**"Effect of Polyherbal and Mineral Supplementation on Serum Mineral Profile and Metabolic Regulation in Delayed Pubertal Crossbred Heifers" (Area of the study)**

**Abstract**

This study evaluated the effects of dietary supplementation with polyherbal and mineral mixtures on the serum mineral profile of delayed pubertal crossbred heifers over a 28-day period. Serum levels of calcium, sodium, phosphorus, and magnesium were measured at different time points to assess the influence of supplementation. A significant variation (P < 0.05) in serum calcium levels was observed among groups, with Group 3 (polyherbal and mineral mixture) exhibiting the highest calcium concentrations at days 21 and 28, followed by Group 1 (polyherbal mixture) and Group 2 (mineral mixture). This indicates a synergistic effect of combined supplementation in improving calcium metabolism. Phosphorus levels also showed significant improvement (P < 0.05) across all supplemented groups, with Group 3 demonstrating the highest phosphorus levels by day 28 (7.85 ± 0.08 mg/dL), followed by Group 2 and Group 1. These results suggest enhanced phosphorus retention and bioavailability due to supplementation. Magnesium levels increased notably in Groups 2 and 3, while the control group exhibited minimal changes. The highest magnesium levels were observed in Groups 2 and 3 at day 14, reinforcing the importance of mineral supplementation in maintaining magnesium homeostasis.The study concludes that polyherbal and mineral supplementation significantly improves serum mineral profiles and metabolic status, with combined supplementation yielding the most pronounced benefits. These findings underscore the potential of dietary interventions in optimizing mineral metabolism, thereby contributing to improved reproductive performance in delayed pubertal heifers.

 **Keywords:** *Aegal marmelos*, *Murraya koenigii*, *Moringa oleifera*, Minerals, Fertility

**1.Introduction**

 In the dairy industry, anestrus is one of the most concerning conditions that negatively impacts cattle, reducing both their ability to reproduce and the profitability of small and marginal farmers. Reproductive problems are expected to impede the improvement of animal performance in India, which would significantly affect the nation's economy.

Numerous hormonal and non-hormonal therapeutic agents are being used to address this problem; however, dairy producers rejected the majority of synthetic drugs due to their high cost and inconsistent outcomes. Many therapeutic plants have been found in India since ancient times. Since modern medicinal plants are safer, more effective, and less expensive than more costly hormones, they hold promise for the future in the treatment of a range of reproductive disorders of farm animals (Mehrotra *et al*., 2005).

Well-known medicinal plants include *Murraya koenigii* and *Aegal marmelos.* were employed separately to increase fertility in laboratory animals. Curry leaves, or *Murraya koenigii*, are a common plant in India that contain a variety of free amino acids and alkaloids. The leaves of *Murraya koenigii* improve fertility and cause oestrous, which raises the amounts of calcium and phosphorus in the blood (Kumar and Punnamurthy, 2005; Jhondale *et al*., 2009). Beneficial substances such carbohydrates, proteins, fatty acids, amino acids, phytochemicals, and vitamins A, B1, B2, B3, and C are abundant in the leaves of both *Murraya koenigii* and *Aegle marmelos.* Calcium (Ca), phosphorus (P), magnesium (Mg), potassium (K), iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), cobalt (Co), chromium (Cr), and other important minerals are also present in them. After being treated with *A. marmelos* leaves, Kumar (2008) observed increased fertility in anestrus goats and buffaloes. Aeglin, rutin,mameline, β-sitosterol, γ-sitosterol, O-isopentenyl glycoside, cuminaldehyde, citronellal, phenylethyl cinnamamides, eugenol, and marmesinin are among the active substances found in the leaves. There are also several coumarins, alkaloids, steroids, and essential oils. The one that shows estrogen-like activity among these is β-sitosterol-D-glucopyranoside, which also has oestrogenic effects when combined with calcium salt. Mehrotra et al. (2005) found that ethanolic extracts of *M. koenigii* (1000 mg/kg) significantly enhanced follicular development in rats, increasing the number of large surface, preantral, and antral follicles This implies that *M. koenigii* might cause an increase in follicle-stimulating hormone (FSH), which would encourage the early recruitment of follicles that respond to FSH and expand the population of big follicles. Mehrotra et al. (2005) found an increase in ovarian steroidogenic activity, particularly in the generation of oestradiol 17-β. Because oestradiol is known to cause granulosa cells to undergo mitosis, the treated rats' follicular development was probably enhanced. By encouraging follicular growth and oestrus, *M. koenigii* has been demonstrated to improve reproductive performance in lab rats, as well as in anestrus goats and cattle (Mehrotra, 2002).

The nutrient-dense plant Moringa oleifera offers vital vitamins, minerals, and phytoestrogens that support better regulation of reproductive hormones and oestrous cyclicity (Sahu *et al*., 2023). It has been demonstrated that adding these herbal bioactives to cattle feeds enhances immunological response, lipid metabolism, and hormone balance, all of which maximise reproductive health.

Mineral supplementation is essential for promoting enzymatic functions, hormone production, and cellular activities necessary for successful reproduction. Sufficient mineral levels support follicular growth and ovulation, lower the risk of early embryonic loss, and boost immune defenses, thereby enhancing uterine health and increasing conception success (Sahoo, 2021). Considering the wide-ranging advantages of both herbal bioactives and essential minerals, their combined use is believed to have a synergistic effect on metabolic health and the serum mineral profile. This study is designed to investigate the effects of dietary supplementation with *Aegle marmelos*, *Murraya koenigii*, and *Moringa oleifera*, alongside a mineral mixture, on the metabolic status and serum mineral levels in crossbred heifers.

**2. Materials and techniques**

* 1. **Collection of Plant Material**:

 Fresh, mature green leaves of *Murraya koenigii* (July-August), *Aegle marmelos* (March to July), and *Moringa oleifera* (Winter and Autumn) were gathered from their natural habitat. The leaves were cleaned thoroughly with water to separate contaminants.

* 1. **Processing and Drying:**

The leaves were subsequently shade-dried at room temperature for 4 to 7 days to preserve their bioactive compounds. After detaching them from the stems, the dried leaves were individually stored in plastic bags until the grinding process. They were then ground into a fine powder using a mechanical grinder and kept in airtight containers at room temperature until further use.

* 1. **Preparation of Polyherbal Mixture:**

 The polyherbal mixture was prepared by blending the powdered *Murraya koenigii* (100g), *Aegle marmelos* (100g), and *Moringa oleifera* (50g)leaves .

**2.4Experimental Design and Animal Selection**

 A total of 24 clinically healthy crossbred (Jersey × Assam Local) heifers, aged between 24 and 32 months and exhibiting delayed puberty, were selected for the study. Selection criteria included a similar good body condition score (BCS) as per Mishra et al. (2016), absence of cyclic ovarian structures, no detectable clinical abnormalities in the reproductive tract, and no prior signs of oestrus. The heifers were randomly divided into four groups, each subjected to a different supplementation regimen.

Twenty-four crossbred heifers with delayed puberty were selected for the study and randomly divided into four experimental groups, each consisting of six animals. The Control group was given a basal diet following the nutritional recommendations of ICAR (2013) without any additional supplementation. Group 1 received the basal diet along with a daily oral dose of a polyherbal mixture comprising *Aegle marmelos* (100 g), *Murraya koenigii* (100 g), and *Moringa oleifera* (50 g), total 250 g per animal per day. Group 2 was provided with the basal diet supplemented with an area-specific mineral mixture (AAUVETMIN) at a daily dose of 50 g per animal. Group 3 was given both the polyherbal mixture (250 g/day) and the mineral mixture (50 g/day) in addition to the basal diet. The supplementation in all treatment groups continued for 30 days or until the onset of estrus, whichever occurred first. The effectiveness of the supplementation was assessed based on estrus induction, pregnancy establishment, and overall reproductive performance. Pregnancy diagnosis was carried out through rectal palpation 60 days after artificial insemination (AI).

**2.5 Blood Collection, Serum Separation Methodology**

**2.5.1 Blood Collection Procedure**

 Blood samples were collected weekly from the cattle following supplementation, using the jugular vein as the sampling site. Around 10 ml of venous blood was drawn using a sterile 18-gauge needle and transferred into vacutainer tubes without anticoagulant. The tubes were gently inverted to mix, then left to clot before being centrifuged at 3000 rpm for 10–15 minutes to separate the serum. The clear serum was carefully extracted using a sterile micropipette and stored in cryovials at -20°C for subsequent biochemical and mineral analyses.

**2.6 Statistical Analysis**

 The data were analyzed using SPSS software, with comparisons between groups performed through two-way ANOVA. A p< 0.05 was considered statistically significant.

**3. Results and discussion**

**3.1Qualitative Phytochemical Screening of *Murraya koenigii*, *Aegle marmelos,* and *Moringa oleifera* leaves**

The phytochemical analysis of the herbal powder revealed the presence of phenols, flavonoids, tannins, alkaloids, terpenoids, and saponins, while glycosides were not detected in any of the samples. These results are consistent with the findings of Chakma et al. (2024), who observed unsaturated steroids, triterpenes, alkaloids, flavonoids, and tannins in *Murraya koenigii* and *Aegle marmelos*. Likewise, Dhandapani and Sabna (2008) reported the presence of alkaloids, flavonoids, tannins, saponins, steroids, and phlobatannins in *A. marmelos* leaves. In addition, Gupta et al. (2012) identified alkaloids, glycosides, terpenoids, saponins, tannins, flavonoids, and steroids in crude extracts of *A. marmelos*, while Bristy et al. (2017) confirmed the presence of glycosides in various *A. marmelos* extracts.

**Table 1: PHYTOCHEMICAL ANALYSIS OF *MURRAYA KOENIGII*, *AEGLE MARMELOS* AND *MORINGA OLEIFERA* (QUALITATIVE SCREENING)**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Phytochemicals** | **Test** | ***A. marmelos*** | ***M. koenigii*** | ***Moringa oleifera*** |
| Steroids | Salkowski Test | + | + | + |
| Alkaloids | Dragendorff's Test | + | + | + |
| Mayers Test |
| Wagner's Test |
| Hager's Test |
| Tannins | Ferric Chloride Test | + | + | + |
| Flavonoids | Ferric Chloride Test | + | + | + |
| Glycosides | Sodium Hydroxide Test | - | - | - |
| Benedicts Test | - | - | - |
| Triterpenes | Salkowski's Test | + | + | + |
| Saponins | Foam Test | + | + | + |

**3.2 Serum mineral profile**

The serum calcium concentrations (mg/dL) in heifers subjected to various dietary treatments are showed in fig.1. A significant difference (p < 0.05) was observed in calcium levels among the groups over time. On day 0, calcium levels ranged from 9.69 ± 0.43 mg/dL in Group 3 to 10.26 ± 0.55 mg/dL in Group 1, with an overall average of 9.99 ± 0.16 mg/dL. By day 7, slight variations were noted, with Group 2 recording the lowest level (9.65 ± 0.10 mg/dL) and Group 1 the highest (9.77 ± 0.57 mg/dL). A gradual increase in calcium concentration was evident by day 14, particularly in Group 3 (10.70 ± 0.43 mg/dL), followed by Group 2 (10.53 ± 0.25 mg/dL), whereas Group 1 showed a minor decline.

A significantly high (p < 0.05) was observed on day 21, where Group 3 reached the highest calcium level (11.57 ± 0.39 mg/dL), with Group 1 following closely (11.18 ± 0.18 mg/dL). On day 28, calcium levels appeared to stabilize, with Group 3 again showing the highest value (11.56 ± 0.15 mg/dL), and Group 2 (11.08 ± 0.07 mg/dL) trailing behind.

Though all groups experienced some degree of fluctuation, Group 3 (receiving both polyherbal and mineral supplementation) consistently maintained the highest calcium levels, especially on days 21 and 28, suggesting a synergistic interaction between the supplements. Group 1 (polyherbal only) exhibited a general upward trend, likely attributable to enhanced mineral metabolism driven by plant-derived bioactive compounds. In contrast, Group 2 (mineral supplementation only) showed a steady increase, indicating efficient calcium retention and utilization. These results emphasize the effectiveness of dietary interventions in supporting calcium metabolism, with the combined use of polyherbal and mineral supplements providing the most significant improvement.

Phosphorus levels increased across all supplemented groups, with Group 3 (receiving both polyherbal and mineral mixtures) consistently displaying the highest average phosphorus concentration (6.74 ± 0.23 mg/dL), followed by Group 2 (mineral mixture) and Group 1 (polyherbal mixture). On day 28, Group 3 recorded the peak phosphorus level (7.85 ± 0.08 mg/dL), while the control group, which was provided only a concentrate diet, maintained the lowest phosphorus values throughout the study (5.77 ± 0.18 mg/dL).

These results indicate that dietary supplementation has a significant impact on phosphorus metabolism in heifers. Group 1 showed a progressive rise in phosphorus levels, suggesting that plant-derived bioactives may enhance phosphorus bioavailability. Group 2 exhibited higher phosphorus values, supporting the effectiveness of mineral supplementation in improving phosphorus absorption and retention. Group 3, which consistently recorded the highest phosphorus levels, indicates a synergistic effect of combining polyherbal and mineral supplements in optimizing phosphorus metabolism. Overall, these findings underscore the potential of dietary strategies to improve phosphorus balance, which could contribute to better growth and bone development in heifers.

Supporting studies have shown similar outcomes. Kumar and Punniamurthy (2009) reported a significant rise (p > 0.05) in serum inorganic phosphorus (i-P) levels in anestrus heifers supplemented with 100 g/day of *M. koenigii* leaves for 30 days. According to Razo et al. (2020), while serum calcium remained unaffected by polyphenol and flavonoid intake, phosphorus levels increased with higher doses of these compounds. Dhami et al. (2019) found significant increases in plasma calcium, i-P, and trace minerals such as zinc, iron, and copper when cows were supplemented with a blend of *M. koenigii* and *A. marmelos*. Similarly, Baitule et al. (2016) observed significantly elevated (p > 0.05) serum mineral levels in anestrus buffaloes fed bael and curry leaf powder.

Sathiskumar (2005) noted that an imbalanced calcium-to-phosphorus (Ca:P) ratio and deficiencies in these minerals could lead to pituitary and gonadal dysfunction in anestrus heifers. Both calcium and phosphorus play vital roles in the absorption and utilization of other microminerals and enhance the sensitivity of reproductive hormones through various enzymatic pathways. Calcium is involved in steroid synthesis in organs such as the ovaries, testes, and adrenal glands, suggesting that low serum calcium may be linked to the suboptimal reproductive performance of the animals in the study. Phosphorus deficiency has been associated with reproductive issues including anestrus, subestrus, irregular estrous cycles, and delayed puberty. As phosphorus is a critical component of nucleic acids, nucleotides, phospholipids, and certain proteins, hypophosphatemia can negatively affect numerous cellular functions. It is also essential for energy transfer, phospholipid metabolism, and enzyme activation. Previous studies have reported enhanced estrous cyclicity in anestrous cows following supplementation with area-specific mineral mixtures (ASMM), which aligns with the present findings (Mohapatra *et al*., 2012).

Calcium significantly affects the release of GnRH and LH from the pituitary gland and plays a crucial role in the gonadotropic regulation of ovarian steroid hormone production (Carnegie and Tsang, 1984). A deficiency in phosphorus can result in irregular estrous cycles, anoestrus, reduced ovarian activity, and a lower conception rate (Pugh, 1985). Serum Magnesium was found to be higher in the polyherbal and mineral mixture supplemented group. While magnesium does not directly influence reproductive functions, it is essential for numerous enzymatic reactions involving ATP-dependent enzymes. Additionally, it aids in the absorption of both calcium and phosphorus (Sharma *et al*., 2004).

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**Fig 1: Serum CALCIUM (mg/dL) level in experimental animals supplemented with polyherbal mixture and minerals**

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**Fig 2: Serum Phosphorus(mg/dL) level in experimental animals supplemented with polyherbal mixture and minerals**



**Fig3: Serum Magnesium (mg/dL) level in experimental animals supplemented with polyherbal mixture and minerals**

**3.3 Serum metabolic profile**

**3.3.1Serum total protein**

Serum total protein was monitored over 28 days across different dietary treatments. Total protein levels remained statistically nonsignificant. Group 3 animals exhibited significantly higher serum total protein levels compared to those in Group I. Maintaining an optimal level of total protein in the blood is essential for proper expression of estrus in cows. Inadequate protein intake can result in weak or poorly expressed estrus and, in some cases, may even lead to a temporary halt in estrous activity (Hafez and Hafez, 2000). A low protein diet can cause a negative nitrogen balance, which can severely disrupt normal estrus behavior in animals (Vhora *et al*., 1995).



**Fig 4: Serum Total Protein(g/dL) level in experimental animals supplemented with polyherbal mixture and minerals**

**3.3.2 Serum Glucose (mg/dL)**

Over the 28-day period, serum glucose levels (mg/dL) in heifers showed a rising trend in Group 1 (polyherbal mixture) and Group 2 (mineral mixture), with Group 3 (polyherbal and mineral mixture) recording the highest glucose concentration by Day 28. This suggests a synergistic effect when both supplements are combined. The elevated glucose levels observed in the treatment groups, compared to the control, could be linked to bioactive compounds present in Bael, Curry leaves, and Moringa, which are known to improve insulin sensitivity and promote glucose uptake (Chakma *et al*., 2024).

Furthermore, minerals like chromium and zinc contribute to insulin regulation and glucose metabolism, likely accounting for the observed pattern in Group 2. These results are consistent with earlier findings that reported increased glucose levels in dairy cattle and non-cyclic goats supplemented with *Murraya koenigii* and *Aegle marmelos* (Dutt *et al*., 2010; Dhami *et al*., 2022). In contrast, Jaiswal et al. (2009) observed that *Moringa oleifera* reduced fasting glucose levels in diabetic rats by enhancing insulin secretion and promoting GLUT4 expression. Elevated plasma glucose levels indicate sufficient energy availability, which is closely linked to the reproductive health of the cows being studied. Increased glucose levels can enhance progesterone production by stimulating the release of luteinizing hormone (LH) (Kumar *et al*., 2003). On the other hand, low blood glucose (hypoglycemia) may trigger negative feedback to the hypothalamus, leading to reduced secretion of gonadotropins from the pituitary gland. This, in turn, can suppress ovarian cell activity, particularly in anoestrus animals.



**FIG. 5: SERUM GLUCOSE (mg/dL) LEVELS IN EXPERIMENTAL ANIMAL SUPPLEMENTED WITH POLYHERBAL MIXTURE AND MINERALS**

**3.3.3 Total cholesterol**

Serum total cholesterol levels (mg/dL) in heifers subjected to various dietary treatments were monitored over a 28-day period. The control group, which received only concentrate feed, showed relatively stable cholesterol values throughout, ranging from 141.17 ± 6.19 mg/dL on day 0 to 142.04 ± 4.86 mg/dL on day 28. In contrast, Group 1 (polyherbal mixture) displayed a downward trend, with cholesterol decreasing from 138.16 ± 4.84 mg/dL at day 0 to 131.19 ± 3.84 mg/dL at day 28. Group 2 (mineral mixture) also showed a decline, from 142.17 ± 4.49 mg/dL to 134.70 ± 1.68 mg/dL. Group 3 (polyherbal + mineral mixture) recorded an initial reduction in cholesterol levels, reaching 133.02 ± 2.65 mg/dL by day 14, and then stabilized at 136.63 ± 4.49 mg/dL by day 28. Although fluctuations were noted between groups, the differences among treatment means were not statistically significant (p > 0.05).

These observations suggest that dietary supplementation, particularly with polyherbal and mineral blends, may affect serum cholesterol levels in heifers. The presence of phytochemicals such as flavonoids and polyphenols in Bael, Curry leaves, and Moringa may contribute to reduced cholesterol biosynthesis or improved lipid metabolism. The decline in cholesterol in Group 2 also points to the potential role of mineral supplementation in regulating lipid profiles, with elements like zinc and chromium being known to support lipid metabolism. Elevated plasma cholesterol levels support the earlier onset of estrus in dairy cows, as cholesterol serves as a precursor for the synthesis of gonadal steroid hormones (Westwood *et al*., 2002).

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**FIG. 6: SERUM TOTAL CHOLESTEROL (mg/dL) LEVELS IN EXPERIMENTAL ANIMAL SUPPLEMENTED WITH POLYHERBAL MIXTURE AND MINERALS**

**3.3.4 NEFA (Non-Esterified Fatty Acids)**

Fig. 7. illustrates the serum NEFA (Non-Esterified Fatty Acids) concentrations in cattle across different time intervals (0, 7, 14, 21, and 28 days). Initially, on Day 0, NEFA levels were comparable among all groups, ranging from 0.40 to 0.45 mmol/L. Throughout the study, the control group displayed minor fluctuations but remained relatively stable, with NEFA values between 0.39 and 0.46 mmol/L. In contrast, all treatment groups (Group 1, Group 2, and Group 3) showed a consistent decline in NEFA levels over time. Group 3 (polyherbal and mineral supplementation) experienced the most significant reduction (p<0.05), dropping from 0.42 mmol/L on Day 0 to 0.25 mmol/L on Day 28, indicating the synergistic effect of the combined supplementation in lowering NEFA concentrations.NEFA is a key biomarker of lipid metabolism and energy status in cattle, with elevated levels often indicating increased fat mobilization due to energy deficits, potentially compromising animal health. In this trial, the control group’s stable NEFA levels suggested that the basal diet alone had minimal influence on fat metabolism. Group 1, receiving herbal supplements (Bael, Curry leaves, and Moringa), showed a moderate decrease in NEFA levels, pointing to the potential lipid-modulating effects of phytoconstituents. Group 2 (mineral mixture) also exhibited a decline, though less marked than in Group 3. The lowest NEFA level (0.28 mmol/L at Day 28) observed in Group 3 underlines the effectiveness of combined herbal and mineral supplementation in supporting metabolic health. This improvement may be associated with enhanced energy balance, reduced fat mobilization, better lipid metabolism, and lowered oxidative stress.Furthermore, polyphenols and flavonoids present in herbal formulations are known to regulate lipid metabolism by modulating lipid synthesis, degradation, and transport in the body. These compounds can also suppress NEFA release from adipose tissue and promote lipid clearance from circulation, contributing to reduced serum NEFA concentrations (Hashemzadeh Cigari *et al*., 2014; Baghbadorani *et al*., 2022). Enhanced dietary energy utilization due to these supplements may explain the lowered circulating NEFA levels. Additionally, the hepatoprotective properties of these herbs are believed to support liver function and metabolism, which plays a central role in lipid processing. A healthier liver facilitates improved lipid metabolism, ultimately leading to a reduction in NEFA release into the bloodstream (Adewuyi *et al*., 2005). Supporting these findings, Baghbadorani et al. (2022) reported significantly lower (p<0.01) NEFA levels in cows supplemented with herbal extracts.



**FIG. 7: SERUM NEFA (mmol/L) LEVELS IN EXPERIMENTAL ANIMALS SUPPLEMENTED WITH POLYHERBAL MIXTURE AND MINERALS**

**Conclusion**

This study demonstrated that dietary supplementation with polyherbal and mineral mixtures significantly influenced the serum mineral profile of crossbred heifers. The combination of polyherbal and mineral supplementation (Group 3) consistently resulted in the highest levels of calcium, phosphorus, and magnesium, suggesting a synergistic effect in enhancing mineral metabolism. The polyherbal mixture (Group 1) contributed to moderate increases, likely due to bioactive compounds improving mineral absorption, while the mineral mixture (Group 2) showed a steady rise in mineral levels, indicating effective retention and utilization. These findings highlight the importance of dietary interventions in optimizing mineral homeostasis, which may positively impact reproductive efficiency and overall health in delayed pubertal heifers.

**Availability of data and material**

Not applicable

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