Impact of feeding Murraya koenigii and Aegle marmelos leaves on metabolic and reproductive performance in crossbred cows

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Abstract

The study was carried out to ascertain the effect of herbal formulation on metabolic profile and reproductive performance of crossbred cows. Crossbred cows (24) were randomly assigned to four treatment groups. Cows in CON (control) group were fed a diet comprised of green fodder, concentrate and wheat straw, while, the cows in HP (herbal powder), HPL (herbal pellets) and HEX (herbal extract) groups were offered CON diet having herbal powder (200gd^{-1}), herbal pellets (200gd^{-1}) and methanolic herbal extract (equivalent to 200g herbal powder d^{-1}), respectively. The cows were fed on treatment diets for 7 d and continued with CON diet up to one month. Hb levels were analogous among the treatments; however, haematocrit values were significantly higher ($P < 0.05$) in the HEX group relative to CON. Total protein, albumin, globulin, A:G ratio, serum glucose, total cholesterol, AST and ALT were comparable across the dietary treatments. Serum NEFA, urea and creatinine were lower ($P < 0.01$) in treatment groups relative to CON. Serum minerals viz. i-P, Cu, Zn, Mn and iron were significantly ($P < 0.01$) higher in all the treatments than CON. The conception at first insemination was considerably higher in HPL, HEX and HP groups relative to CON. Spinnbarkeit and pH values of cervical mucus were noticeably higher in HP, HPL and HEX groups. The typical fern pattern of cervical mucus exhibited higher conception rate in treatment groups. It may be concluded that the herbal formulation positively influenced metabolic parameters and reproductive performance in post-partum crossbred cows.

1. Introduction

Natural products derived from plants have garnered significant attention within the scientific community and among the general public due to their potential role in treating, preventing, and enhancing health. The accessibility and affordability of these medicinal herbs have contributed to their popularity as a reasonably priced source of therapeutic compounds (Abeysinghe et al. 2021). In the context of livestock management, reproductive efficiency is a pivotal determinant of a sound and profitable enterprise. Reproductive diseases in dairy animals pose a substantial challenge, leading to poor reproductive efficiency and subsequent reductions in productivity (Kuralkar and Kuralkar, 2021). Achieving a phase of transition free of diseases, increased artificial insemination (AI), and higher pregnancy rates per service becomes imperative to augment reproductive efficiency in bovines. Despite advancements in breeding, feeding, and management practices, the reproductive efficiency of dairy animals has witnessed a decline over the past few decades, contrasting with increased production levels. The use of therapeutic agents, both hormonal and non-hormonal, has been extensive in attempts to enhance reproductive efficiency in dairy animals, albeit with varying degrees of success (Jadhav and Bhutani, 2005; Khade et al. 2011; Lone et al. 2012; Purkayastha et al. 2015; Dutta et al. 2019; Kumar et al. 2019; Verma et al. 2019). However, hormonal treatments have raised concerns about potential adverse effects on neuroendocrine and physiological functions, along with implications for public health due to residues in food animals and their products (Dutt et al. 2018).

The need for alternative, affordable methods is highlighted by the disadvantages of utilizing synthetic hormones and medications for animal reproductive management, such as their high costs, variable outcomes, and difficulty in obtaining commercial formulations. Approaches to reduce disease incidence and enhance reproductive function are being developed in order to minimize financial losses in dairy animals (Kumar et al. 2014). India, known as the "Botanical Garden of the World," boasts a diverse range of medicinal plants, positioning it as the leading producer of medicinal herbs globally. These herbs have significant implications for both human and animal health. Recent attention has been directed towards several herbs, including garlic, fenugreek, raspberry leaves, shatavari, thyme, black pepper, ashwagandha, and clove, which have demonstrated extraordinary therapeutic potential in combating various reproductive diseases and disorders in dairy animals (Mishra et al. 2002; Kabir et al. 2001; Das et al. 2002; Kumar et al. 2008; Rajkumar et al. 2008; Kumar et al. 2018; Chakma et al., 2020; Shafi et al. 2020). Commercially available herbal remedies have shown variable success rates in restoring cyclicity in dairy animals, stimulating ovarian function and improving reproductive health. Notably, the past two decades have seen systematic studies on the use of medicinal plants, such as pipal (*Ficus religiosa*), curry (*Murraya koenigii*), and bel (*Aegle marmelos*), to enhance fertility in dairy animals (Jondhale, 2007; Kumar, 2008; Jondhale et al. 2009a; b).

Researches have demonstrated that extracts and powders of A. marmelos and M. koenigii, either alone or in combination, can lower inflammation and uterine infections while concurrently improving lipid profiles, insulin, glucose, and antioxidant enzyme activity (Sabu and Kuttan, 2004; Upadhya et al. 2004; Xie et al. 2006; Sharma et al. 2007; Abdallah et al. 2017). Recent research has demonstrated encouraging outcomes in enhancing reproductive parameters, antioxidant responses, and immunological functions in rats through the supplementation of herbal extracts from *M. koenigii*, *M. oleifera*, and *A. marmelos* (Dhivagar, 2022). The abundance of phytochemicals in bel and curry leaves, such as marmelosin, tannins, limonene, aegelin, marmesinin, marmelin, koenigine, mukonicine, murrayacinine, isomahanine, and more, underscores their therapeutic potential (Bansal and Bansal, 2011; Naczk and Shahidi, 2004; Reddy, 2008; Jain et al. 2012; Snehlata et al. 2018; Ganesan et al. 2013; Chakma et al., 2024). These phytochemicals have been associated with various health benefits, including antioxidant, antimicrobial, antidiabetic, anticholesterol, anticancer, and hepatoprotective properties. Among the studied herbs, curry (*M. koenigii*) and bel (*A. marmelos*) stand out as significant plants examined for ovarian dynamic investigations in both laboratory and farm animals. Their effects have been evaluated in different reproductive disorders including anestrus, repeat breeding, and endometritis, showing promising results as therapeutics (Hegde et al. 2002; Mehrotra et al. 2003; Satheshkumar and Punniamurthy, 2009; Nandini et al. 2010; Dutt et al. 2011). Supplementation with powder or extract of *M. koenigii* and *A. marmelos* for 5 to 9 days has been observed to improve genital health, increase estrus expression, and enhance conception rates in dairy animals facing reproductive challenges (Baitule et al. 2016; Kumawat et al. 2016; Tiwari et al. 2021; Dhami et al. 2022).

In light of these findings, it may be possible to turn powdered herbs into extracts and pellets with the goal of lowering dosages, increasing utilization, and creating formulations based on active ingredients. Therefore, the present study hypothesizes using curry and bel leaves as a safe, potent, and cost-effective alternative strategy to improve the reproductive efficiency of crossbred cows during the postpartum period. The research will specifically address metabolic profiles, antioxidant indices, immune responses, and reproductive performances in the pursuit of sustainable and effective strategies for livestock management.

2. Material and methods

The research was conducted at the cattle and buffalo farm of the ICAR-Indian Veterinary Research Institute (IVRI) in Izatnagar, Uttar Pradesh, India, situated at the coordinates 28.4059ºN and 79.4332ºE. In this region, the integration of crops and livestock, known as mixed farming, has been a long-standing tradition. Despite the importance of livestock, small mixed farms often suffer from low productivity due to the prevalence of non-descript breeds and inadequate nutrition. The main crops cultivated in this area are rice, wheat, and sugarcane, with cereal straws and stovers constituting the primary diet for ruminants. All experimental protocols received approval from the Institutional Animal Ethics Committee and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

2.1. *Preparation of the plant material*

2.1.1. *Collection, drying and storage of plant material*

The *M. koenigii* and *A. marmelos* leaves were collected in a single batch from the ICAR-IVRI, Izatnagar in September 2022. After being air-dried at a room temperature of 27ºC, the leaves were ground using an electric grinder. The dried and ground leaves were then combined in equal parts, creating a 1:1 ratio herbal formulation.

2.1.2. Preparation of herbal pellets

The herbal powder was further used to prepare pellets, which underwent processing in a feed pelleting unit. Within the pellet-making machine, this mixture was passed through the die which was of 10 mm size, the mixture was churned and pressed, transforming it into pellet form. The resulting pellets averaged 4–5 cm in length and had a diameter of 10 mm.

2.1.3. Methanolic extract preparation

In a Stoppard conical flask, 100 g of dried herbal powder underwent extraction with 1000 mL of methanol-water solution (70:30 ratio) at room temperature. The mixture was agitated for 12 h using an electric shaker. Subsequently, the extracts were filtered using a cotton wool plug and filter paper. The filtrate was then dried at 39°C for future use. The resulting dried powder was crushed and stored in bottles at 4°C, as described by Begashaw et al. (2017).

2.2. Animals and treatments

Twenty-four crossbred cows (60 d post-partum) of 2nd to 4th parity were distributed into four treatment groups consisting of six animals in each in a CRD. The cows in all the treatment groups were provided iso-nitrogenous and iso-caloric diets. The experimental cows were randomly divided into 4 treatment groups (CON, HPL, HEX and HP). CON (control) group animals were fed a diet comprised of green fodder, concentrate mixture and wheat straw, while, the experimental cows in HP (herbal powder), HPL (herbal pellets) and HEX (herbal extract) groups were offered CON diet having herbal powder (200gd^{-1}), herbal pellets (200gd^{-1}) and methanolic herbal extract (equivalent to 200g herbal powder d^{-1}), respectively. The experimental cows were fed on treatment diets for 7 d and continued with CON diet up to one month, further after one month of treatment the non-responsive cows (not exhibited oestrus) were given treatment again in different groups and monitored for another one month to assess the response in respect of oestrus induction and pregnancy establishment, metabolic profile, health, production and reproduction performance. The pregnancy diagnosis was done per rectal after 60 d of AI. The total duration of the experiment was of 120 d.

2.3. Collection of blood and separation of serum

The blood samples were collected from jugular venipuncture on 0, 3, 7, 14 and 21 day of feeding by following standard protocol with with clean, sterilized needles, early in the morning before feeding and watering. About 10 mL blood was collected, out of which 2 mL was placed into vacutainers containing anticoagulant (EDTA) for estimation of Hb and haematocrit. Remaining 8.0 mL blood was dispensed into serum vacutainers and allowed to clot at ambient temperature for 45 minutes by keeping at slanting position. The tubes were spun at 2,000 xg for 10 minutes using a refrigerated centrifuge. The collected sera were transferred into Eppendorf tube with Pasteur pipette and stored in deep freeze at -20^{0}C for further serum-biochemical analysis. Haemoglobin (Hb) and haematocrit were measured in whole blood immediately after blood collection. Haemoglobin (Hb) was measured by using the cyanomethaemoglobin technique (Dacie and Lewis, 1969) and hematocrit was measured using Wintrobe’s tube. The serum glucose, NEFA, total proteins, albumin, urea, creatinine, total cholesterol, aspartate transaminase (AST) and alanine aminotransferase (ALT) were analyzed spectrophotometrically (MultiskanTM FC Microplate Photometer with incubator, Thermo Scientific Ltd.) by using diagnostic kits manufactured by Coral Clinical Systems (Goa, India) following the standard protocol provided with the kits. The major minerals (Ca and P) were analyzed by using the method of Talapatra et al. (1940) and trace minerals such as Cu, Zn, Mn and iron were analyzed by Atomic Absorption Spectroscopy (AAS) method

2.4. Chemical analysis

Samples of the concentrate mixture, wheat straw, green fodder, herbal pellets, herbal extracts and herbal powder were milled to pass through a 1.0 mm sieve and then examined for proximate principles (AOAC, 2012). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured following the methods of Van Soest et al. (1991).

2.5. Gene expression study

For gene expression study, already published primer of progesterone (PGR) TAATCTGTGGGGATGAAGCA-F and TAATCTGTGGGGATGAAGCA-R was standardized and used (Driver et al. 2009). The PCR conditions were optimized by amplifying the cDNA with specific primers at various annealing temperatures. The specificity of each primer was verified before performing the qRT-PCR analysis. The relative change in gene expression was analyzed using the 2-ΔΔCT method, as detailed by Livak and Schnittger (2001).

Relative fold change = $2^{-\Delta\Delta CT}$, where, $\Delta\Delta CT$= (C_T, target gene- C_T, reference gene) test – (C_T, target gene- C_T, reference gene) housekeeping gene.

2.6. Reproductive performance

Oestrus detection was done for all the lactating crossbred cows twice a day throughout the study period with the help of a trained teaser bull. All the postpartum crossbred cows were inseminated artificially with frozen semen which were exhibited in oestrus. All the non-return cows were diagnosed for pregnancy after 60 d of insemination by trans-rectal palpation. The different postpartum reproductive parameters like onset of oestrus, services per conception and conception rate was recorded. All the experimental cows were closely monitored every day for any types of disease or disorders such as mastitis, metritis etc.

2.6.1. Cervical mucus characteristics

Cervical mucus was obtained prior to artificial insemination using mid-cervical aspiration utilizing sterile blue sheaths and a universal artificial insemination gun via the recto-vaginal method. The blue sheath was attached to the universal artificial insemination gun to act as an extended syringe. The insemination gun's piston functioned as a plunger, facilitating the extraction of the cervical mucus. Following the collection, 5 mL of the cervical mucus was transferred immediately to the laboratory in a sterile petri dish for analysis of the specified parameters:

2.6.1.1. Spinnbarkeit value

Few drops (2–3 drops) of cervical mucus were placed on a grease-free slide, with another clean slide positioned on top. The slides were then gently pulled apart to stretch the mucus until it broke. The distance between the two slides at the point of breakage was measured using a centimeter scale mounted on a wall. (Panigrahi, 1964).

2.6.1.2. Arborization pattern and pH

To analyze the arborization pattern, 2–3 drops of cervical mucus were placed on a grease-free glass slide and spread evenly. The sample was air-dried and examined under a low-power objective (10X) of a microscope. Fern patterns were classified into typical, atypical, and absent categories. pH was determined by using a pH indicator stick (full range, pH 1.0 to 14.0) - Qualigens (#Q38141, Thermo Fisher Scientific) which was saturated with a drop of cervical mucus placed on a clean slide. At least 2 sticks with overlapping pH ranges (5.4 to 7.0 and 6.4 to 8.0) were used to test the same sample.

2.7. Statistical analysis

The results obtained were statistically analyzed using IBM, SPSS (20.0) and through Duncan's Multiple Range test, means were categorized accordingly. The parameters from periodic collections were scrutinized by implementing repeated measures protocol through GLM of SPSS; which encompassed between-subjects principal effect of treatments, within-subjects principal effect of period and interaction amid the treatment and period. The $P < 0.05$ was used to declare the means to be significant. All the statistical procedures were used as per Snedecor and Cochran (1994).

3. Results

3.6. Chemical composition of feedstuffs offered (% DM basis)

The chemical composition and fibre fractions of wheat straw, green, concentrate, pellets, extract and powder supplements are mentioned in Table 1. The chemical composition of herbal pellets and powder was found to be comparable, however, herbal extract showed lower values of EE, TA, NDF and ADF as compared to herbal pellets and powder.

Table 1
Chemical composition of feeds offered (% DM basis)

Attributes (%)	Wheat straw	Green fodder	Concentrate mixture	Herbal pellets	Herbal extract	Herbal powder
DM	91.82	10.60	86.82	92.94	90.16	94.11
OM	93.17	87.44	92.68	87.33	85.42	88.15
CP	2.72	10.82	17.86	11.25	13.41	12.14
EE	1.06	1.33	2.48	2.81	3.50	1.21
TA	6.83	12.57	7.32	12.67	14.58	11.85
NDF	85.42	75.61	43.53	53.68	31.56	51.16
ADF	51.21	41.24	16.85	32.38	23.45	29.36
[DM: Dry matter; OM: Organic matter; CP: Crude protein; EE: Ether extract; TA: Total ash; NDF: Neutral detergent fibre; ADF: Acid detergent fibre]						

3.7. Metabolic profile

3.2.1. Hb and haematocrit

The haematocrit (%) and Hb (gdL^{-1}) levels are presented in Table 2. The levels of Hb (gdL^{-1}) were analogous ($P > 0.05$) among the treatment groups. Mean values of haematocrit were significantly higher ($P < 0.05$) in the HEX group relative to CON, while HPL and HP groups had intermediate response between CON and HEX.

3.7.1. Serum biochemical parameters

The results related with serum glucose, NEFA, total protein and its fractions, serum urea, total cholesterol, creatinine, AST and ALT are presented in Table 2. The serum glucose ($mgdL^{-1}$) levels were comparable ($P > 0.05$) amongst the dietary treatments, though serum NEFA ($mmolL^{-1}$) values were significantly ($P < 0.01$) lower in HPL group followed by HEX, HP and CON groups. Serum NEFA was significantly reduced ($P < 0.01$) from 3rd d onwards. The mean values of total protein, albumin, globulin (gdL^{-1}), A: G ratio and total cholesterol ($mgdL^{-1}$) were parallel ($P > 0.05$) amongst the dietary treatments. The serum urea level was significantly ($P < 0.01$) lower in HPL and HEX groups relative to CON, however, HP group had an intermediate position among HPL, HEX and CON. The serum creatinine (mg %) concentration was significantly ($P < 0.01$) lower in HPL, HEX and HP as compared to CON. Activities of serum enzymes viz. AST and ALT were similar among the treatment groups.

Table 2
Effect of herbal formulations supplementation on metabolic profile of crossbred cows

Attributes	Treatments				SEM	P-value
	CON	HPL	HP	HP		
Hb, gdl^{-1}	11.57	12.18	12.27	11.67	0.22	0.213
Haematocrit, %	34.73^{a}	36.56^{ab}	36.82^{b}	35.03^{ab}	0.32	0.049
Glucose, $mgdl^{-1}$	47.27	48.15	48.02	48.25	0.34	0.740
NEFA, $mmolL^{-1}$	0.43^{c}	0.37^{a}	0.39^{b}	0.39^{b}	0.00	< 0.001
Total protein, gdl^{-1}	7.75	7.78	8.08	7.87	0.05	0.137
Albumin, gdl^{-1}	3.30	3.30	3.41	3.38	0.02	0.260
Globulin, gdl^{-1}	4.45	4.47	4.67	4.49	0.05	0.400
A: G ratio	0.75	0.74	0.74	0.79		
Total cholesterol, $mgdl^{-1}$	142.47	140.84	141.4	141.55	0.96	0.964
Urea, $mgdl^{-1}$	35.96^{b}	32.48^{a}	31.79^{a}	33.55^{ab}	0.55	0.02
Creatinine, mg%	1.23^{b}	1.10^{a}	1.11^{a}	1.11^{a}	0.01	0.00
ALT, UL^{-1}	32.08	31.80	32.47	32.17	0.31	0.903
AST, UL^{-1}	90.65	92.14	88.62	89.91	0.53	0.135

^{abc} and ^{xyz} means bearing different superscript in a row and column differ significantly

3.7.2. Serum mineral profile

Serum mineral such as Ca, i-P, Cu, Zn, Mn and iron are presented in Table 3. The mean values for serum Ca ($mgdL^{-1}$) were parallel (P > 0.05) among the treatment groups, however, serum i-P ($mgdL^{-1}$) levels were significantly (P < 0.01) higher in HPL, HEX and HP in relative to CON. The mean for serum Ca were significantly (P < 0.05) higher at 3rd d, however, i-P values were increased 3rd d onwards. The mean values of Cu, Zn, Mn and iron were significantly (P < 0.01) higher in treatment groups as compared to CON. Serum Cu and Zn levels were noticeably (P < 0.01) higher in HPL followed by HEX and HP and CON. Serum Mn values were significantly (P < 0.01) higher in HPL followed by HEX, HP and CON group. The levels of serum iron were significantly (P < 0.01) higher in HPL and HP groups relative to CON, while HEX had an intermediate position between CON and HP groups. The values of all the trace minerals were increased significantly (P < 0.01) from 3rd d onwards. The treatments and periods interactions were also significantly (P > 0.01).

Table 3
Serum mineral profile of crossbred cow supplemented with herbal formulations

Treatments	Period (s)					Treatment mean	SEM	P-value		
	0	3	7	14	21			T	P	TxP
Ca, $mgdl^{-1}$										
CON	11.83	12.45	10.02	10.30	10.81	11.08	0.22	0.213	0.015	0.828
HPL	11.66	12.53	10.55	11.13	13.85	11.94				
HEX	11.93	12.77	12.02	11.03	12.58	12.07				
HP	10.65	13.45	10.03	10.36	10.62	11.02				
Period mean	11.52^{xy}	12.80^{y}	10.66^{x}	10.71^{x}	11.96^{xy}					
i-P, $mgdl^{-1}$										
CON	5.86	7.03	7.22	6.97	7.17	6.85^{a}	0.08	0.025	0.003	0.752
HPL	6.94	7.65	7.51	7.08	7.77	7.39^{b}				
HEX	6.75	7.73	7.81	7.20	8.07	7.51^{b}				
HP	6.95	7.58	7.25	7.73	7.10	7.32^{b}				
Period mean	6.63^{x}	7.50^{y}	7.45^{y}	7.24^{y}	7.53^{y}					
Cu, μgml^{-1}										
CON	0.74	0.75	0.78	0.79	0.74	0.76^{a}	0.01	0.000	0.000	0.002
HPL	0.80	1.27	1.23	1.20	1.21	1.14^{c}				
HEX	0.74	1.03	1.13	1.10	1.09	1.02^{b}				
HP	0.70	1.07	1.09	1.11	0.99	0.99^{b}				
Period mean	0.75^{x}	1.03^{y}	1.06^{y}	1.05^{y}	1.01^{y}					
Zn, μgml^{-1}										
CON	0.70	0.72	0.79	0.82	0.83	0.77^{a}	0.01	0.000	0.000	0.053
HPL	0.74	1.04	1.29	1.24	1.31	1.12^{c}				
HEX	0.74	1.02	1.17	1.07	1.10	1.02^{b}				
HP	0.74	1.00	1.15	1.14	1.19	1.05^{b}				
Period mean	0.73^{x}	0.95^{y}	1.10^{z}	1.07^{z}	1.11^{z}					

^{abc} and ^{xyz} means bearing different superscript in a row and column differ significantly

Treatments	Period (s)					Treatment mean	SEM	P-value		
	0	3	7	14	21			T	P	TxP
Mn, $\mu g ml^{-1}$										
CON	0.25	0.25	0.26	0.27	0.30	0.27^{a}	0.00	0.000	0.000	0.018
HPL	0.26	0.35	0.38	0.39	0.39	0.35^{d}				
HEX	0.25	0.35	0.36	0.37	0.37	0.34^{c}				
HP	0.25	0.30	0.33	0.35	0.36	0.32^{b}				
Period mean	0.25^{w}	0.31^{x}	0.33^{xy}	0.34^{yz}	0.35^{z}					
Iron, $\mu g ml^{-1}$										
CON	3.48	3.37	3.54	4.00	3.67	3.61^{a}	0.03	0.000	0.000	0.000
HPL	3.44	4.07	4.57	4.80	5.14	4.40^{c}				
HEX	3.50	4.05	4.53	4.68	4.58	4.27^{bc}				
HP	3.50	4.05	4.24	4.44	4.58	4.16^{b}				
Period mean	3.48^{w}	3.88^{x}	4.22^{y}	4.48^{z}	4.49^{z}					

^{abc} and ^{xyz} means bearing different superscript in a row and column differ significantly

3.8. Reproductive performance

Supplementation of herbal formulation in different forms showed considerable improvement on confirmed pregnancy rate in crossbred cows. The conception at first insemination was considerably higher in HPL, HEX and HP groups relative to CON, while, conception at subsequent insemination was found higher in HPL, HEX and CON than HP group. The cows in treatment groups came into heat (first oestrus) earlier ($P < 0.01$) as compared to CON. However, 66.7, 16.67, 16.7 and 33.3% of cows in CON, HPL, HEX and HP did not show any sign of oestrus and were non-responsive. The number of AIs per conception were higher ($P < 0.01$) in CON relative to treatment groups (Fig. 1).

3.3.1. Physical characteristic of cervical mucus

The pH and spinnbarkeit values (cm) of cervical mucus were significantly higher ($P < 0.05$) in the HP, HPL, and HEX groups compared to the CON (Figs. 2 and 3). The arborization pattern (fern pattern) of cervical mucus with typical fern pattern exhibited higher conception rate in all the treatment (HP, HPL and HEX) groups in relation to atypical fern pattern in CON group is depicted in Table 7.

Table 7 Conception rate in relation to fern pattern of cervical mucus in crossbred cows fed herbal formulation

Treatments	Arborization pattern (fern pattern)	Conception rate (%)
CON	Typical	16.66
	Atypical	16.66
HPL	Typical	66.66
	Atypical	16.66
HEX	Typical	83.33
	Atypical	0.00
HP	Typical	50.00
	Atypical	16.66

3.9. Relative mRNA expression of PGR genes

The data pertaining to gene expression of progesterone (PGR) is depicted in Fig. 4. The fold expression of PGR gene was significantly higher ($P < 0.01$) in HPL, HEX and HP groups compared to CON.

4. Discussion

4.6. Chemical composition of feeds

The chemical composition of wheat straw and green fodder offered to experimental cows was within the normal range as reported by previous workers (Negi et al. 2015; Kumar et al. 2019; Wadhwa and Bakshi, 2023; Yatto et al. 2023). The DM, OM, CP, EE, TA, NDF and ADF of the concentrate mixture was also observed to be consistent with the results obtained by earlier workers (Choudhary et al. 2022; Patir et al. 2023; Singh et al. 2023). The composition of herbal powder and pellets was similar, while herbal extract had lower EE, TA, NDF and ADF values.

4.7. Hb and haematocrit

The present investigation demonstrates a noteworthy increase ($P < 0.05$) in haematocrit levels in the HEX group following administration of herbal formulation, while levels of Hb remained consistent across all treatment cohorts. These findings align with the established normal range for cattle, as noted by Kaneko et al. (2008). This elevation in haematocrit may be attributed to the potential enhancement of blood circulation facilitated by the herbal mixtures, ensuring efficient transportation of red blood cells throughout the body. The observed rise in haematocrit values could also be associated with the presence of flavonoids and quercetin, known for their hematopoietic properties (Raja et al. 2011). Furthermore, it's noteworthy that the herbal formulation contains volatile compounds (Mendoza et al. 2019; Sánchez et al. 2021). Our findings corroborate those reported by Nanda et al. (2013), who documented a significant increase ($P < 0.05$) in haematocrit concentration in goats following supplementation with a polyherbal formulation. Similarly, Barkakati and Kalita (2020) observed a significant elevation in Hb levels with polyherbal formulation supplementation compared to the CON group in cattle. Choudhury and Sinha (2015) observed a significant increase ($P < 0.05$) in haematocrit concentration in rats with the addition of *M. koenigii* leaves extract at doses of 250–500 mg/kg BW compared to the CON group. Lee-Rangel et al. (2022) also reported a significant increase in haematocrit concentration in calves treated with a polyherbal phytogenic source.

4.8. Serum glucose and NEFA

The synthesis, breakdown, and transport of lipids in the body have all been demonstrated to be influenced by herbal formulations containing polyphenols and flavonoids, which may decrease the release of NEFA from adipose tissue and increase lipid clearance from the bloodstream, thus lowering circulating NEFA levels (Hashemzadeh- Cigari et al. 2014;

Baghbadorani et al. 2022). Furthermore, the decreased NEFA concentration could be due to the presence of bioactive compounds in herbal formulations (*M. koenigii* and *A. marmelos*) that enhanced energy metabolism and nutrient absorption in cows (Lopreiato et al. 2020). The improved energy utilization could lead to a reduction in circulating NEFA levels, as the cow's body is better able to use energy sources from the diet. Nevertheless, these herbs are known to have hepatoprotective properties, supporting the liver's function and metabolism. A healthier liver might contribute to better lipid metabolism, reducing the release of NEFAs into the bloodstream (Adewuyi et al. 2005). The present results are in concurrence with Barjibhe et al. (2019), who reported that polyherbal supplementation to cows significantly reduced the NEFA concentration in serum. In addition, cows supplemented with herbal extract in the treatment group had a lower ($P < 0.01$) concentration of serum NEFA (Baghbadorani et al. 2022). Razo Ortiz et al. (2020) reported that supplementation of polyherbal mixture (Ashwagandha, tulsi, gooseberry and giloy) to lambs did not have any significant effect to glucose level. Lozano-Sánchez et al. (2021) also observed that there was no significant effect on serum glucose with supplementation of polyherbal supplement in finishing lambs. Hashemzadeh-Cigari et al. (2015) observed that glucose level was not affected, but serum NEFA reduced in cows supplemented with herbal mixture as compared to the CON group, despite their analogous energy balance. Although the precise mechanisms responsible for the lower NEFA levels in cows supplemented with the herbal mixture remain unclear, it is proposed that the herbal mixture may inhibit lipolysis, thereby improving lipid metabolism and enhancing metabolic status in periparturient cows by increasing insulin sensitivity.

4.9. Total protein, albumin, globulin and A: G ratio

Blood metabolites such as total protein, albumin, globulin, and A:G ratio were assessed as indicators of the general health and vitality of the animals. The results revealed that these blood metabolites did not show significant differences ($P > 0.05$) among the various dietary treatments. These observation align with previous research conducted by Birudu et al. (2020), who similarly found no significant changes in A/G ratio and albumin levels in rats treated with methanolic leaf extracts of *A. marmelos* compared to control groups. Adebajo et al. (2006) also observed no significant impact on serum total protein, albumin, and globulin levels in Swiss Albino rats when supplemented with methanolic extracts of *M. koenigii* at different dosage rates. However, contrary results were reported by Gandhi et al. (2012), who noted a significant reduction in total protein levels in rats treated with *A. marmelos*. Furthermore, Dhami et al. (2022) reported varying plasma protein concentrations among different animal categories upon bi-herbal therapy with *M. koenigii* and *A. marmelos*, with lowered levels observed in buffaloes and infertile heifers compared to cows. Rivero et al. (2012) observed an increase in plasma protein levels after adding an extract rich in phenols to lamb diets, consistent with the findings of Lee-Rangel et al. (2022), who observed increased serum protein concentration in calves treated with a polyherbal phytogenic source. Furthermore, Dorantes-Iturbide et al. (2022) found that lambs fed a dietary supplement with a polyherbal additive containing hydrolyzable tannins, flavonoids, and essential oils exhibited a significant increase in serum albumin concentration. The analogous values of serum proteins observed in our study may be attributed to the relatively short duration of the treatment (7 days) compared to previous experiments, which were conducted for varying durations.

4.10. Total cholesterol

The feeding of the herbal formulation to dairy cows did not show a significant effect ($P > 0.05$) among the treatments. In this study, supplementation with the herbal formulation had no effect on serum cholesterol levels, suggesting that the bioactive metabolites did not impact intestinal absorption or lipid synthesis in the cells of cows (Dorantes-Iturbide et al. 2022). However, Razo Ortiz et al. (2020), who also reported that supplementation of polyherbal mixture (*Ashwagandha, tulsi, gooseberry and giloy*) to lambs do not have any significant effect to serum cholesterol level. In contrast to our findings, Satheesh and Pari, (2008) reported that saponins extracted from A. marmelos reduce blood cholesterol levels by competing with cholesterol for binding sites or by interfering with cholesterol biosynthesis in the liver. Priyadarshinee et al. (2021) observed that *M. koenigii* and *A. marmelos* leaves supplementation in cows significantly reduced the total cholesterol in treatment groups. The comparable cholesterol concentration in present study may be attributed to the short duration (7 d) of herbal treatment.

4.11. Serum urea, creatinine and serum enzymes

A significant ($P < 0.01$) reduction was observed in serum urea concentration in the HEX and HPL groups, indicating a decrease in rumen protein breakdown. This suggests enhanced EAA absorption in HEX, HPL, and HP groups (Pathak et al. 2017; Wankhede et al. 2022). Moreover, the decrease in serum urea indicates minimal protein catabolism and normal kidney function (Roseler et al. 1993; Hosten, 1990; Shavit et al. 2012). The serum creatinine value was also significantly ($P < 0.01$) lower in treatment groups as compared to CON. This beneficial effect may be due to the presence of polyphenols, tannins, and flavonoids in the herbal formulations. These compounds exhibit antioxidant activity and anti-inflammatory effects, and provide cardiovascular health benefits, potentially aiding kidney health by reducing oxidative stress and inflammation in renal tissues. Previous studies have associated these compounds with potential protection against kidney damage (Arulselvan et al. 2006). The findings are consistent with the observations of Arulselvan et al. (2006), who found that blood urea and plasma creatinine decreased in treatment groups when experimental rats were treated with *M. koenigii*, which provides addition al evidence to prove the anti-diabetogenic property of *M. koenigii* leaf extract.

The primary serum markers for assessing liver changes include AST, ALT, total protein, and total albumin were unaffected by the treatment involving herbal formulation mixture, indicating no liver damage (Díaz Galván et al. 2021). Furthermore, its polyphenols might provide an antioxidant benefit (Biswas and Giri, 2015), along with other advantageous effects on liver function due to this nutrient (Mehedint and Zeisel, 2013).

4.12. Serum mineral profile

Macro and micro elements play a crucial role in maintaining immune function and overall health. The high magnesium content in curry leaves suggests that they could serve as an excellent source of magnesium, which is known to activate various enzyme systems (Olusanya et al. 2008). Ca and P are also critical, as they influence an animal's ability to utilize other micronutrients and can modulate reproductive hormone sensitivity through various enzyme systems (Dutta et al. 2001). This could explain why the supplementation of curry leaves might lead to higher serum Ca and P levels and, in turn, support ovarian activity in anestrous heifers. Additionally, both *M. koenigii* and *A. marmelos* leaves are rich in essential minerals such as C, P, iron, zinc, manganese, and copper (Shantala and Prakash, 2005; Igara et al. 2016; Vijayalakshmi and Venkatalakshmi, 2017; Kujur et al. 2022). Our results align with Orzuna-Orzuna et al. (2021), who found that serum Ca levels among treatment groups were within the normal range for ovine, indicating that herbal mixtures do not disrupt mineral balance or nutritional status in lambs. In a similar vein, Sathesh Kumar and Punniamurthy (2009) observed that daily supplementation with 100 gd^{-1} of *M. koenigii* leaves for 30 days significantly improved serum inorganic i-P levels and induced estrus in anestrous heifers. Similarly, Razo Ortiz et al. (2020) found that serum calcium levels were not affected by polyphenol and flavonoid doses, but serum phosphorus concentrations rose with increased doses of these compounds. Dhami et al. (2019) reported that supplementing cows with a mixture of *M. koenigii* and *A. marmelos* led to significant increases in plasma Ca, i-P, and other micro-minerals such as Zn, iron, and Cu. It is obvious that the mineral composition of the tree foliage is superior to that of tropical grasses. Goodchild and McMeniman (1994) reported that inclusion of tree foliage in ruminants' diet increased the supply of minerals to the animals and rumen microbes and ultimately improved their performance. However, in contrary to our findings, Baitule et al. (2016) noted significant increases in serum Ca levels on day 9, the day of estrus, and on day 8 after estrus onset in treated groups, possibly due to supplementation with *A. marmelos* and *M. koenigii*. Likewise, Kumawat et al. (2014) found significantly higher serum Ca levels on days 4, 6, 8, and 10 after treating delayed pubertal heifers with dried leaf powder from *A. marmelos* and *M. koenigii*, compared to non-treated groups. This study also observed that the effect of these leaves on serum P levels was significant, with marked variations on days 2, 4, and 8 after treatment compared to non-treated heifers.

4.13. Reproduction

Supplementation of herbal formulation in different forms showed considerable improvement on confirmed pregnancy rate in crossbred cows (Patton et al. 2007; Ambrose, 2021). The beneficial effects of herbal formulations (*A. marmelos* and *M. koenigii*) supplementation could be due to the presence of phytoestrogens, minerals, and antioxidants. These components might stimulate gonadotropin hormones from the anterior pituitary, potentially contributing to the restoration of cyclicity in anestrus animals (Dutt et al., 2010; 2011). The antioxidants present may have enhanced folliculogenesis by preventing ROS-induced apoptosis of oocytes (Vijayalakshmi and Venkatalakshmi, 2017). The phytoconstituents might have stimulated steroidogenic activity in the hypothalamus, promoted follicular growth, and facilitated steroid production, leading to the initiation of cyclicity (Jondhale et al. 2009a; Nandini et al. 2010). Additionally, the presence of bioactive compounds in the herbal formulation may have contributed to follicular development, particularly during the dominance phase leading up to ovulation, resulting in the resumption of estrus. In the present study, though the conception rate was significantly higher in all the treatment groups relative to CON, however, HPL and HEX groups showed better conception rate and established pregnancy than HP (herbal powder) group, which could be probably due to the less wastage and complete consumption of herbal powder in pellets forms as compared to powder (Wanapat et al. 2013). The findings of the current study align with those of Patel and Gupta (2020), who reported the highest conception rate (71.42%) in delayed pubertal heifers supplemented with *M. koenigii*, *A. marmelos*, and a mineral mixture. Additionally, *M. koenigii*, whether used alone or in combination with a mineral supplement and a low dose of GnRH injection, was found to be equally effective in inducing fertility in anestrus buffaloes (Umashanker et al. 2006). Delayed pubertal heifers have shown induced estrus behavior after being supplemented with *A. marmelos* combined with *M. koenigii* (Kumar et al. 2016; Kumawat et al. 2016). Baitule et al. (2016) found 75% oestrus induction after supplementing *A. marmelos* and *M. koenigii* for 9 days in anestrous buffaloes. Satheshkumar and Punniamurthy (2009) also studied that supplemental effect of *M. koenigii* leaves for a period of 30 days and found induced oestrus in 60% of the anestrus heifers. Mehrotra (2002) have also documented that *M. koenigii* has been found to enhance ovarian function in rats and improve the therapeutic response in acyclic goats and heifers, particularly regarding oestrus and conception. Tiwari et al. (2021) reported that the oestrus response was 83.33% in postpartum buffaloes when supplemented with a mixture of *A. marmelos* and *M. koenigii*. However, Das et al. (2012) observed 92.90% oestrus induction in delayed pubertal buffalo heifers after feeding of *A. marmelos* and *M. koenigii* herbs for 9 days. Das et al. (2016) and Kumawat et al. (2016) reported that 90.9% and 92.3% of delayed pubertal heifers, respectively, began cycling after being fed a herbal mixture. Additionally, they observed a pregnancy rate of 54.1% in the group treated with the herbal mixture, compared to 16.7% in the control group. Therefore, it may be speculated that favourable effects of herbal formulations (mixture of *M. koenigii* and *A. marmelos*) in terms of estrus induction, ovulation, established pregnancy and shorter time period taken in induction of estrus are exhibited as compared to individual plant treatment which could be a reflection of synergistic actions of herbs active principles (Nandini et al. 2010; Hedge et al. 2002; Mehrotra et al. 2005). The active principles seem to operate either through mimicking gonadotrophins activity or stimulating the central mechanism for endogenous release of gonadotrophins along with possibility of local action (Dutt et al. 2018).

4.14. Physical characteristic of cervical mucus

It is believed that the herbal formulations (mixture of *M. koenigii* and *A. marmelos* leaves) are having hormonal regulatory properties that might have supported the oestrous cycle and overall reproductive health cows. Since, this herbal formulation having antioxidant and anti-inflammatory properties which might have helped in reducing inflammation in the reproductive tract, promoting better cervical mucus production and a favourable environment for sperm transport (Dhankhar et al. 2011; Gahlawat et al. 2014; Nigam and Nambiar, 2015; Rautela et al. 2018). Our findings align with those of Dhami et al. (2022), who observed that administering a bi-herbal treatment (*M. koenigii* and *A. marmelos*) @ 200 g daily for 5 days resulted in improved mucus quality at subsequent next oestrus in infertile animals. On contrary, Rahi et al. (2013) reported that on feeding of herbal extract containing polyphenols have reduced the pH which may be due to decline in bacterial load and inflammatory process in uterus after treatment.

The fertility is associated with an arborization pattern, as in our study we found that typical fern pattern was having higher conception rate in treatment groups thereby it was observed higher conception rate with typical fern pattern in all the treatment groups. The enhanced conception rate in crossbred cows with typical fern pattern indicates higher sperm penetration (Rangnekar et al. 2002; Layek et al. 2013). Alkaline pH of cervical mucus is favourable for higher sperm motility. The increase in pH in the treatment groups had higher fertility rate and also suitable for uterine environment with maximum sperm penetration in alkaline pH (Pattabiraman et al. 1967; Rangnekar et al. 2002). Similarly, the treatment groups exhibited a higher conception rate, correlated with an increased spinnbarkeit value. Additionally, the conception rate was influenced by the arborization pattern, as a typical arborization pattern is indicative of a proper hormonal balance, which supports higher conception rates (Alena et al. 2008).4.10. *Relative mRNA expression of PGR genes*

The progesterone gene's expression was associated with the progesterone hormone, which was likewise shown to be significantly greater in the treatment groups. The fold expression of PGR gene was significantly higher ($P < 0.01$) in HPL, HEX and HP groups as compared to CON. Progesterone is a crucial ovarian steroid that regulates the estrous cycle, maternal recognition of pregnancy, and gestation in farm animals (Spencer et al., 2004). Beyond these roles, progesterone is also vital for various reproductive functions, including the behavioral expression of estrus, ovulation, maintenance of uterine quiescence, embryo survival and normal parturition.

Conclusions

In conclusion, the herbal formulation exhibited a positive influence on both metabolic parameters and reproductive performance in postpartum crossbred cows. The observed improvements in blood parameters, and early onset of estrus suggest promising prospects for integrating herbal supplements in dairy cattle management. These findings underscore the potential of herbal formulations as a supportive measure for enhancing the overall health and fertility of postpartum cows.

Declarations

Ethical approval

Before conducting the experimental trails, the animals (crossbred cattle) studies were approved by institutional ethics committee IAEC, ICAR-IVRI, Izatnagar-243122, Bareilly, Uttar Pradesh (Regd No: IAEC/07.07.2022/B6), and was conducted in accordance with the ethics standards as applicable international, national and/or institutional guidelines.

Conflict of interest

The authors do not have any conflict of interest regarding this research.

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Authors contributions

J. C: conducted the research as a part of her Ph.D. research work. She has conducted the experimental trial deligently along with handling and management of the cows and laboratory tests including laboratory and molecular works. She also wrote the main manuscript text.

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Availability of data and materials

Data provided within the manuscript is complete and can be provided on request to the authors.

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Figures

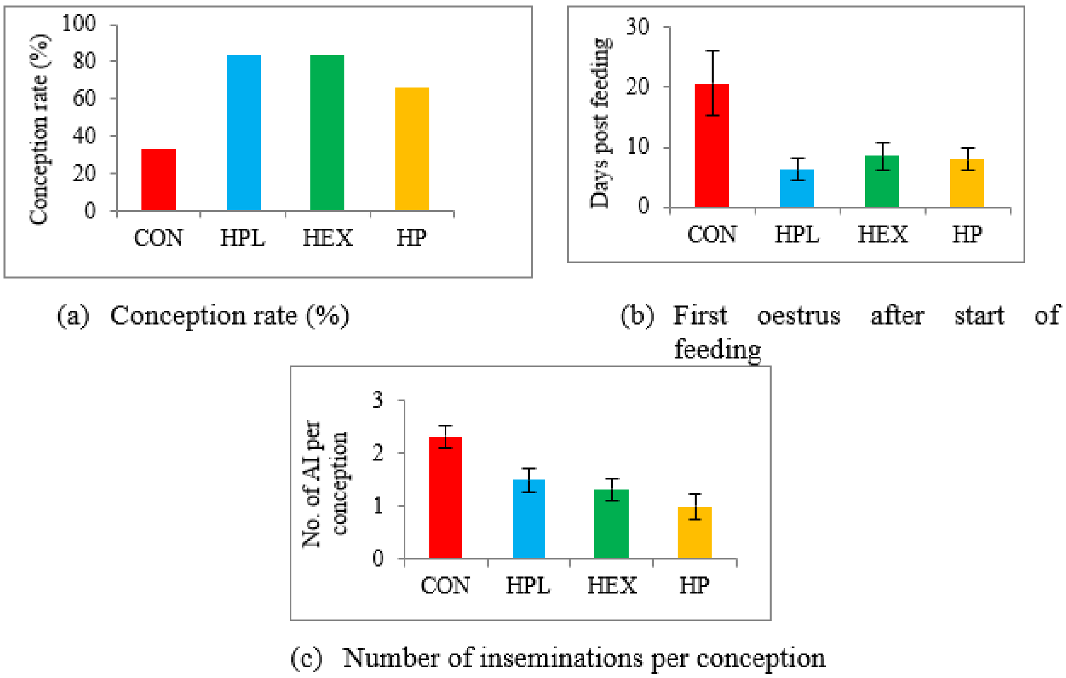
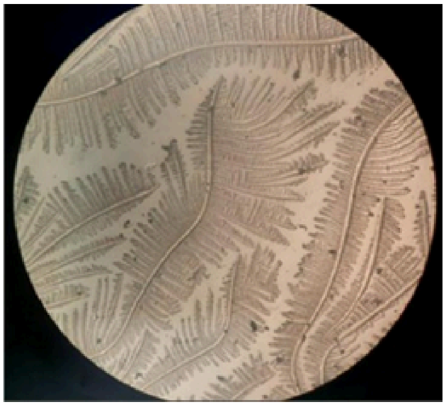
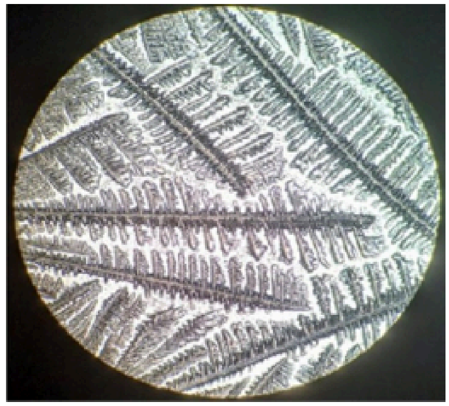


Figure 1

Reproductive performance of crossbred cows supplemented with herbal formulations (a) conception rate (b) first oestrus after start of feeding (days) and (c) number of inseminations per conception


(a) Typical fern pattern at 10X

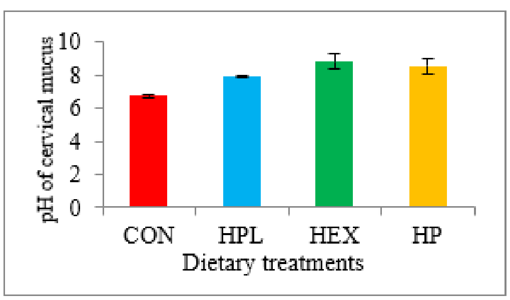

(b) Typical fern pattern at 4X


(a) Atypical fern pattern at 10X

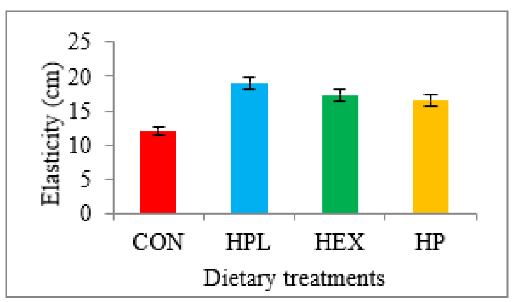

(b) Atypical fern pattern at 4X

Figure 2

Fern pattern of the cervical mucus



(a)



(b)

Figure 3

Effect of herbal formulations on pH (a) and spinnbarkeit values (b) of cervical mucus in crossbred cows

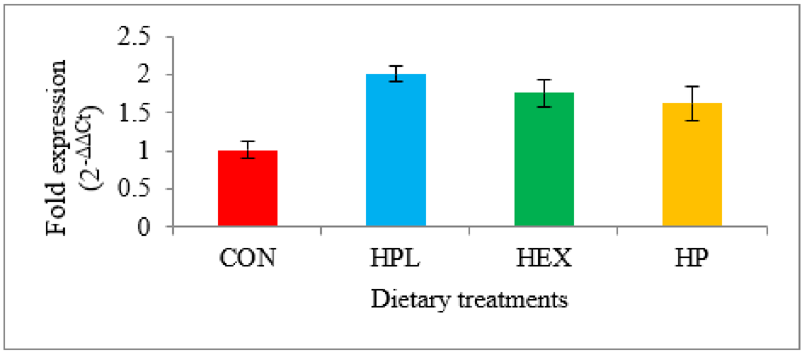


Figure 4

mRNA expression of progesterone gene