). The GC-MS analysis of the aqueous leaf extract of *P. daemia* revealed 20 major peaks, some with small retention time and also revealing presence of several phyto-estrogenic compounds.

Conclusion: The present study effectively demonstrated that aqueous leaf extract of PD enhanced milk production in mice and might be used as an alternative strategy for increasing milk production and can be extrapolated to farm animals and human beings.

Key words: GC-MS, hormones, lactation, phytochemicals, *Pergularia daemia*

Słowa kluczowe: GC-MS, hormony, laktacja, związki bioaktywne, *Pergularia daemia*

INTRODUCTION

Food intake, digestibility, stimulation of the immune system, and increasing milk yield are some of the criteria necessary for the development of the young. Due to endocrine anomalies and stress, milk production often decreases in lactating/nursing mothers [1, 2]. There are numerous synthetic galactagogues but their efficacy was never tested. They are also not easily available to people of low socio-economic status and living in remote areas. Continuous milk supply is essential for neonatal growth. People believe that in addition to herbal galactagogues, consumption of watery foods and green vegetables increases lactation. Galactagogues are substances, foods, or herbal complements used to initiate, continue, or increase breast milk production. Currently, some herbal galactagogues have gained prominent acceptance in the scientific community as a substitute for allopathic drugs for increasing milk production [3-8]. Traditional folk use diverse parts of plants as galactagogues but they have not been validated. However, their use is still prevalent in many countries and are also commonly cultivated.

In India, the tribal people constitute about 8.6% of total population. These tribes live in small habitats in remote areas with no proper access to education and healthcare. They are considered to be the weakest segment in terms of socio-economic status with their socio-cultural pattern varying from tribe to tribe and from region to region. Purulia district of West Bengal is predominantly inhabited by tribal communities which include Santhals, Bhumij, Munda, Lodha, Kora, and Mahali. These tribal communities use various plant parts to increase milk production in farm animals. Tribal quacks usually administer them also to humans. *Pergularia daemia* (Forssk.) Chiov. is used as a galactagogue by various tribes but lacks scientific basis and has no literature available in various scientific databases. In such a set-up, there is a necessity to estimate its safety and efficiency as an herbal galactagogue.

Pergularia daemia (PD) belongs to the *Asclepiadaceae* family, vernacularly known as “Uttaravaruni” in Sanskrit, “Chagulbanti” in Bengali (locally), and “Utranajutuka” in Hindi. Several parts of PD were stated to have parasitocidal and antipyretic potential and often used for treatment of diarrhoea in infants and also intermittent fevers [9]. Furthermore, the aerial parts of PD have been reported for its various pharmacological properties: hepato-protective, anti-fertility, anti-diabetic, antimicrobial, anti-inflammatory, and wound healing potentials [10-15]. Other investigators reported the potential protective effect of this plant to counter snake bites and in the treatment of induced arthritis in experimental mice model [16, 17]. It was described by others that the shoots of PD contain cardenolides, alkaloids, saponins, triterpenes and steroidal compounds [18, 19]. However, after intensive examination of various biological databases (PubMed, Google Scholar) we did not encounter any articles that investigated the galactagogic property of the leaf extract of *Pergularia daemia*. Hence, the current investigation aimed to find the potential of aqueous leaf extracts of PD in promoting milk production in mice and to identify its phyto-constituents which were responsible for such effect.

MATERIALS AND METHODS

Chemicals and reagents

Concentrated HCl, magnesium ribbon, concentrated H_2SO_4 , ethanol, NH_4OH , $CHCl_3$, ferric chloride, and l-ascorbic acid were purchased from Merck (India) Ltd. Folin-Ciocalteu, glacial acetic acid, and methanol were procured from Merck, Germany. DPPH was obtained from Himedia, India. Gallic acid, sodium carbonate, and sodium bicarbonate were purchased from Sisco Research Laboratories, India. The prolactin assay kit was procured from Kruse Pathline, Ahmedabad, India. Entire chemicals, reagents and kits procured/used in the current investigation were of analytical grade.

Preparation of the leaf extract

The plant parts were collected near the village of Ghatal, a flood prone area in West Bengal (22.67 °N, 87.72°E), Midnapore district in September and October 2018, then they were identified. A plant specimen voucher was submitted to the Botany department (V256SKBU/2018) for the record. Sun dried leaves were extracted in Millipore grade water (the ratio of plant material to solvent was 1:10 m/v. Extraction was conducted at room temperature (25°C) and stirred constantly for 24 hours. The obtained extract's moisture was evaporated to a dry condition and stored at 4°C till needed. The yield of the aerial part was 15.20% calculated by the equation:

$$\text{yield (g/100g dry plant leaf sample)} = W1 \times 100 / W2,$$

where W1 represents the mass of the extract after evaporation of the solvent and W2 was the mass of the dry plant sample, respectively.

Preliminary phytochemical examination

Phytochemical examination to find reduced sugars, flavonoids, tannins, alkaloids, carbohydrates, steroids, and glycosides was conducted by routine procedures described elsewhere [20].

DPPH free radical scavenging activity of extract

For the possession of the antioxidant properties of *P. daemia* extract, the methodology of Braca *et al.* was used, depending on radical scavenging properties of DPPH [21]. For diluting the working solution of the extract, ethanol was used, while ascorbic acid was used as a standard in various concentrations (50, 100, 150, 200, 400, 600, 800 µg/ml). 0.002% DPPH was dissolved in C₂H₅OH and 1 ml of this was mixed to 1 ml of working solution of the extract and the standard (ascorbic acid) separately, incubated for 45 minutes in the dark. The blank was made by mixing 1 ml of C₂H₅OH with 1 ml of 0.002% of DPPH, then the absorbance was documented at 517 nm (Shimadzu UV-1800) and % inhibition was measured.

Total phenolic content

The method of Kim *et al.* with slight alterations was employed for estimation of total phenolic content of the aqueous leaf extract [22]. Briefly, 1 ml

of leaf extract was meticulously mixed with 1 ml of Folin-Ciocalteu reagent. After 5 minutes, 7% Na₂CO₃ (10 ml) and 10 ml of deionized water were added and mixed thoroughly. Then, the solution-mixture was kept in dark for about 90 minutes at a room temperature and absorbance was taken at 750 nm, against an appropriate blank. Various concentration of gallic acid solution was used for preparation of the standard curve. Total phenolic content was represented as mg of gallic acid equivalents per gram of the dried leaf sample.

Ultraviolet visible absorption analysis (UV)

300 mg of dry leaf powder was placed in 20 ml of Millipore water, kept overnight with continuous stirring at a temperature of 37°C and then filtered. A part of the filtered material was scanned from 190-500 nm to detect the distinctive wavelengths by using UV-visible Spectrophotometer (Shimadzu, UV-1800, Japan).

GC/MS analysis of leaf extract

GC/MS analysis of the aqueous leaf extract of *Pergularia daemia* was conducted by Gas chromatograph coupled to a mass spectrophotometer equipped with a fused capillary column, (HP-5MS, 0.25 mm × 30 m × 0.25 µm) Model No: Agilent 190915-433. For GC/MS recognition, ionization energy of 69.9eV was applied to the electron ionization system. The carrier gas was Helium (99%) with a continuous stream rate of 1 ml/min. The volume of the sample injected was 5 µl with GC grade ethanol at an average velocity of 37 cm/sec. Initial temperature of the column was 50°C for 5 min which was then programmed to 280°C with the total running time of the GC being 28 minutes.

Experimental animals

All experimentations with animals were done following "Principles of laboratory animal care" (NIH publication No. 85-23, revised in 1985) and Animal Ethics Guideline issued by Govt. of India in 1995, as well as following specific Indian laws on "Protection of Animals" under the supervision of approved investigators. All animals were housed and *in vivo* experiments were performed at Animal house facility of Sidho-Kanho-Birsha University, with due permission from Institutional Animal Ethics Committee [IAEC]

(No 1973/GO/Re/S/17/CPCSEA; Committee for the Purpose of Control and Supervision of Experiments on Animals, Exp No: IAEC/ZOO/SJB-1). The study was conducted on random inbred female albino mice (*Mus musculus*) whose weight varied between 23 and 28 g. Nearly 30 lactating females having minimum of 5 pups, were selected for the study. Oral acute and sub-acute toxicity studies were conducted following OECD guidelines.

Mice were segregated into 5 groups. Group I was the normal control without any treatment (placebo mice received only water since the leaf extract was dissolved in double distilled water, 60 μ l) whereas mice in groups II, III, IV and V received 50 mg/kg b. wt., 100 mg/kg b. wt., 150 mg/kg, and 200 mg/kg b. wt. of leaf extract of PD (aqueous, 60 μ l) orally. These doses were selected after conducting acute and sub-acute toxicity investigations. Dam milk production was measured by a commonly used protocol [23]. In brief, the weights of the pups were taken at 8.00 AM and allocated (W1). Then the pups were kept isolated away from the mother for 6 hours and their weight was taken again which was considered (W2). Then the pups were returned back to their mothers to be fed for 1 hour and subsequently weighed again which was assigned (W3). The daily milk yield was adjusted for the loss of weight caused due to various metabolic processes in the pups. The value considered for the experiment was $W2-W1/6$, the number obtained was multiplied by the number of hours the pups suckled per day and added to the daily suckling gain. The everyday gain in pups' weight was measured from W2. Blood was collected from the tail vein of mice and the serum was isolated and kept in -20°C until further use. At the end of the experiment the mice were administered with a combination of ketamine-dexmedetomidine (intra-peritoneal, the dose 80 mg/kg of ketamine + 1 mg/kg dexmedetomidine to induce anaesthesia) and then sacrificed by cervical dislocation.

Prolactin levels

Serum prolactin levels were analysed by the Tietz method, following manufacturers protocol using Elisa kits (Kruise Pathline, Ahmedabad, India) [24].

Statistical analysis

Results were given as a mean $\pm$ SD of each group. One-way ANOVA and Dunnet's test were employed to find the significant difference among groups. A probability of $p < 0.05$ was considered to be statistically significant.

RESULTS

The aqueous extract had 120 $\mu\text{g/ml}$ of total phenolic content and the qualitative assay showed presence of flavonoids, alkaloids, tannin, glycosides, reducing sugars and steroids which are presented in table 1.

Table 1.

Qualitative data on phytoconstituents present in the aqueous leaf extract of *Pergularia daemia*.

Sl No	Chemical compounds	Intensity
1.	Flavonoids	++++
2.	Alkaloids	+++
3.	Carbohydrate	++
4.	Tannin	+
5.	Reducing sugar	++
6.	Glycosides	+
7.	Steroids	+++
8.	Phenolics	+++
9.	Carotenoids	++

+ minute, ++ less abundant, +++, and ++++ much abundant

Aqueous leaf extract of PD at all four doses showed a dose-dependent suppression of DPPH activity by about 23 to 29% at concentrations of 400 to 800 $\mu\text{g/ml}$, compared to the standard in figure 1. UV-Vis spectrophotometric data revealed the presence of 5 major peaks when the aqueous extract was scanned from 190 nm to 500 nm and most peaks were between 400 to 500 nm. The major peak we found was between 230 and 300 nm due to the incidence of flavonoids and phenolic compounds. The flavonoid spectra characteristically consist of two greatest absorptions in ranges of 230–285 nm (band I) and 300–350 nm (band II), while the absorption maxima in a range of 420 nm and 444 nm could be due to α -carotene and lutein (tab. 2, fig. 2).

Figure 1.

Figure of percentage inhibition of aqueous leaf extract of *Pergularia daemia* against standard ascorbic acid (AA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n – non-significant

Figure 2.

UV-vis spectrophotometric data of the extract revealing various absorbance and the peaks

Table 2.

Table showing the Uv-vis spectrophotometric data of aqueous extract of *Pergularia daemia*

Sl No	Wavelength	Absorbance	Peak number
1.	483.00	0.037	3
2.	444.00	0.060	4
3.	426.00	0.162	6
4.	270.50	0.164	8
5.	266.00	0.163	16

The GC-MS study revealed 16 peaks of various compounds that reportedly act against various ailments. In the present study we found that linoleic acid, ethyl ester, 1-monolinoleoylglycerol trimethylsilyl ether, β -sitosterol, α -amyrin, β -amyrin and phytol is present in the extract and they have considerable percentage peak areas and some had large retention time (tab. 3).

In the present study, the acute and subacute toxicity studies revealed no death after 96 hours of treatment while one mortality was reported after 30 days of treatment (tab. 4). Acute and subacute toxicity studies revealed that even at higher doses, aqueous leaf extract of PD was relatively non-toxic to mice, as they all survived. Neither their weight decreased nor did they reveal any toxic symptoms (tab. 4). However, administration of the leaf extract at 400 mg/kg and 500 mg/kg revealed minor salivation, occasional corner sitting, increased sniffing, and drowsiness which were noted on the table side observation of the animals. Based on these studies, doses of 50 mg/kg, 100 mg/kg, 150 mg/kg and 200 mg/kg body weight were selected for subsequent galactagogic studies.

When the lactating female mice were administered with various doses of aqueous leaf extract it was found that 150 and 200 mg/kg body weight of PD leaf extract showed a considerable increase in milk production when compared to normal lactating mice from the 3rd to 10th day which was statistically significant ($p < 0.05$ through $p < 0.01$). Furthermore, when the group taking 150 mg/kg b. wt.

Table 3.

GC-MS analysis of the extract of *Pergularia daemia* showing different phytoconstituents

No	RT	Name of compound	Mol. Formula	Mol. weight	Peak area [%]
1	8.04	<i>trans</i> -13-Octadecenoic acid	C ₁₈ H ₃₄ O ₂	282	0.51
2	8.56	2-Hexadecanol	C ₁₆ H ₃₄ O	242	1.6
3	8.71	9-Hexadecenoic acid	C ₁₆ H ₃₀ O ₂	254	0.57
4	10.17	2-Pentanone	C ₅ H ₁₀ O	142	0.29
5	11.03	Diethylphthalate	C ₁₂ H ₁₄ O ₄	222	2.84
6	11.35	9-Tetradecenoic acid	C ₁₄ H ₂₆ O ₂	226	0.63
7	13.44	Cyclopropanetetradecenoic acid	C ₂₆ H ₅₀ O ₂	394	1.47
8	13.57	2,3-Dihydroxypropyl elaidate	C ₂₁ H ₄₀ O ₄	356	0.84
9	16.04	Coumatetralyl isomer	C ₂₀ H ₁₈ O ₃	306	4.48
10	16.81	4-Oxazolecaboxylic acid, 4,5-dihydro-2-phenyl-1-methylethyl ester	C ₁₃ H ₁₅ NO ₃	233	11.85
11	17.02	Linoleic acid ethyl ester	C ₂₀ H ₃₆ O ₂	308	2.02
12	35.58	1-Monolinoleoylglycerol trimethylsilyl ether	C ₂₇ H ₅₄ O ₄ Si ₂	498	1.85
13	37.25	β -Sitosterol	C ₂₉ H ₅₀ O	414	1.47
14	37.42	α -Amyrin	C ₃₀ H ₅₀ O	426	0.51
15	38.27	β -Amyrin	C ₃₀ H ₅₀ O	426	0.36
16	52.16	Phytol	C ₂₀ H ₄₀ O	296	7.17

Table 4.

Showing acute and sub-acute toxicity studies, mortality and symptoms of mice treated with various concentration of aqueous extract of *Pergularia daemia*

Sl No	Dose/day	Mortality	Symptoms after administration of PD
Acute toxicity (96 hr)			
1	25 mg/kg	0/6	Nil
2	50 mg/kg	0/6	Nil
3	100 mg/kg	0/6	Nil
4	200 mg/kg	0/6	Nil
Sub-acute toxicity (30 days)			
5	400 mg/kg	0/6	corner sitting, sniffing
6	500 mg/kg	1/6	drowsiness for about an hour after treatment, sniffing, salivation

Figure 3.

Effect of aqueous extract of *Pergularia daemia* on milk production (mg/pup). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n – non-significant

Figure 4.

Figure represents percentage weight gain in pups in different treated and control sets from day 1 to day 15 treated with various doses of *Pergularia daemia* (PD). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n – non-significant

was compared with the dams fed with 50, 100, and 200 mg/kg bodyweight of aqueous leaf extract it revealed that milk production improved which was also statistically significant ($p < 0.01$). Doses of 50 mg/kg and 100 mg/kg b. wt. of the leaf extract did not improve milk production performance compared to the normal control (fig. 4).

Similarly, a slow increase in weight was observed in pups from the initial days at all fixation intervals but the results were statistically significant on the 12th, 13th, 14th and 15th day in groups of mice fed with 150 and 200 mg/kg body weight of PD leaf extract compared to suitable control ($p < 0.05$) and groups fed with 50 and 100 mg/kg of aqueous leaf extract (fig. 4).

When the variation in serum prolactin levels was compared between treated and control sets of mice, it revealed that PD leaf extract at a dose of 150 mg/kg showed the highest concentration in blood from 2nd to 12th day. Lactating mothers administered with 200 mg/kg of extract also showed an increase of

prolactin content from 6th to 8th day. However, this decreased on the 10th to 12th day when compared to normal control ($p < 0.05$). The other two doses did not show any increase compared to the normal control mothers (fig. 5). However, we were unable to

Figure 5.

Serum prolactin level (pg/ml) variation in mice treated with various doses of *Pergularia daemia* (PD), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

analyse serum prolactin levels for a few more days due to low serum levels and as we felt that regular drawing of blood might harm both the dam and pups.

DISCUSSION

In India, a substantial part of the population still lives in tribal areas and depends solely on quacks and traditional healers for primary healthcare. Often tribal healers administer their farm animals and tribal people with plant parts to increase milk production. There is limited data on such galactagogues regarding their safety and efficacy. There are reports that milk production increases due to tactile stimulation which is sufficient to raise the blood oxytocin levels above the threshold limit [25, 26]. Inadequate milk supply is one of the important problems that lactating mothers face, hence, herbs have been routinely used to increase lactation, which is prevalent even today among various tribal populations. Their use is primarily based on prior understanding and practice. There are only limited investigations that effectively demonstrate the galactagogic properties of herbs, their safety, and effectiveness. Throughout the world women have used many approaches to increase milk production such as special diets, and use of herbal or natural substances. Mothers of different social and traditional backgrounds often choose various methods for the purpose according to their tradition or experience [27, 28]. Some investigators reported that few herb aids in lactation are *Trigonella foenum-graecum* [29, 30], *Cnicus benedictus* [31], *Silybum marianum* [32, 33], *Galega officinalis* [34, 35], *Althaea officinalis* [36, 37], *Camellia sinensis* [38] and *Nigella sativa* [39]. Many of these herbal medicines have gained popularity in the Western and other Asian countries as galactagogues. In our study, the increase of milk production in lactating mice might be due to proliferation and activity of mammary epithelial cells which in turn is due to the action of the extract on mammary glands and hormones involving in lactation like prolactin [40-42].

We also found that there were some complexes with potent antioxidant properties and are phyto-estrogenic in nature, such as β -sitosterol. PD contains several phytochemical compounds such as steroids, triterpenoids, tannins, flavonoids, coumarins, and reducing sugars which explains its utilization in traditional medicine. Toxicity studies revealed that the water extract of the plant is virtually non-toxic and harmless for use when administered orally even at

higher doses such as 400 mg/kg and 500 mg/kg for 30 days. Others have also reported that flavonoids, alkaloids, saponins, and tannins are responsible for the galactagogic activity and their activity decreases the dopamine antagonist, a competitive PRL antagonist. Therefore, inhibition of dopamine synthesis may increase milk production in the rodents.

In the present study the plant showed strong antioxidant activity which may be due to the occurrence of several phytoconstituents that appeared in the GC-MS study, such as phytol, hexadecenoic acid, tetra-decenoic acid, etc. Furthermore, the existence of phenols, carotenoids, and flavonoids may be accountable for the augmentation of antioxidant activity proven in the qualitative assays used in the present study. Our outcomes show that secretion of prolactin plays a key role in lactation. Milk production was significantly increased in mice treated with aqueous leaf extract in various doses compared to untreated control. The upsurge in milk production in lactating mice could be due to the increased production/proliferation of cells present in the mammary glands. Milk ingestion is responsible for the physiological development of new-born pups and the surge in body weight observed in pups revealed that aqueous extract in various doses could be helpful. However, pups of the dams getting a dose of 150 mg/kg body weight of aqueous extract increased weight significantly when linked to the other three doses and the control ($p < 0.001$). The percentage weight gain in pups was strongly co-related to the quantity of the milk available when they were being suckled which was also more in the group which received 150 mg/kg aqueous extract in the present investigation. Furthermore, phyto-estrogenic compounds such as β -sitosterol could also be responsible for the formation of the oestrogen hormone which helps in developing milk ducts, alveoli of mammary glands and also increases the concentration of oestrogen receptors [24]. The existence of numerous compounds in the present study such as phytol (derivative of chlorophyll), hexadecenoic acid (palmitic acid), octadecanoic acid (stearic acid), and flavonoids might be responsible for the galactagogic properties owing to the action of a single compound or conjoint action of all compounds. Investigation conducted by other investigators also put forward that some of the phytoconstituents are accountable for increased milk yield in farm animals which is also in line with our findings [43-48]. Further research is required on mouse mammary cell lines and in other mammalian models to come to a definite conclusion. Our investigation suggests that aqueous

PD leaf extract has the potential for use as a galactagogue and can raise confidence regarding its safety and efficacy.

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