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Therapeutic efficacy of bromelain in treatment of subclinical endometritis in buffaloes

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Abstract
The study aimed to assess the effectiveness of Bromelain in treatment of subclinical endometritis in buffaloes. Buffaloes (n=60) with a history of repeat breeding were screened through per-rectal examination of the reproductive tract, followed by evaluation of cervical-vaginal mucus score and pH. Buffaloes with subclinical endometritis were randomly allotted to Bromelain (Group I) and *E. coli* LPS (Group II) groups treated by intrauterine route. Haemato-biochemical findings revealed that differences in neutrophils, lymphocytes and serum cholesterol levels in Group I and II buffaloes were significantly ($p<0.01$) lower while, serum calcium levels were significantly ($p<0.01$) higher at subsequent estrus after treatment compared to pre-treatment estrus. Haemoglobin, PCV, serum total protein and phosphorus levels increased non-significantly ($p>0.05$) whereas AST and ALT levels decreased non-significantly at subsequent estrus after treatment. In conclusion, recovery rates of Group I and II were recorded as 75.00 and 85.50 percent, respectively.

Keywords: Subclinical endometritis, bromelain, *E. coli* LPS, haemato-biochemical parameters

1. Introduction
Subclinical endometritis is characterized by inflammation of the uterus typically identified through cytology, without evidence of clinical symptoms such as the presence of pus or purulent material in the vagina. Diagnosis involves assessing the proportion of neutrophils in mucus samples collected from the uterine lumen. The clear mucus discharge containing more than 5% polymorphonuclear cells (PMN) indicates the presence of subclinical endometritis (Kasimanickam *et al*., 2004) [11]. Various hematological and serum biochemical parameters have been analyzed to establish correlations with inflammatory and infectious conditions of the bovine endometrium (Bajaj *et al.*, 2016) [3]. Different treatment protocols have been employed for endometritis in buffaloes, including antibiotics, antiseptics, hormones, and anti-inflammatory medications. However, the indiscriminate use of antibiotics, with irregular and inadequate dosage, has led to the emergence of microbial resistance, posing a significant challenge under one health concept. Recent approaches to treating endometritis involve intrauterine infusion of immunomodulators such as *E. coli* LPS, proteolytic enzymes, Oyster glycogen, and Lysozyme, providing an alternative to traditional intrauterine and parenteral antibiotic treatments (Sarma *et al*., 2012) [17]. Bromelain a proteolytic enzyme exhibits medicinal properties such as anti-inflammatory, anti-thrombotic, fibrinolytic and anti-cancer effects in cancer patients by direct impact on cancer cells and their micro-environment, as well as in the modulation of immune, inflammatory and haemostatic systems in humans (Chobotova *et al.* 2010) [4]. With these considerations in mind, the current study was taken up to assess the efficacy of Bromelain in treatment of subclinical endometritis in buffaloes.

2. Materials and Methods
The present investigation was undertaken in buffaloes presented to the Large Animal Gynaecology Unit, Department of Veterinary Gynaecology and Obstetrics, NTR College of Veterinary Science and Veterinary Dispensaries and Rural Livestock Units of Animal Husbandry Department, Govt. of A.P. that were located in and around Gannavaram, Andhra Pradesh. Repeat breeder buffaloes were diagnosed for subclinical endometritis using detailed

clinico-gynaecological examination by per-rectal examination, followed by assessment of rheological characteristics of cervico-vaginal mucus like colour, consistency based on spinbarkeit value (Verma *et al*. 2023) [24] and pH by using pH paper strips with indicator ranging from 7.5 to 9.0 (Merck Ltd., PCLBL, Worli, Mumbai) (Tsiligianni *et al*. 2001) [22]. For which the cervico-vaginal mucus (CVM) was collected by aspiration technique at the time of estrus before treatment and at subsequent estrus after treatment in both groups. Also, the presence of uterine infection (subclinical and clinical endometritis) was detected by leucocyte esterase strip test (SHENHUA URS-11G) and uterine discharge cytology from the smears as per the procedure described by Gahlot *et al.* (2017) [8]. Blood samples were collected from subclinical endometritis affected buffaloes of Group I and Group II in EDTA vacutainers to assess the haematological parameters, while blood collected in clot-activating vacutainers was utilized to obtain the necessary serum for assessing biochemical parameters. Haemoglobin (Hb), Packed cell volume (PCV) and Differential leukocyte count (DLC) (neutrophil, lymphocyte counts) were determined as per the standard procedures described by Jain (1993) [10]. Estimation of biochemical parameters such as serum metabolites (total protein, cholesterol), serum enzymes (Aspartate aminotransferase and Alanine aminotransferase) and serum minerals (calcium and phosphorus) was done by using MULTISKAN GO, Thermo Scientific Instruments with the help of commercially available kits manufactured by Erba Transasia Biomedicals, Solan, HP. The statistical analysis of the data was done by "paired t-test" as per the procedures outlined by Snedecor and Cochran (1994) [19].

3. Results and Discussion

3.1 Occurrence of subclinical endometritis based on per-rectal examination

A total of 60 buffaloes with the history of repeat breeding were screened to assess the consistency, symmetry, tonicity of uterine horns and revealed that 35 buffaloes exhibited normal, symmetrical uterine horns at the time of estrus, whereas 25 buffaloes exhibited thickened uterine horns with extra curvature. Based on the findings of per-rectal examination the occurrence of subclinical and clinical endometritis was 58.33 and 41.67 percent, respectively.

3.2 Occurrence of subclinical endometritis based on cervico-vaginal mucus score

The buffaloes, which exhibited normal, symmetrical uterine horns (n=35) during the per-rectal examination at the time of estrus, were evaluated for cervico-vaginal mucus score. The results revealed that 27 (77.14%) buffaloes showed clear cervico-vaginal mucus (score 0), while 8 (22.86%) buffaloes showed abnormal mucus discharge (score 1-2). Among the buffaloes with abnormal mucus discharge, 14.29 percent (5/35) of buffaloes had mucus with flakes of pus (score 1) and 8.57 percent (3/35) exhibited muco-purulent discharge (score 2).

3.3 Categorization of subclinical endometritis based on pH of cervico-vaginal mucus

The buffaloes which had cervico-vaginal mucus score 0 (n=27) were utilized for the estimation of pH. The pH of cervico-vaginal mucus was recorded by using pH paper strips (within range of 6.5-9.0). Out of 27 subclinical endometritis the cervico-vaginal mucus of 22 (81.48%) buffaloes showed pH range from 7.5 to 8.5 and 5 (18.52%) buffaloes expressed pH range from 8.5 to 9.0.

3.4 Diagnosis of subclinical endometritis

3.4.1 Leucocyte esterase strip test (LEST)

The buffaloes with cervico-vaginal mucus score 0 (n=27) were further screened with leucocyte esterase test (LEST). The uterine discharge from these buffaloes was subjected for LEST and the colour changes were suggestive of leucocyte esterase activity, which was scored from 0 to 4 depending on the degree of infection or leucocyte cell count. The uterine discharge of 27 buffaloes upon leucocyte esterase strip test revealed that 8 (29.62%), 15 (55.56%), 3 (11.11%) and 1 (3.70%) uterine discharges showed traces (1) low (2), moderate (3) and high (4) number of leucocytes, respectively. From the present results it was interpreted that uterine discharges with LEST score of 1 and 2 i.e traces (±) and low number of leucocytes (+) were positive for subclinical endometritis while LEST scores of 3 (++) and 4 (+++) were considered to be positive for clinical endometritis.

3.4.2 Uterine discharge cytology

The buffaloes, which exhibited clear cervico-vaginal mucus with score 0 were evaluated for uterine discharge cytology by aspiration technique. Aspiration of the uterine discharge at estrus could be easily done with the help of blue sheath to obtain good quantity of uterine discharge without any contamination. The cytosmears stained with Field's stain could be easily evaluated for the percentage of PMN cells. The percentage of PMN cells in the present study were recorded in the range of >5% to 17% in 77.78 percent (21/27) and >18% in 22.22 percent (6/27) of buffaloes, respectively.

Buffaloes having the PMN counts >18% were diagnosed as to be suffering from clinical endometritis based on uterine discharge cytology and were excluded from the present study. Whereas, the subclinical endometritis affected buffaloes (n=16) with >5% PMN cells (range 6-17%) were randomly divided into 2 groups and subjected for therapeutic trials *viz*., Bromelain (n=8) and *E. coli* LPS (n=8) and the efficacy of treatments was compared in terms of haemato-biochemical variations, recovery and conception rates.

3.5 Haemato-biochemical changes in subclinical endometritis affected buffaloes

3.5.1 Haematological changes

The haemoglobin (gm/dL) and PCV (%) concentrations increased non-significantly (p>0.05) at subsequent estrus after treatment as compared to pre-treatment estrus (Table 1; Plate 1). The present findings were in concurrence with the earlier reports of Thangamani (2022) [20] and Verma *et al.* (2023) [24] who also found a non-significant variation in values of Hb and PCV. On the contrary, the findings of Kekan *et al*. (2005) [12] suggested that the mean Hb concentration and packed cell volume were significantly lower in repeat breeders with uterine infection as compared to healthy bovines which could be due to mild anaemia. In the present study, the possible variations in the Hb and PCV levels could be due to the immunomodulatory effect of treatment molecules under the study, which might have resulted in overall improvement of uterine health and elimination of bacterial infection within the uterus.

The difference in neutrophils count (%) and lymphocytes count (%) in Group I and Group II at subsequent estrus after treatment were significantly (p<0.01) lowered as compared to pre-treatment estrus (Table 1; Plate 1). The present findings

were in close agreement with the reports of Thangamani (2022) [20] and Verma *et al.* (2023) [24] who also recorded significant variations in % of neutrophils and lymphocytes. On the divergent, the reports of Rao (2010) [15] cited that neutrophil (%) and lymphocyte counts (%) differed non-significantly after treatment. The variation in the current study could be due to the degree of leucocytosis which depended upon several factors such as severity of infection, nature of the causative agent or predisposing factors, susceptibility of animals and localization of inflammation (Kumar *et al*., 2018) [13].

3.5.2 Biochemical changes

The serum total protein concentration (gm/dL) was increased non-significantly ($p>0.05$) in both the groups after treatment and was within the normal reference range (Table 2; Plate 2). The findings in the present study were in close agreement with the report of Gahlot *et al.* (2017) [8] and Tilakrao (2018) [21] who also found a non-significant variation in the concentration of total protein. On the contrary, Rao (2010) [15], recorded lowered serum total protein (gm/dL) concentrations in endometritic/repeat breeder buffaloes compared to normal healthy buffaloes. A non-significant increase in the levels of protein could be due to better uptake of amino acids, which were required for the biosynthesis of gonadotropins and gonadal hormones and might have resolved the reproductive hormonal disturbances due to recovery from subclinical uterine infection.

The mean concentration of cholesterol (mg/dL) in serum of Groups I and II showed a highly significant decline ($p\leq0.01$) at post-treatment estrus as compared to the pre-treatment value (Table 2; Plate 2). The present findings were in concurrence with the earlier reports of Gahlot *et al.* (2017) [8] who also reported significantly higher cholesterol concentration in buffaloes affected with sbclinical endometritis (SCE). The higher levels of cholesterol in the SCE affected buffaloes of the present study might be due to cellular changes in the endometrium which attracted the immune cells (neutrophils) to promote inflammatory responses, including augmentation of toll-like receptor (TLR) signalling (Gilbert *et al*., 1993) [9].

A non-significant decrease in the levels of mean serum AST and ALT was recorded in Group I and Group II buffaloes at pre-treatment and post-treatment estrus (Table 2; Plate 2). The present findings are in close agreement with the reports of Salzano *et al*. (2020) [16], Afreen (2021) [1] who also recorded non-significant variations in the concentration of AST and ALT which might be due to lower intensity of uterine infection with minimal epithelial damage in the endometrium and it might not have been manifested in the peripheral circulation (Tilakrao, 2018) [21].

The mean mineral concentrations recorded in the SCE affected buffaloes revealed that the serum calcium (mg/dL) of Group I and Group II buffaloes showed a significant increase ($p\leq0.01$) at post-treatment estrus as compared to the pre-treatment value(Table 2; Plate 2). The concentrations of the mean serum phosphorus (mg/dL) in Groups I and II showed a non-significant ($p>0.05$) increase at subsequent estrus after treatment compared to pre-treatment values. These findings were in agreement with the earlier observations of Das *et al*. (2012) [5] and Amle *et al*. (2014) [2] who reported that mean serum calcium and phosphorus concentrations (mg/dL) were significantly lower in repeat breeding bovines affected with SCE. These lower levels might have predisposed to occurrence of uterine infection in buffaloes as myometrium contractability was one of the major components of uterine defence mechanisms leading to expulsion of the uterine contents, which were influenced by lack of exercise and hypocalcaemia (Sheldon and Dobson, 2004) [18].

3.6 Clinical recovery and conception rate

The assessment of clinical recovery from subclinical endometritis following treatment with intrauterine infusion of Bromelain (Group I; n=8) and *E. coli* LPS (Group II; n=8) was evaluated before treatment and at subsequent estrus after treatment based on variations in the haematological and biochemical profiles, pH of cervico-vaginal mucus and uterine discharge cytology (PMN cell %).

The mean pH value revealed a significant ($p<0.01$) decline from pre-treatment to post-treatment estrus in Group I and Group II buffaloes treated with Bromelain and *E. coli* LPS, respectively (Table 3). The present findings were in agreement with the observations of Venkatesh (2019) [23] who also recorded a similar trend. In the current study, the higher pH before treatment could be due to increased concentration of bacterial metabolites that damaged the endometrium (Kumar *et al*., 2004) [14]. Results of pH recorded after treatment implied that recovery from subclinical infection had occurred as the pH reduced from alkaline values to nearly neutral values due to clearing of the uterine infection.

A significant reduction ($p<0.01$) in the PMN cell percentage was recorded in uterine discharge of Group I and II treated buffaloes at subsequent estrus after treatment as compared to the pre-treatment values (Table 4) which might be due to immunomodulatory action of Bromelain and *E. coli* LPS, which improved the uterine defence mechanism that culminated to reduction in the PMN cell count as stated by Bajaj *et al*. (2016) [3] and Venkatesh (2019) [23].

In the present study, the recovery rate of Group I buffaloes after therapy was 75.00 percent (6/8) while the same in Group II was 87.50 percent (7/8) (Table 5). The clinical recovery was based on presence ≤5% PMN cells on evaluation of uterine discharge cytology in buffaloes of both the treatment groups at subsequent estrus after treatment revealed that the recovery rates of both the treatment groups varied non-significantly ($p>0.05$). From the results, it could be interpreted that Group I buffaloes showed similar recovery rate as compared to Group II buffaloes as the variation in recovery rate was statistically non-significant. Hence, the efficacy of Bromelain was similar with that of *E. coli* LPS and could be used as an effective alternative to obtain better recovery rate in subclinical endometritis affected buffaloes. Several actions of Bromelain *viz.* modulation of both T cell and B cell immunity (Engwerda *et al*., 2001) [6], anti-inflammatory and analgesic actions in addition to its anti-oedematous, antithrombotic and fibrinolytic effects, anti-cancer effects (Chobotova *et al*., 2010) [4] with proven antioxidant activity (Esam and Taloki, 2020) [7] might have resulted in clinical recovery in subclinical endometritis affected buffaloes.

The conception rate following treatment with Bromelain and *E. coli* LPS was 66.66 (4/6) and 71.43 (5/7) percent, respectively from the first service conception rates performed on the recovered buffaloes (Table 6). Further, in the present study a higher conception rate was recorded in subclinical endometritis buffaloes treated with *E. coli* LPS (Group II) (71.43%) compared to treatment with Bromelain (Group I) (66.66%) and the difference was statistically non-significant ($p>0.05$).

Table 1: Haematological values (Mean± SE) of subclinical endometritis buffaloes before treatment and after treatment.

Group	Treatment	Hb (gm/dL)	PCV (%)	Neutrophils (%)	Lymphocytes (%)
Group I (Bromelain) (n=8)	Before treatment	11.43±0.26	35.57±0.38	41.81±0.59	53.37±0.41
	After treatment	12.01±0.12	36.28±0.12	33.06±0.31**	42.75±0.49**
Group II (*E. coli* LPS) (n=8)	Before treatment	11.40±0.28	35.53±0.37	42.37±0.57	52.12±0.71
	After treatment	12.02±0.13	36.23±0.11	32.81±0.45**	42.00±0.59**

Means bearing different superscripts **($p \leq 0.01$) differ significantly within a column for before treatment and after treatment

Table 2: Serum biochemical values (Mean± SE) of subclinical endometritis buffaloes before treatment and after treatment

Group	Treat ment	TP (gm/dL)	Cholesterol (mg/dL)	AST (IU/L)	ALT (IU/L)	Ca (mg/dL)	P (mg/dL)
Group I (Bromelain) (n=8)	Before	6.37±0.13	112.75±1.39	69.87±0.58	22.80±0.40	10.37±0.31	6.35±0.10
	After	6.55±0.07	108.50±1.43*	67.56±0.59	21.81±0.50	11.00±0.26*	6.60±0.09
Group II (*E. coli* LPS) (n=8)	Before	6.35±0.11	110.87±1.36	69.62±0.75	22.68±0.64	9.96±0.23	6.21±0.15
	After	6.52±0.05	108.12±1.34*	67.25±0.64	21.62±0.92	11.05±0.12*	6.52±0.07

Means bearing different superscripts *($p \leq 0.05$) differ significantly within a column for before treatment and after treatment.

Table 3: The pH of cervico-vaginal mucus (Mean± SE) at estrus in subclinical endometritis buffaloes before treatment and at subsequent estrus after treatment.

Groups	No. of buffaloes	Before Treatment	At subsequent estrus
Group I (Bromelain)	8	8.22±0.11	7.52±0.15**
Group II (*E. coli* LPS)	8	8.06±0.12	7.10±0.14**

Means bearing (**) different superscripts differ significantly ($p \leq 0.01$) within a row for before treatment and at subsequent estrus

Table 4: The percentage of PMN cells (Mean± SE) in uterine discharge at estrus in subclinical endometritis buffaloes before treatment and at subsequent estrus after treatment

Groups	No. of buffaloes	Before Treatment	At subsequent estrus
Group I (Bromelain)	8	9.50±0.56	4.87±0.51**
Group II (*E. coli* LPS)	8	9.25±0.59	4.62±0.41**

Means bearing (**) different superscripts differ significantly ($p \leq 0.01$) with in a row for before and at subsequent estrus

Table 5: Recovery rate in buffaloes affected with subclinical endometritis based on uterine discharge cytology

Treatment Group	No of animals treated	No of animals recovered	Recovery (%)	Chi-square value
Group I (Bromelain)	8	6	75.00	1.00^{NS}
Group II (*E. coli* LPS)	8	7	87.50	

NS Non-significant difference ($p>0.05$) observed for uterine discharge cytology in between the treatment groups.

Table 6: Conception rate in buffaloes recovered from subclinical endometritis

Treatment Group	No of animals treated	No of animals recovered	No of animals conceived	Conception rate (CR) on animals recovered (%)	Chi-square value
Group I (Bromelain)	8	6	4	66.66	1.00^{NS}
Group II (*E. coli* LPS)	8	7	5	71.43	

NS non-significant difference ($p>0.05$) was observed between the
Groups I and Group II for conception rates

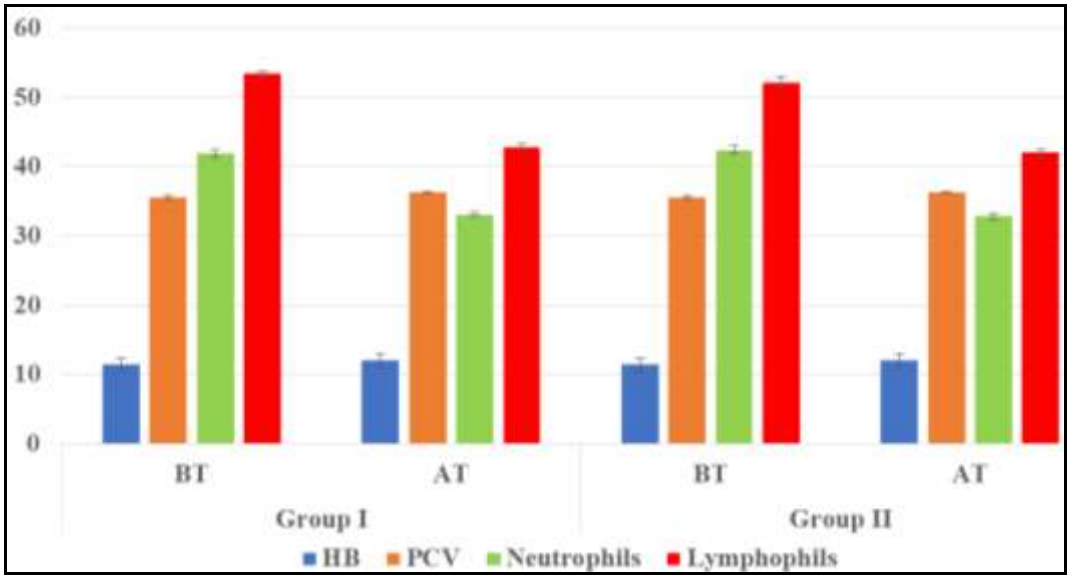

Plate 1: Mean Haematological values in Group I and Group II before and after treatment

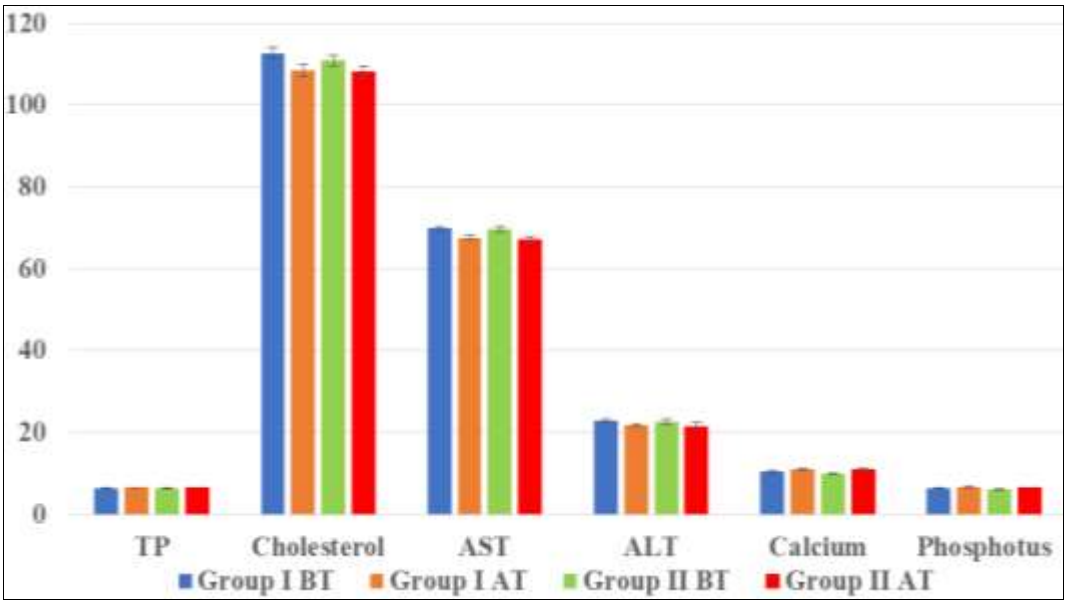

Plate 2: Mean Biochemical values in Group I and Group II before and after treatment

4. Conclusion

From the results, it could be interpreted that Group I buffaloes showed a similar first service conception rate as compared to Group II buffaloes as the variation in conception rate was statistically non-significant. Hence, the efficacy of Bromelain was similar with that of *E. coli* LPS and could be used as an effective alternative to antibiotic therapies improve the recovery and conception rates in buffaloes affected with subclinical endometritis. Further, the cost of the treatment for subclinical endometritis in buffaloes could be mitigated with more readily available Bromelain replaced with *E. coli* LPS which was difficult to procure with higher initial cost at the time of purchase.

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