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Influence of Tanniferous Multinutrient block supplementation on reproductive performance of Murrah buffalo heifers

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Abstract
The present study was planned to evaluate the effect of Multi-nutrient block (MBs) and Tanniferous Multi-nutrient block (TMBs) on reproductive performance of Murrah buffalo heifers. A total of 21 Murrah buffalo heifers were selected and divided into three experimental groups (T_1, T_2 and T_3) having seven heifers in each group, based on body weight (247±2.29 kg) and age (40±04 months). Control (T_1) group was fed basal diet, comprising of wheat straw (*ad lib*), green fodder and concentrate to ful fill nutrient requirement of animals (ICAR, 2013). T_2 group was fed basal diet with only 60% of concentrate mixture of T_1 group supplemented with MBs (*ad lib*). T_3 group was fed basal diet with only 60% of concentrate mixture of T_1 group supplemented with TMBs (*ad lib*). Progesterone and estradiol concentration increased between intervals in all groups. MBs and TMBs supplemented buffalo heifer exhibited highest percentage of estrus during trial while highest conception rate was found in T_3 group. Based on the findings of the present study, it is concluded that MB's and TMBs supplementation lead to equal reproductive performance Thus MBs and TMBs can safely replace 40% concentrate mixture which improve reproductive performance of Murrah buffalo heifers.

Keywords: Tanniferous, Multi-nutrient block, Reproductive, Murrah buffalo, Heifer

Introduction
India is blessed with 109.85 million buffaloes sharing more than 50% milk production (DAHD, 2019) [6] in the country. Heifer rearing is the most expensive part of the buffalo production with feed cost contributing 63% to 84% of the total cost (Razaquee *et al.*, 2010) [18] representing a large expense to the overall farm operations. The feeding of livestock in India is largely based on agricultural byproducts but there is a huge gap between demand and supply. On all India basis, the overall deficit of concentrate, dry fodder and green fodder is 28.9%, 23.4% and 11.24%, respectively (Roy *et al.,* 2019) [19]. High nutritive value feed is prioritized to productive animals thus feeding of growing animals is compromised because small and marginal farmers are unable to afford high-cost rearing of buffalo heifer. Buffaloes are traditionally regarded as a poor breeder because of relatively poor reproductive efficiency. They exhibit many of the known reproductive disorders including delayed onset of puberty. Buffalo usually attain puberty when they reach about 60% of their adult body weight (250 to 400 kg), but the age at which they attain puberty may be highly variable, ranging from 18 to 46 months (Warriach *et al*., 2015) [22]. Poor nutritional quality of feed resources is one of the major limiting factors in delayed puberty of buffalo heifers but the age at first calving can be reduced through proper nutritional management of growing heifers (Zicarelli, 2010) [24].
Tannins are secondary plant polyphenols with great diversity. They have a high affinity for proteins and polysaccharides. Tannins produced by different plants, by different parts of the plants or in particular seasons can have different physical and chemical properties. Condensed tannins or proanthocyanidins, are a diverse group of polymeric flavanoids that readily complex with carbohydrates and proteins (Hagerman, 1992) [7]. Consequently, the tannin-protein reaction has been widely used to improve protein metabolism in ruminants. The anthelmintic mechanisms of plant tannins have been suggested through "direct" action of tannins on parasite cells by reducing establishment of the infective third-stage larvae in the host thereby reducing the host invasion, reducing excretion of nematodes eggs by the adult worms, and also by reducing development of eggs to third-stage larvae and through "indirect" action by improving the host's resistance to nematodes (Singh *et al.*, 2016) [20].

However, similar to their antimicrobial activities, the anthelmintic effects of tannins vary greatly depending on chemical composition and structure of tannins, the parasite species or growth stages and/or the hosts' species. Tamarind (*Tamarindus indica*) is an economically important tree, found in many countries in Asia, Africa and South America. India is the world's largest producer of tamarind and it is estimated that 300,000 T are produced annually. The tree mostly grows wild, although it is cultivated to a limited extent. It is particularly abundant in Indian states of Madhya Pradesh, Bihar, Andhra Pradesh, Karnataka, Tamil Nadu and West Bengal (Rao *et al*., 2015) [17]. Tamarind seed husk is a very economic agricultural byproduct having 14% condensed tannin (Bhatta *et al.,* 2001) [3] which is known to improve the host nutrition by protecting the dietary proteins from rumen degradation and also having anti parasitic property.

Urea is a good and economic source of nitrogen for ruminants and in order to supply urea in a safe way, several methods have been tried where urea molasses mineral block has proven to be the most convenient and economic. Several hundred formulas, with or without molasses, have been developed and tested according to the local availability, quality and price of ingredients. Potential source and optimum level of condensed tannins in form of tanniferous Multinutrient block, to be used in the diets of buffalo heifer to reduce GI parasitic load warrants investigation. This may provide three-way advantage to conquer the limitations of ruminants by reducing endoparasites, and strategic use of tanniferous byproducts and health status which otherwise remain under-utilized as animal feed. Hence, the present study was carried out using tanniferous multinutrient block to evaluate its effect on reproductive performance of Murrah buffalo heifers.

Materials and Methods

Place of work

The experiment was conducted on 21 Murrah buffalo heifers, divided into three groups based on body weight (247±2.29kg), available at livestock farm, N.D.V.S.U., Jabalpur. While, other works like data processing, analysis etc. were conducted in the Department of Animal Nutrition, College of Veterinary Science and Animal Husbandry, N.D.V.S.U., Jabalpur, Madhya Pradesh. Experiment was conducted for a period of one year. Feeding trial was conducted for six months.

Production of MB and TMB

Two types of multi-nutrient block were involved in the present study. A cold process technology was employed for the preparation of Multi-nutrient and Tanniferous multi-nutrient block. The common ingredients used for making MB and TMB were molasses, deoiled rice bran, urea, salt, mineral mixture, tamarind seed husk (in TMB), mustard oil cake, wheat flour, calcium oxide and guar gum, as a binder All the ingredients were thoroughly mixed. The whole mass was thoroughly stirred with the help of a rod by adding gradual amount of molasses in the metal basin. After that whole mixed feed was transferred into the wooden block making mould and pressed with help of handle. The blocks were air dried in the shed for 05-07 days until they are hard enough for easy handling, transport, hanging, and for licking by the experimental animals. Buffalo heifer (n=21) were selected and based on their body weight and age, randomly divided into three groups (T1, T2 and T3), having seven heifers in each group. Heifers in all the three groups were fed a similar basal diet, comprising of wheat straw, green fodder and concentrate to meet the nutrient requirements (ICAR, 2013) [8]. The buffalo heifers were housed in a well-ventilated shed having cemented floor with individual feeding and watering arrangement. Standard management practices were followed in the shed. Heifers were offered weighed quantity of concentrates followed by roughage (dry + green) feeding in morning and evening. The heifers were let loose for about 2-3 hours daily in the surrounded paddock for exercise. Clean fresh water was made available ad libitum.

The feeding trial was conducted at the Livestock Farm, Adhartal (LSF). For this purpose, twenty-one Murrah buffalo heifers were randomly allocated three treatment groups. The composition of concentrate mixture.

T_1: (Control): Concentrate mixture + green fodder + wheat straw *(ad lib).*

T_2: Concentrate mixture (60% concentrate mixture of T_1) + MBs + green fodder + wheat straw *(ad lib).*

T_3: Concentrate mixture (60% concentrate mixture of T_1) + TMBs + green fodder + wheat straw *(ad lib).*

Reproductive parameters

To see the effect of feeding MB and TMB, following parameters were recorded:

- Onset of 1st estrus was recorded.
- No. of services/conception were recorded.
- Progesterone in serum samples collected at 0, 90th and 180th days interval was determined by using commercial ELISA kit (plate 12).
- Estradiol concentration in serum samples collected at 0, 90th and 180th days interval was determined by using commercial ELISA kit (plate 11).

Estradiol ELISA

Principle of the test

The Monocent, Inc.'s E2 ELISA Test System is based on the principle of Delayed competitive binding assay between E2 in the test specimen and E2 enzyme conjugate for a constant amount of anti-Estradiol monoclonal antibody epitopes (Biotin reagent). In the incubation, anti-E2 antibodybiotin reagent, E2 standards, controls, and samples are incubated for 45 minutes at room temperature (RT), then E2 enzyme conjugate is added on the top of the reaction mixture and incubation continues is added for 45 minutes more. During the incubation, a fixed amount of HRP- labeled E2 competes with the endogenous E2 in the standard, sample, or quality control serum for a fixed number of binding sites of the specific E2 antibody. E2 peroxidase conjugate immunologically bound to the well progressively decrease as the concentration of E2 in the specimen increases. Unbound of anti-Estradiol Biotin Reagent and E2 peroxidase conjugate is then removed and the wells are washed. Next, a solution of TMB Reagent is added and incubated at room temperature for 20 minutes, resulting in the developed of blue color. The color development is stopped with the addition of stop solution, and the absorbance is measured spectrophotometric ally at 450nn. A standard curve is obtained by plotting the concentration of the standard versus the absorbance.

Materials and Components

1. Microwells coated with Streptavidin12x8x1
2. Estradiol Standards set: 6 vials (Ready to use) 0.5 ml

3. Estradiol Biotin Conjugate, 1 bottle (ready to use)7ml
4. Estradiol Enzyme conjugate Concentrate, 20x, 1 vial0.7 ml
5. Assay Diluent, 1 bottle (Ready to use)12ml
6. TMB Reagent, 1 bottle (Ready to use)12ml
7. Stop Solution, 1 bottle (Ready to use)12ml
8. Wash Concentrate 20x: 1 Bottle25ml

Materials required but not provided
1. Distilled or deionized water.
2. Precision pipettes.
3. Disposable pipette tips.
4. ELISA plate reader capable of reading absorbance at 450nm.
5. Graph paper.

Reagent preparation
1. 20X Enzyme conjugate: Prepared 1X working solution at 1:20 with assay diluent (e.g., Add 0.1 ml of the E2 enzyme conjugate concentrate to 1.9 ml of assay diluents)
2. 20X Wash buffer: Prepared 1X Wash buffer by adding the contents of the bottle (25ml, 20X) to 475ml of distilled or deionized water.

Test procedure
1. All reagents were brought to room temperature (20-25ºC) before use.
2. Secured the desired number of coated wells in the holder.
3. Dispensed 25ml of standards, specimens and controls into appropriate wells.
4. Dispensed 50ml of working solution of Estradiol Biotin Reagent into each well.
5. Mixed well by placing on shaker for 10-20 seconds.
6. Incubated at (20-25 ºC) for 45 minutes.
7. Dispensed 100ml of Estradiol Enzyme Reagent to all wells and mixed well by placing on shaker for 10-20 seconds.
8. Incubated at (20-25 ºC) for 45 minutes.
9. Liquid form all wells was removed. Washing of the wells three times with 300 ml of 1X wash buffer was done. It blotted on absorbance paper towel.
10. Dispensed 100ml of TMB reagent into each well and incubated at (20-25 ºC) for 20 minutes.
11. The reaction was stopped by adding 50 ml of stop solution to each well.
12. Gently mixed for 30 seconds. It was made sure that all the blue color changes to yellow color completely.
13. Absorbance was read at 450 nm with a microplate reader within 15 minutes.
14. Calculation of results was done

Progesterone ELISA
Principle of the test
The Calbiotech, Inc progesterone is a solid phase competitive ELISA. The samples, working progesterone-enzyme (HRP). Conjugate and anti-progesterone-Biotin reagent are added to the wells coated with Streptavidin. Progesterone in the patient's specimen complete with a progesterone HRP conjugate for binding sites. Unbound Progesterone and Progesterone enzyme conjugate is washing buffer. Upon the addition of the TMB substrate, the intensity of colour is inversely proportion to the concentration of Progesterone in the samples. A standard curve is prepared relating color intensity to the concentration of the Progesterone.

Materials provided
1. Microwells coated with Streptavidin
2. Progesterone Standard set: 6 viral (ready to use)
3. Progesterone Enzyme Conjugate (20X)
4. Progesterone Biotin Conjugate, 1 Bottle (ready to use)
5. Assay Diluent, 1 bottle (ready to use)
6. TMB Substrate: 1 bottle (ready to use)
7. Stop Solution: 1 bottle (ready to use)
8. Wash concentrate (20X): 1 bottle

Materials not provided
1. Distilled or deionized water.
2. Precision pipettes.
3. Disposable pipette tips.
4. ELISA reader capable of reading absorbance at 450nm.
5. Absorbance paper or paper towel.
6. Graph paper.

Reagents preparation
1. Progesterone-enzyme Conjugate Solution- Dilute the Progesterone enzyme conjugate1:21 with assay diluents in a container. (A slight excess of solution is made).
2. Wash Buffer- Prepared 1X Wash Buffer by adding the contents of the bottle (25ml, 20X) to 475 ml of distilled or deionized water and stored at room temperature.

Assay procedure: Prior to assay, all reagents were brought to room temperature.
1. The desired number of coated strips are placed into the holder.
2. 20 ml of Progesterone standards, control and serum samples are pipetted.
3. Added 100 ml of working Progesterone Enzyme Conjugate to all wells.
4. Added 50 ml of Progesterone Biotin Conjugate to all wells.
5. Incubated for 60 minutes at room temperature (20-25º C)
6. Liquid from all wells was removed, Washing of wells was done three times with 300 ml of 1X wash buffer and blotted on absorbent paper towels.
7. Added 100 ml of TMB substrate to all wells.
8. It was then incubated for 15 minutes at room temperature.
9. Added 50 ml of stop solution to all wells. The plate was shaken gently to mix the solution.
10. The absorbance was read on ELISA Reader at 450 nm within 15 minutes after adding the stop solution.
11. The results were calculated.

Results and Discussion
The reproductive hormone profile was assessed by assaying progesterone and concentration in the serum samples and the result is presented in Table 01. The mean serum progesterone (P4) concentration (ng/ml) was comparable (p>0.05) between treatment groups at 0, 90th and 180th day. The mean serum concentration of progesterone, irrespective of treatments, was significantly (p<0.01) different at 0, 90th and 180th days, with the highest values on 180th day. The mean serum progesterone concentration was 0.83±0.14, 1.14±0.16 and 1.20±0.10 (ng/ml) 180th day for T_1, T_2 and T_3 group respectively.

Table 1: Effect of MBs and TMBs supplementation on progesterone (ng/ml) in Murrah buffalo heifers

Day	Treatment 1	Treatment 2	Treatment 3	P value
Day-0	0.68±0.13	0.48^b±0.06	0.46^c±0.06	0.207
Day-90	0.86±0.11	0.92^a±0.10	0.83^b±0.06	0.756
Day-180	0.83±0.14	1.14^a±0.16	1.20^a±0.10	0.162
P value	0.584	<0.0001	<0.0001	

Means bearing different superscripts differ significantly between treatments (uppercase) & between intervals (lowercase) (p<0.05)

Effect of MBs and TMBs supplementation on estradiol (pg/ml) in Murrah buffalo heifers

At the 0, 90th, and 180th days, the experimental Murrah buffalo heifer's estradiol (pg/ml) concentration was measured to determine the effects of MBs and TMBs supplementation. The results are shown in Table. The mean serum estradiol concentration (pg/ml) was comparable (p>0.05) between treatment groups at 0, 90th and 180th day. However, the mean estradiol concentration was found significantly different at 0, 90th and 180th days, within the group.The mean serum estradiol concentration was 29.14±1.94, 29.57±2.64 and 31.86^a±0.63 (pg/ml) on 180thday for T_1, T_2 and T_3 group respectively.

Table 2: Effect of MBs and TMBs supplementation on estradiol (pg/ml) in Murrah buffalo heifers

Day	Treatment 1	Treatment 2	Treatment 3	P value
Day-0	18.86^b±2.40	18.14^b±1.56	18.71^c±1.74	0.963
Day-90	23.57^{ab}±1.15	23.14^{ab}±2.34	25.29^b±1.02	0.620
Day-180	29.14^a±1.94	29.57^a±2.64	31.86^a±0.63	0.573
P value	0.005	<0.0001	<0.0001	

Means bearing different superscripts differ significantly between treatments (uppercase) & between intervals (lowercase) (p<0.05)

The overall mean serum progesterone (P4) concentration was comparable (p>0.05) in T_1, T_2 and T_3 groups respectively. The mean serum concentration of progesterone, irrespective of treatments, was significantly (p>0.05) different at 0, 90th and 180th days, with the highest values on 180th day. Similarly, the overall mean serum estradiol concentration was also found comparable (p>0.05) in all groups.

The blood level of P4 >1 ng/ml is considered a good indicator of the beginning of puberty in heifers (Terzano *et al*., 2012 and Khan *et al*., 2015) [21, 11]. The initial level of progesterone was <1 ng/ml and the estradiol concentration varied from 9.95-12.15 (pg/ml) among all groups on 0 day, indicating all heifers to be in their prepubertal stage, Jain and Pandey (1983) also reported P4 level <1 and estradiol level between 6.8-12.8 pg/ml in prepubertal buffalo heifers during the first 12 month of age. The progesterone and estradiol level was found similar among all groups, but concentration of progesterone and estradiol on 0, 90th and 180th days period differed significantly (p<0.05) with the advancement of age in heifers. The increasing trend of progesterone (>1 ng/ml) and estradiol in supplemented groups indicated that heifers started attaining puberty early to bring the heifers to cyclicity (Jain and Pandey, 1983) [9]. Khan *et al.* (2015) [11] reported that supplementation of Vitamin E and mineral caused early initiation of cyclicity (32 days postpartum) compared to control group (35 days postpartum) which was evident from progesterone concentration (>1 ng/ml). Campbell and Miller (1998) [5] also reported similar results in dairy cows supplemented with vitamin E and Zn. The increase in the level of estradiol is reflected as a transformation of the follicles into the interstitial tissues that augments the potential capacity of ovary to form estrogen during the prepubertal stage (Baker, 1972) [1]. The estradiol concentration show two peaks i.e one before the day of oestrus while second during days 10-11 of the cycle (Baruselli *et al*., 1997; Manik *et al.*, 1998) [2, 14], because of these fluctuations estradiol is not considered as reliable as progesterone level in detecting the onset of puberty in prepubertal animals.

Heat detection record

The heat detection records of buffalo heifers in all groups is presented in Table 03. The first heat detection record obtained from 180 days study showed that 71.4 percent heifers exhibited heat symptoms in T_2 and T_3 groups with the 80 and 60 percent conception rate respectively.

Table 3: Heat detection and conception records in Murrah buffalo heifers

S. No.	Attributes	T_1	T_2	T_3
1.	Number of heifers	7	7	7
2.	Number of heifers exhibited estrus	4/7	5/7	5/7
3.	Percentage of estrus (%)	57.14	71.4	71.4
4.	Heifers Bred	4/4	5/5	5/5
5.	Number of heifers conceived	2/4	4/5	3/5
6.	Avg. Service per conception	2	1.75	1.67
6.	Overall conception rate (%)	50	80	60
7.	Number of heifers not exhibited estrus	3	2	2

An efficient reproductive process is a prerequisite for profitable dairy farming. Reproductive well-being and performance of farm animals is largely dependent on their nutritional status, which is often compromised in developing countries, coupled with the limited purchase capacity of smallholder and landless farmers. Anestrous or delayed estrus is one of the most commonly occurring reproductive problems in cattle and buffalo in India, thus affecting livestock productivity and economics to a great extent.

In our 180 days study the heat detection record showed that 71.4 percent heifers exhibited heat symptoms in T_2 and T_3 groups. This showed the supplementation of MBs and TMBs had potential for early onset of estrus in buffalo heifers. Yadav *et al.* (2012) [23] reported postpartum estrus was significantly (P<0.05) lower (89.18 days) in UMMB group than control without supplement (136.24 days) which showed that UMMB supplementation along with balance concentrate mixture helped in early induction of estrus in cows, which was in agreement with the finding of Ramesh *et al.* (2009) [15].

In postpartum animals, a higher proportion (71 percent) of the supplemented buffaloes displayed estrus within 50 days postpartum, compared with only 14 percent in the controls (Randhawa, 2002) [16]. Khadda *et al.* (2014) [10] also reported significant reduction in postpartum estrus period and service period in UMMB group than the control group.

However, some studies revealed that UMMB supplementation did not appear to affect the onset of first behavioral oestrus, which could probably be related to factors other than nutritional status of an animal (Butler, 2000) [4]. Similar observation was made when UMMB supplementation in buffaloes on larger-scale organized urban dairy farms had no effect on oestrus induction (Kumar, 2001) [12], probably due to the already better nutritional status of buffaloes (Makkar and Saijpaul, 1996) [13].

Conclusion
It is concluded that MBs and TMBs supplementation lead to improve in reproductive performance of Murrah buffalo heifers.

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