ORIGINAL ARTICLE

TamaFlex™—A novel nutraceutical blend improves lameness and joint functions in working horses

Sandesh Jain[1] [iD] | Satyajit G. Patil[2] [iD] | Gopichand Chinta[2] [iD] | KrishnaRaju Venkata Alluri[2] [iD]

[1]Vins Bioproducts Ltd., Hyderabad, Telangana, India

[2]Laila Nutraceuticals R & D Centre, Vijayawada, Andhra Pradesh, India

Correspondence
KrishnaRaju Venkata Alluri, Pharmacology & Clinical Research, Laila Nutraceuticals R & D Centre, Vijayawada, Andhra Pradesh, India.
Email: avkr@lailanutra.in

Abstract

Background: Lameness is one of the major causes of reduced physical performance and early retirement in working horses. TamaFlex™ (NXT15906F6) is a standardized synergistic anti-inflammatory botanical formulation containing *Tamarindus indica* seed extract and *Curcuma longa* rhizome extract at a 2:1 ratio.

Methods: We conducted a 12-week single-center, randomized, blinded, placebo-controlled trial demonstrating the efficacy of NXT15906F6 in horses with lameness grade 2–4 on the American Association of Equine Practitioners (AAEP) scale. Twenty-two lame horses were supplemented with NXT15906F6 (2.5 gram/day) or placebo over a period of 84 days. Improvement in lameness over placebo was the primary endpoint, and changes in the levels of rheumatoid factor (RF), anti-nuclear antibody (ANA), and anti-cyclic citrullinated peptide (ACC-peptide) in serum, and pro-inflammatory cytokines including interleukin (IL-1β and IL-6), tumor necrosis factor-α (TNF-α) and prostaglandin-E$_2$ (PGE$_2$) in serum and synovial fluid were the secondary endpoints.

Results: NXT15906F6 exhibited significant relief from lameness in a time-dependent manner. NXT15906F6 also reduced levels of ANA, PGE$_2$, IL-1β, TNF-α and IL-6. Moreover, NXT15906F6 supplementation is safe and tolerable in alleviating joint pain in lame horses, and protects the joints from further degradation by reducing pro-inflammatory mediators.

Conclusion: NXT15906F6 significantly reduces the lameness during walking and trotting, leading to an improvement in their joint flexibility, health, and working performances.

KEYWORDS
AAEP scale, ACC peptide, lameness, laminitis, rheumatoid factor, TamaFlex

1 | INTRODUCTION

Lameness is defined as an abnormal stance or gait caused by either a structural or a functional disorder of the locomotor system. Lameness is characterized by the manifestation of pain, mechanical disruption, and alteration of stance or gait. These alterations may be mild during rest but debilitate the physical performance of equines. Horses experience high-intensity pain, obstruction in movement, and

altered gait during walking, trotting, or exercise in their daily life (Kufa, 2019).

Lameness is a widespread cause of reduced physical performance and early retirement of horses. Over 70% of horses suffer from lameness in their lifetime (Niemelä et al., 2016). A retrospective study conducted on 30,741 horses from 47 countries during 2008–2011 showed that 29.8% of horses were eliminated from rides due to lameness (Nagy et al., 2014). In the United Kingdom, 33% of horses had been lame during their career (Murray et al., 2010). A field study conducted on 227 horses from India and Pakistan found that 98% of the working horses had a gait abnormality in all four limbs, and 87% had at least one limb scoring 3 or 4 on a lameness scale of 0–4. Lameness and pathological abnormalities are often responsible for specific pain responses in the feet, limbs, and spine (Broster et al., 2009). Lameness can be caused by laminitis, trauma (caused by single or repetitive work), developmental defects, infection, congenital or acquired anomalies, metabolic disturbances, and circulatory and neuronal disorders (Baxter et al., 2020). A major cause of lameness is laminitis, a severely debilitating disease caused by inflammatory and pathologic sequelae, as well as advancing age. Cold hosing and NSAIDS, such as phenylbutazone and flunixin meglumine, are the first-line therapies for the management of lameness in horses. Interleukin-1 receptor antagonist protein, platelet-rich plasma, and exogenous corticosteroids such as triamcinolone acetonide are the second-line agents (Contino, 2018). Despite their therapeutic ability, these agents have their own adverse effects on prolonged use. In recent years, researchers have shifted their focus toward standardized botanical extracts to cure joint discomfort and pain. Earlier studies revealed the anti-inflammatory efficacy and pain alleviation by diverse nutraceuticals, including *Curcuma longa*, *Boswellia serrata*, passion fruit peel, pycnogenol, and L-carnitine (Liu et al., 2018). Botanicals have a long history of safe use in food and traditional medicine in pain alleviation (Di Lorenzo et al., 2013).

Tamarind (*Tamarindus indica* L., Fabaceae) leaves and fruits are well-known sources of food ingredients. Tamarind seed kernel is known to have high antioxidant and anti-inflammatory activities (De Caluwé et al., 2010). The anti-inflammatory and anti-arthritic efficacy of *T. indica* seed extract was demonstrated against Freund's adjuvant (FCA)-induced arthritis in rats (Sundaram et al., 2015). Turmeric (*C. longa* L., Zingiberaceae) is a rhizomatous herbaceous perennial plant. Curcumin, the principal component in turmeric, is well known for its potent anti-inflammatory activities by inhibiting TNF-α-dependent NF-κB activation (Hatcher et al., 2008). Furthermore, curcumin down-regulates inducible cyclooxygenase-2 enzyme expression and inhibits proinflammatory 5-lipoxygenase (5-LPO) production (C. V. Rao, 2007).

TamaFlex™ (NXT15906F6) is a clinically proven synergistic botanical formulation containing ethanol and aqueous extracts of *T. indica* seeds combined with an aqueous ethanol extract of *C. longa* rhizome. NXT15906F6 supplementation over a period of 90 consecutive days improved knee flexibility and offered substantial relief from knee pain after physical activity in healthy male and female subjects (P. S. Rao et al., 2019). In vitro cell-based experiments and a pre-clinical model of monosodium iodoacetate-induced osteoarthritis in Sprague Dawley rats showed that NXT15906F6 acts as a synergistic anti-inflammatory formulation to reduce pain, inflammation, and osteoarthritis symptoms (unpublished observation).

We hypothesized that NXT15906F6 might alleviate lameness and joint pain and improve joint flexibility and functions in horses with lameness.

In this randomized, blinded, placebo-controlled study, we assessed the safety and efficacy of NXT15906F6 (dose 2.5 g/day) in alleviating joint pain in horses with lameness. We evaluated rheumatoid factor (RF), anti-nuclear antibody (ANA), anti-cyclic citrullinated peptide (ACC-peptide) in serum and interleukin-1β (IL-1β), IL-6, tumour necrosis factor-α (TNF-α), and prostaglandin-E$_2$ (PGE$_2$) in serum and synovial fluid of the horses at baseline and at the end of the study. Total blood chemistry parameters exhibited broad spectrum safety.

2 | MATERIALS AND METHODS

2.1 | Study design

This study was designed as a single-centre, randomized, blinded, placebo-controlled clinical trial in 22 male and female horses (age 5–7 years) over a period of 84 consecutive days. The study protocol was reviewed, recommended by the Institutional Animal Ethics Committee, and approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (Approval No. 25/4/2019-CPCSEA). The principal investigator, a trained veterinarian, obtained written and informed consent form from the owner/authorized person of each horse participating in this study after an adequate explanation of the aims, methods, objectives, and potential hazards of the protocols of the study.

2.2 | Test item

TamaFlex™ (NXT15906F6) is a novel patented 2:1 combination of extracts derived from *T. indica* seeds and *C. longa* rhizomes. NXT15906F6 contains six parts (w/w) of *T. indica* seed extract, three (w/w) of parts *C. longa* rhizome extract and one part of excipient mixture (80% [w/w] microcrystalline cellulose powder and 20% [w/w] Syloid 244FP). NXT15906F6 is standardized to contain a minimum of 65% proanthocyanidins and 3% total curcuminoids. The detailed methods of preparation and standardization of NXT15906F6 were described earlier (Badmaev et al., 2018).

2.3 | Horse population

Twenty-eight male and female horses (22 males; 6 females) of age 5–7 years suffering from varying degrees of lameness (for one to two months) as identified by their caretakers, confirmed by a senior equine veterinarian were assisted by a team of qualified veterinarians. Medical history was screened to obtain 22 horses with lameness grades 2–4 according to the American Association of Equine Practitioners (AAEP)

TABLE 1 Inclusion/exclusion criteria

Inclusion criteria	Exclusion criteria
Horses of either sex (age 4–7 years)	Horses with AAEP grade 5 lameness
Lameness grade 2–4 according to the AAEP scale.	Horses with defects in musculoskeletal joints acute soft tissue injuries and synovitis
Lameness present for 1—2 months prior to enrolment	History of serious illness or disease at time of diagnosis
Horse with good health except lameness	Pregnant and lactating horses
	Horses on therapy with any other joint supplements or provided with glucosamine, chondroitin, mussel extract, polysulfated methylsulfonylmethane, curcumin, fish oil/omega-3 during the 10 weeks prior to enrolment
	Horses on corticosteroid or NSAIDS in last 6 weeks
	Horses medicated with multiple treatments of pentosane polysulphate or polysulphated glycosaminoglycan or hyaluronic acid and biphosphonate during last 6 months
	Horses underwent any regenerative, therapy in the past 6 months

Abbreviation: AAEP, American Association of Equine Practitioners.

scale. The AAEP scale ranges from zero to five, with zero being no perceptible lameness and five being the most extreme lameness (Mike, 2010). The inclusion and exclusion criteria are mentioned in Table 1. The study was conducted over a period of 12 weeks from May to September 2019. Twenty horses completed the study. Horses were housed in natural environmental conditions and sheltered in a covered area. Standard animal husbandry practices were followed during the study. The animals were fed green and dry fodder and commercially available pellets (Kamdhenu Agrovet, Sangli, Maharashtra, India), mash feed along with a vitamin–mineral mixture (Zenley Corporation, Ambala, Haryana, India), and filtered drinking water was provided to the animals ad libitum.

2.4 | Randomization and supplementation

Selected horses were randomized in a 1:1 ratio with either NXT15906F6 or placebo by providing randomized supplements in the order of recruitment. The computerized randomization code was generated by a biostatistician using a block randomization procedure (PROC PLAN, SAS 9.4 for Windows). Prior to supplementation, the manufacturer of the test item provided pre-labelled investigational product (active and placebo sachets [IP]) as per the randomization codes generated by QA personnel. The master list of randomization codes was stored in the control officer's locked office, and it was kept in strict and secure surveillance. The boxes containing the IPs were then stored in the designated area until shipment to the study site. The IPs were shipped to the study site along with the IP shipment log. At screening, complete physical examination (general attitude and demeanour, appetite, drinking, manure amount, manure appearance) and serum and urine analysis were conducted to rule out horses with abnormal baseline values.

Synovial fluid samples were collected from selected horses before the supplementation and at the end of the study. On days 0, 14, 28, 56, and 84 of supplementation, horses were evaluated for lameness scores, health, and adverse events. Blood and urine samples were collected at

baseline and at the end of the study to observe any changes in equine health parameters.

An earlier clinical study showed that daily supplementation with 400 mg of NXT15906F6 is efficacious in reducing joint pain and inflammation and improving knee function in human adults (P. S. Rao et al., 2019). Based on comparative body weight calculations between humans and horses, an approximate dose of 2.5 g/day was proposed and tested in a pilot study. The chosen dose of NXT15906F6 was 2.5 g/day administered orally to horses once daily over a period of 84 consecutive days.

2.5 | Blood, urine, and synovial fluid collection

Blood, urine, and synovial fluid samples were collected in the morning between 8 and 11 AM. Blood samples were collected aseptically from the jugular vein. Synovial fluid samples were collected by synoviocentesis aseptically by a trained veterinarian using a 22-gauge needle from the affected joint from selected horses for analysis.

2.6 | Primary efficacy measures

2.6.1 | Lameness scores

Lameness was evaluated at baseline and on days 14, 28, 56, and 84 of supplementation using the AAEP scale 0–5. Lameness at a walk (LWMEW) and lameness at a trot (LAMET) were determined by experienced veterinarians who were blinded to the study groups (Mike, 2010). Each horse was observed on loose lead walking in a straight line and during trot on a rough surface in a U-shaped pattern consisting of 40 m in a straight line, 20 m in a half-circle, and 40 m in a straight line. The clinicians assigned a numerical rating of 0–5. The AAEP score '0' indicates lameness is not perceptible under any circumstances; '1' difficult to observe lameness and is not consistently apparent, regardless of circumstances; '2' lameness is difficult to observe at a walk or when

trotting in a straight line but consistently apparent under certain circumstances (e.g., circling); '3' lameness is consistently observable at a trot under all circumstances; '4' lameness is obvious at a walk and '5' lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move.

2.7 | Secondary efficacy measures

2.7.1 | Inflammatory biomarkers

Blood samples were collected from the jugular vein at baseline and day 84 of supplementation to assess the effect of NXT15906F6 on the levels of RF, ANA, ACC peptide, and proinflammatory cytokines, including IL-1β, IL-6, TNF-α and PGE$_2$. The synovial fluid samples were collected from selected horses (eight animals from each group, including three females) by synoviocentesis aseptically using a 22-gauge needle from the affected joint for analysis of routine cytology and for the estimation of cytokines, including IL-1β, IL-6, TNF-α, and PGE$_2$. Biomarkers in serum and synovial fluid were analyzed using commercially available kits (Rheumatoid factor: #Cat MBS006299, ACC peptide: #Cat MBS9344438, and IL-1β: #Cat MBS282065, MyBioSource, San Diego, USA, ANA: #Cat E1384541 [Type II], SiNCERE Biotech, New Taipei City, Taiwan, IL-6: #Cat EKC41235, Biomatik, Wilmington DE, USA, TNF-α: #Cat LS-F32050-1, LS Bio, Seattle, WA, USA, and PGE$_2$, #Cat KGE004B: R&D Systems, Minneapolis, MN, USA). The assay sensitivities of ACC peptide, IL-1β, IL-6, TNF-α, RF, ANA, and PGE$_2$ were 0.1 ng/ml, 15.6 pg/ml, 0.78 pg/ml, 9.375 pg/ml, 1.0 ng/ml, 0.054 ng/ml, and 41.4 pg/ml, respectively.

2.8 | Safety evaluations and follow-up procedures

At baseline and at the end of the study, blood and urine samples were collected and analyzed for routine haematology, biochemistry, and urine analysis. At each visit, changes in eating behaviour, supplement compliance, physical appearance, behavioural signs, and adverse events associated with supplementation were recorded. In addition, assessments such as body weight, temperature, pulse, respiration, and blood pressure using the tail-cuff method were recorded at each visit.

As part of the safety assessment, a battery of haematological, serum, and urine biochemical parameters was evaluated at baseline and at the end of the study. In addition, the veterinarian performed the physical assessments, including general attitude and demeanour, appetite, water intake, manure amount, manure appearance, gum colour at each visit.

2.8.1 | Haematological and biochemical measurements

Serum biochemistry included fasting blood glucose (FBG), lipid profile (total cholesterol, triglycerides, HDL and LDL), liver profile (alkaline phosphatase [ALP], aspartate aminotransferase [SGOT], alanine aminotransferase [SGPT], lactate dehydrogenase [LDH], gamma-glutamyl transpeptidase [GGTP], total bilirubin, direct bilirubin, globulin, albumin, albumin and globulin ratio [A/G] and total protein), kidney profile (creatinine, blood urea nitrogen [BUN], uric acid), and electrolytes (sodium, potassium, and chloride). Serum samples were also analyzed for iron, calcium, phosphorus, and creatine kinase (CK) using an automated analyzer (Turbo Chem 100 analyzer; CPC Diagnostics, Chennai, Tamilnadu, India). Vitamins B12 and D were analyzed using commercial kits (Vitamin B12: #Cat E4638-100, BioVision, Milpitas, CA, USA, vitamin D: #Cat KBH501, Krishgen Biosystem, Mumbai, Maharashtra, India). The assay sensitivities of vitamins B12 and D were < 2.5 and < 0.469 ng/ml, respectively. Complete blood count was performed using a Nihon Kohden automated analyzer (Nihon Kohen, Gurgaon, Haryana, India). Free catch urine samples were analyzed for colour, glucose, bilirubin, ketones, specific gravity, blood, urinary gamma-glutamyl transferase (GGT), pH, total protein, creatinine, protein:creatinine ratio, nitrite, leukocytes, epithelial cells, red blood cells, white blood cells, crystals, casts, and bacteria.

2.9 | Statistical data analysis

Data were analyzed by SAS (University edition). Data are presented as the mean $\pm$ SD for continuous data and percentages for categorical data. The comparison between two groups was performed by unpaired t-test for continuous normal data and Mann–Whitney U test for continuous non-normal data (and score data). Log transformations were performed on biomarker data wherever they were appropriate. The comparison between baseline and postbaseline was analyzed by paired t-test for continuous normal data and Wilcoxon signed ranked test for continuous non-normal data (and score data). The comparison between variables was analyzed by the chi-square test/Fisher's exact test for categorical data. All p-values less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Clinical efficacy of NXT15906F6

A total of 28 adult horses (age 5–7 years) were assessed for eligibility criteria before enrolment in the study (Figure 1). Twenty-two horses who met the inclusion and exclusion criteria were enrolled and randomly assigned to either supplement TamaFlex™ (NXT15906F6) or control (placebo) groups. The study endpoints were statistically analyzed. Among the allocated horses, 73% were males ($n = 8$), and 27% ($n = 3$) were females in each group. Among the allocated horses, two horses, one from each group, were hurt during exercise hours, and they were excluded from the study. The remaining 20 horses completed the study. The demographic variables of the intention to treat population are presented in Table 2.
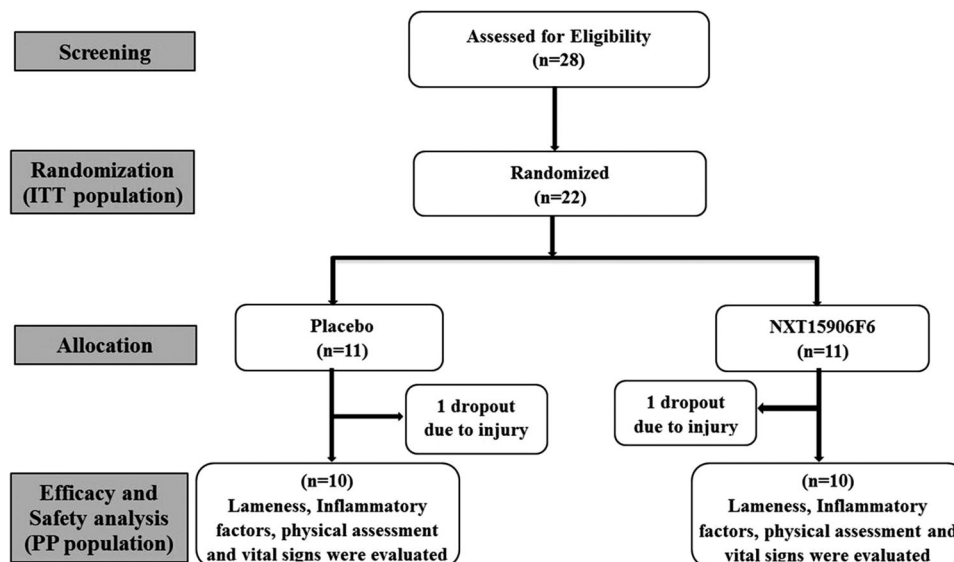
FIGURE 1 Consort flow diagram

TABLE 2 Baseline demographic characteristics

Variables	Placebo (n = 11)	NXT15906F6 (n = 11)	p-Value vs. placebo
Age (years)	5.30 ± 0.48	5.60 ± 0.70	0.2784
Body weight (kg)	230.34 ± 88.61	243.67 ± 69.66	0.7131
Temperature—Higher (°F)	98.87 ± 0.36	98.94 ± 0.12	0.5708
Average pulse (beats/minute)	33.13 ± 3.18	34.67 ± 2.28	0.2328
Respiration (resp/minute)	13.03 ± 2.46	13.13 ± 2.42	0.9280
Average Systolic blood pressure (mmHg)	121.83 ± 17.45	122.83 ± 15.88	0.8949
Average Diastolic blood pressure (mmHg)	92.90 ± 8.99	90.00 ± 11.96	0.5483
Lameness Score	3.3 ± 0.48	3.4 ± 0.52	0.8154

Note: Data are expressed as the mean ± SD, and data were analyzed using an unpaired *t*-test.

At baseline, all demographic variables were comparable and not significantly different. Lameness scores were assessed at baseline and on days 14, 28, 56, and 84 of supplementation. 'Within the group analyses' revealed significant improvements in the baseline scores of lameness in the NXT15906F6 group at 28, 56, and 84 days of supplementation. In contrast, the placebo cohort showed a significant improvement only on day 84. Compared to placebo, a significant reduction in lameness scores was observed in the NXT15906F6 group as early as 28 days of supplementation. The percent reductions in baseline lameness scores were 23.53 ($p = 0.0156$), 47.06 ($p = 0.0020$), and 85.29 ($p = 0.0020$) on days 28, 56, and 84 of supplementation, respectively, in the NXT15906F6 group. In the placebo group, percent reductions in lameness scores of 3.03 ($p = 1.0000$), 15.15 ($p = 0.1250$), and 24.24 ($p = 0.0156$) were observed on days 28, 56, and 84 of supplementation, respectively. NXT15906F6 supplementation led to a significant reduction in lameness score (Figure 2, Table 3). It is interesting to note that the NXT15906F6 group (n = 10) exhibited dramatic improvement in

lameness score (0 on the AAEP scale in six horses, 1 in three horses, and 2 in one horse).

3.2 | Secondary safety and efficacy analysis

Compared to baseline, serum RF decreased by 17% and 11%, respectively, in the NXT15906F6 and placebo groups on day 84. However, these changes were not statistically significant. Serum ANA levels were marginally reduced (9%) when compared to the baseline in the TamaFlex-supplemented group. In contrast, the placebo arm showed a 20% increase from baseline. Compared to placebo, the reduction in ANA levels was significant in the NXT15906F6 group on day 84 of supplementation (NXT15906F6 2.77 ± 0.72 ng/ml, placebo 3.05 ± 0.56 ng/ml, $p = 0.0481$). NXT15906F6 supplementation also reduced serum ACC- peptide levels by 19% ($p = 0.5058$) on day 84 of supplementation compared to placebo (Figure 3).

TABLE 3 Changes in the lameness score from baseline and follow-up visits

Groups	Evaluation days	Lameness score	Change from baseline	% Change from baseline	p, intragroup comparison, (vs. baseline)	p, intergroup comparison (vs. placebo)	p, intergroup comparison of mean change (vs. placebo)
Placebo (n = 10)	Baseline	3.3 ± 0.48	–	–	–	–	–
	Day 14	3.3 ± 0.48	00.00 ± 0.00	0	–	–	
	Day 28	3.2 ± 0.63	−0.10 ± 0.32	3.03	1.0000	–	
	Day 56	2.8 ± 0.79	−0.50 ± 0.71	15.15	0.1250	–	
	Day 84	2.5 ± 0.85*	−0.80 ± 0.63	24.24	0.0156	–	
NXT15906F6 - (2.5 g/day) (n = 10)	Baseline	3.4 ± 0.52	–	–	–	–	–
	Day 14	3.4 ± 0.52	00.00 ± 0.00	0	–	0.6809	1.0000
	Day 28	2.6 ± 0.52*#	−0.80 ± 0.63#	23.53	0.0156	0.0425	0.0085
	Day 56	1.8 ± 0.42*#	−1.60 ± 0.70#	47.06	0.0020	0.0040	0.0052
	Day 84	0.5 ± 0.71*#	−2.90 ± 0.88#	85.29	0.0020	0.0003	0.0002

Note: Data are expressed as the mean ± SD.
*$p < 0.05$ vs. baseline (Wilcoxon signed rank test).
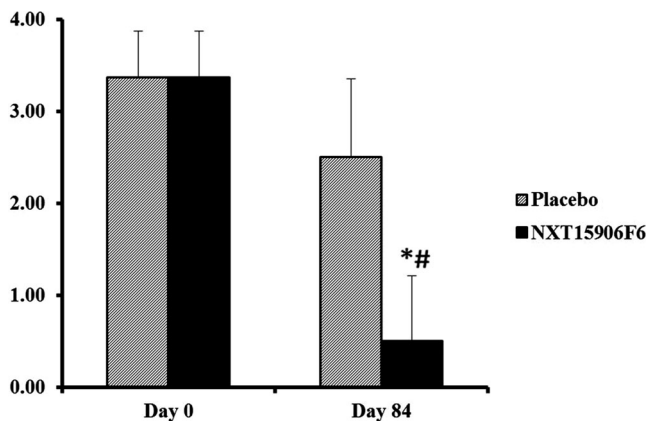#$p < 0.05$ vs. placebo (Mann–Whitney test).

FIGURE 2 Changes in lameness score from baseline to follow-up visits. Data were analyzed using the Mann–Whitney U test. $n = 10$; *$p < 0.05$ vs. baseline, #$p < 0.05$ vs. placebo

At the end of the 84 days of supplementation, serum PGE_2 levels were significantly reduced in the NXT15906F6 group compared to the baseline (69.52 ± 23.35 vs. 161.16 ± 88.79 pg/ml, $p = 0.0075$). In the intergroup analysis, the mean PGE_2 level in the NXT15906F6 group was reduced by 30% ($p = 0.2975$) compared to that in the placebo cohort (Figure 3). In synovial fluid analysis, baseline PGE_2 values were reduced by 284 pg/ml in the NXT15906F6 group. Contrary to the NXT15906F6 arm, baseline values were increased by 254 pg/ml in the placebo cohort. However, these changes were not statistically significant (Figure 4).

In serum, the values of IL-1β were moderately reduced by 13% (10.65 pg/ml) at the end of 84 days of NXT15906F6 supplementation. In contrast, there was a significant rise (49.94 pg/ml) in the placebo cohort from baseline. Compared to placebo, serum IL-1β values in the NXT15906F6 group were significantly reduced ($p = 0.0377$) at the

end of 84 days of supplementation (Figure 3). In the synovial fluid, baseline values were significantly reduced in the NXT15906F6 group after 84 days of supplementation (baseline 49.38 ± 23.41 pg/ml, days 84-25.45 ± 7.04 pg/ml, $p = 0.0331$). Compared to the placebo group, the reduction in IL-1β levels in synovial fluid was significant in the TamaFlex group at 84 days of supplementation (NXT15906F6 25.45 ± 7.04 pg/ml, placebo 38.45 ± 11.78 pg/ml, $p = 0.0445$) (Figure 4).

'Intergroup' and 'Intragroup' analyses revealed that NXT15906F6 intervention did not alter the serum levels of IL-6 compared to baseline and placebo (Figure 3). Interestingly, baseline levels of IL-6 in synovial fluid were significantly reduced after 84 days of supplementation with NXT15906F6 (baseline 2.27 ± 0.38, vs. day 84: 1.70 ± 0.38 pg/ml, $p = 0.0002$). At the end of supplementation, a 12% decrease in IL-6 level was observed in the NXT15906F6 group compared to the placebo (Figure 4).

In serum, TNF-α levels were significantly reduced in the NXT15906F6 group compared to the baseline ($p = 0.0161$) and placebo ($p = 0.0336$) at the end of 84 days of supplementation (Figure 3). Compared to the placebo group, the difference in the average TNF-α level was 24% (178.23 pg/ml) in the NXT15906F6 group at the end of 84 days of supplementation. Changes in synovial fluid TNF-α levels were not statistically significant (Figure 4).

Serum samples were also analyzed for vitamins B12 and D levels. 'Within the group' and 'between the groups' analysis confirmed that, except for a slight increase from baseline in the NXT15906F6 group, no significant differences were observed in the levels of vitamins B12 and D compared to the placebo group (Table 4).

In addition to biomarker analysis, synovial fluid samples were analyzed for neutrophil and leukocyte counts and total protein, lactate, glucose, and pH levels. However, the 'Intergroup' and 'Intragroup' analyses did not reveal any significant differences between the groups at baseline and day 84 of supplementation (data not shown).
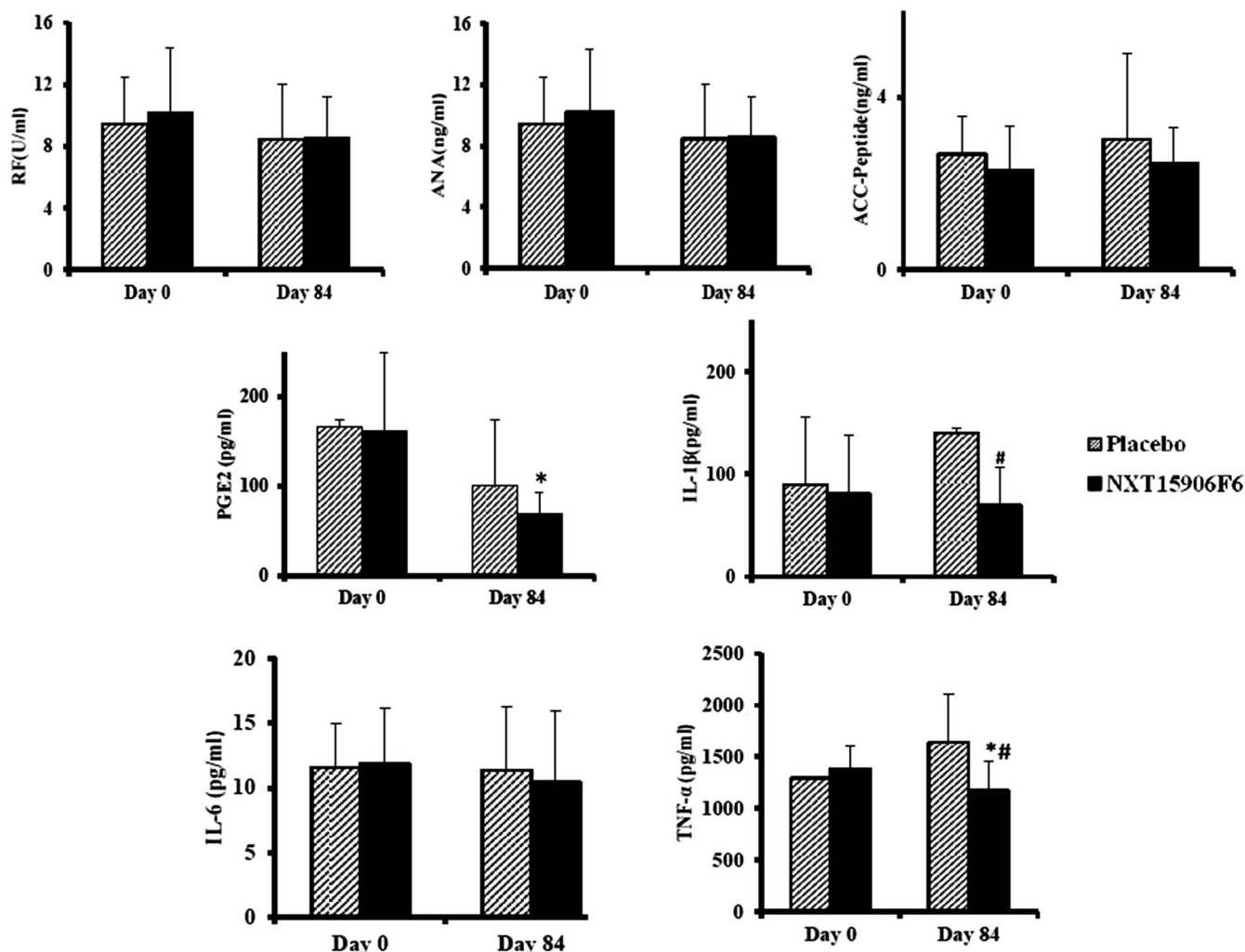
FIGURE 3 Changes in serum biomarkers from baseline to the end of the supplementation. The bar diagram represents the mean ± SD. Data were analyzed using the Mann–Whitney U test. $n = 10$; *$p < 0.05$ vs. baseline, #$p < 0.05$ vs. placebo. Abbreviations: ANA, anti-nuclear antibody; ACC peptide, anti-cyclic citrullinated peptide; IL-1β, interleukin-1β; IL-6, interleukin-6; PGE$_2$, prostaglandin E$_2$; RF, rheumatoid factor; TNF-α, tumour necrosis factor-α

TABLE 4 Changes in vitamins B12 and D from baseline to the end of the study duration

Parameter (units)		Vitamin B12 (pg/ml)	Vitamin D (ng/ml)
Placebo ($n = 10$)	Baseline	23.12±6.53	26.05±9.51
	Day 84	21.59±6.00	25.33±7.91
	p, intragroup comparison (vs. baseline)	0.4834	0.7624
NXT15906F6 (2.5 g/day) ($n = 10$)	Baseline	26.41±8.99	23.59±4.85
	Day 84	28.55±7.91	24.26±3.10
	p, intragroup comparison (vs. baseline)	0.5362	0.6759
	p, intergroup comparison (vs. placebo)	0.0641	0.6988
	p, intergroup comparison of mean change (vs. placebo)	0.3645	0.6245

Note: Data are presented as the mean ± SD. Data were analyzed using a two-tailed t-test with unequal variance.

3.3 | Safety and physical assessments

As part of the safety assessment, a battery of haematological and total blood chemistry parameters and urine analyses were evaluated at baseline and at the end of the study. Physical assessments including general attitude and demeanour, appetite, drinking, manure amount, manure appearance, gum colour, and so forth, were performed at each visit. All horses tolerated the oral supplementation of NXT15906F6,
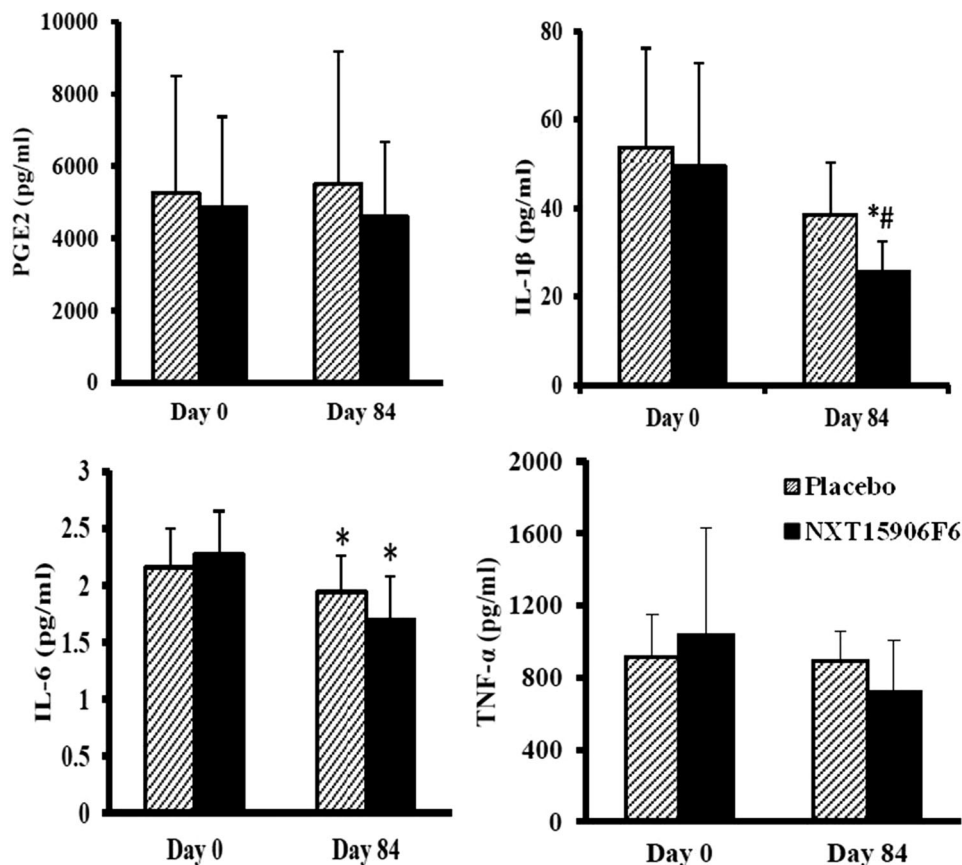
FIGURE 4 Changes in synovial fluid biomarkers from baseline to the end of the supplementation. The bar diagram represents the mean ± SD. Data were analyzed using the Mann–Whitney U test. $n = 10$; *$p < 0.05$ vs. baseline, #$p < 0.05$ vs. placebo. Abbreviations: IL-1β, interleukin-1β; IL-6, interleukin-6; PGE$_2$, prostaglandin E$_2$; TNF-α, tumour necrosis factor-α

with no evidence of supplement-related side effects during the study. The results of complete blood profile, serum biochemistry, and urine analyses were within normal reference ranges during the study period (data not shown).

Body weights, body temperature, pulse rate, and blood pressure of the animals were recorded at baseline and on days 14, 28, 54, and 84 of supplementation. No significant changes in these parameters were observed between the NXT15906F6 and placebo groups (data not shown).

4 | DISCUSSION

This study demonstrates the efficacy of TamaFlex™ (NXT15906F6) supplementation over a period of 84 consecutive days in horses diagnosed with lameness (AAEP scale 2–4) in a single-centre randomized, blinded placebo-controlled clinical trial. Early and significant improvement in lameness was observed in the NXT15906F6 group on day 28 of supplementation. A greater portion of horses in the NXT15906F6 group exhibited superior performance from day 28 until the completion of the study. The percent improvement in lameness in the NXT15906F6 supplementation group was 23.53, 47.06, and 85.29 on

days 28, 56, and 84 of supplementation, respectively. Interestingly, in the NXT15906F6 group, lameness scores were '0' in six horses, '1' in three horses, and '2' in one horse on the AAEP scale, showing improvement in walking and trotting performances after 84 days of supplementation. These observations indicate that NXT15906F6 supplementation benefited the horses in improving their joint flexibility and health.

Studies assessing the welfare of working horses indicate that horses in Afghanistan, Egypt, Ethiopia, India, Pakistan, and Gambia revealed a high prevalence of abnormal gait or lameness, ranging from 90% to 100% (Reix et al., 2015). In particular, 90% of working horses (≥5 years old) present some degree of lameness on the AAEP scale, as they are more prone to intra-articular inflammation and acute or repetitive overload injuries of the metacarpo- or metatarsophalangeal joint. These changes result in synovitis or capsulitis and damage to the articular cartilage, subchondral bone, synovium, and joint capsule, which may lead to degeneration and degradation of cartilage (Niemelä et al., 2016). Therefore, an alternative therapeutic strategy that offers relief from lameness with a better safety profile is warranted to improve the performance of working horses.

Inflammatory processes mediated by proinflammatory cytokines lead to the deterioration of the cartilage matrix, resulting in the loss

of joint function, mobility, and lameness. The inflammatory process begins within the synovium, cartilage, joint capsule, or subchondral bone, where inflammatory mediators create a cascade of events resulting in the gradual degradation of articular cartilage (Goodrich & Nixon, 2006; Vanderweed et al., 2012). IL-6, a proinflammatory cytokine, plays a major role in joint destruction. As a pleiotropic cytokine, IL-6 is markedly up-regulated during tissue inflammation (MA et al., 2017). NXT15906F6 supplementation significantly reduced the IL-6 levels in synovial fluid following 84 days of supplementation. IL-1β, a marker of equine synovitis, also produces destructive mediators such as matrix metalloproteases (MMPs) and PGE$_2$ (Bertugilia et al., 2016). Eicosanoids, such as PGE$_2$, are released from synovial cells and chondrocytes in response to articular injury and are responsible for pain during joint inflammation and lameness. Inflammation, pain, or mechanical injury may result in an abnormal gait characterized by limping and lameness (Ross & Dyson, 2011). The proinflammatory cytokines IL-1β and TNF-α also reduce anabolic processes in chondrocytes, thus decreasing the synthesis of extracellular matrix components (Yimam et al., 2019).

In the present study, reduced levels of the proinflammatory modulators clearly suggest that the herbal blend improved joint inflammation and the associated pain symptoms in the NXT15906F6-supplemented horses. NXT15906F6 is also known to inhibit proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, thereby decreasing proinflammatory responses in the synovium by inhibiting COX-mediated PGE$_2$ production. Furthermore, NXT15906F6 also inhibits the production of nitric oxide and matrix metalloproteinases, mainly MMP3 and MMP13, which are the key contributors to ECM degeneration and OA progression (Kare et al., 2022).

Together, the changes in these inflammatory and pain modulators might explain the basis of the efficacy of NXT15906F6 in improving musculoskeletal function, as indicated by gradual reductions in lameness scores. These changes result in the reduction of laminitis, thereby reducing joint pain during walking and trotting. The feeding behaviour of horses remained unchanged during the 84 days of supplementation. No significant changes in serum proteins and vitamin (B$_{12}$ and D) levels were observed in either study arm. Safety and physical assessments revealed that supplementation with NXT15906F6 over a period of 84 consecutive days is safe and tolerable. Additional studies also showed that this herbal blend was non-mutagenic and non-clastogenic (Badmaev et al., 2018).

One possible limitation of the present study is that this study does not discriminate the lameness of the study horses on an ecological basis, as these horses were suffering from lameness since the past 1–2 months.

In this placebo-controlled investigation, all 10 horses who received TamaFlex™ (NXT15906F6) showed significant improvement from lameness. Overall, TamaFlex supplementation reduces the symptoms of lameness and improves joint flexibility, function, and performance, as well as ameliorates proinflammatory cytokines. Therefore, it is expected that NXT15906F6 supplementation may improve the quality of performance and overall health of horses.

5 | CONCLUSIONS

The present study demonstrates that the anti-inflammatory botanical formulation TamaFlex™ (NXT15906F6) is safe, efficacious, and alleviates joint pain in horses suffering from lameness. Furthermore, NXT15906F6 significantly reduces the proinflammatory mediators IL-1β and TNF-α in horses. TamaFlex promotes quality of life, relieves lameness and joint discomfort and improves gait speed during walking and trotting in equines.

CONFLICT OF INTEREST
The authors declare the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Satyjit G. Patil, Gopichand Chinta, and KrishnaRaju Venkata Alluri are the employees of Laila Nutraceuticals R&D entre, Vijayawada. Sandesh Jain is the employee of Vins Bioproducts Ltd, Hyderabad, Telangana.

ETHICS STATEMENT
The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have adhered to standards for the protection of animals used by the Committee for Purpose Control Supervision of Experiments on Animals (CPCSEA). Ethics committee approval No. # F No 25/4/2019-CPCSEA dated March 20, 2019.

AUTHOR CONTRIBUTIONS
Sandesh Jain, Satyajit G. Patil, and KrishnaRaju Venkata Alluri coordinated the interpretation and reporting of this study. KrishnaRaju Venkata Alluri and Gopichand Chinta performed the data review and interpretation. Satyajit G. Patil and KrishnaRaju Venkata Alluri are involved in the conceptualization and design of the study as per the regulatory requirement. Satyajit G. Patil, Gopichand Chinta, and KrishnaRaju Venkata Alluri contributed to the manuscript drafting and revisions. Sandesh Jain was the principal investigator and carried out the research. Satyajit G. Patil and KrishnaRaju Venkata Alluri monitored the study activities. All authors contributed to and reviewed the manuscript and are accountable for its contents.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW
The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.894.

ORCID

Sandesh Jain https://orcid.org/0000-0003-4693-8689
Satyajit G. Patil https://orcid.org/0000-0002-8468-6307
Gopichand Chinta https://orcid.org/0000-0002-1477-773X
KrishnaRaju Venkata Alluri https://orcid.org/0000-0002-0550-7164

REFERENCES

Badmaev, V., Vik, H., Stohs, S. J., & Galluzzo, F. (2018). Safety and toxicological evaluation NXT15906F6 (TamaFlex®): A food-derived botanical composition containing standardized extracts of *Tamarindus indica* seeds and Curcuma longa rhizomes. *Toxicology Research and Application, 2,* 1–14. https://doi.org/10.1177/2397847317749240

Baxter, G. M., Stashak, T. S., & Keegan, K. G. (2020). Examination for lameness. In G. M. Baxter (Eds.), *Adams and Stashak's lameness in horses* (2nd ed., pp. 67–188). John Wiley & Sons, Inc. Hoboken. https://doi.org/10.1002/9781119276715.ch2

Bertuglia, A., Pagliara, E., Grego, E., Ricci, A., & Brkljaca-Bottegaro, N. (2016). Pro-inflammatory cytokines and structural biomarkers are effective to categorize osteoarthritis phenotype and progression in Standardbred racehorses over five years of racing career. *BMC Veterinary Research, 12*(1), 246. https://doi.org/10.1186/s12917-016-0873-7

Broster, C. E., Burn, C. C., Barr, A. R., & Whay, H. R. (2009). The range and prevalence of pathological abnormalities associated with lameness in working horses from developing countries. *Equine Veterinary Journal, 41*(5), 474–481. https://doi.org/10.2746/042516409x373907

Contino, E. K. (2018). Management and rehabilitation of joint disease in sport horses. *The Veterinary Clinics of North America. Equine Practice, 34*(2), 345–358. https://doi.org/10.1016/j.cveq.2018.04.007

De Caluwé, E., Halamouá, K., & Van Damme, P. (2010). *Tamarindus indica* L.—A review of traditional uses, phytochemistry and pharmacology, *Afrika Focus, 23*(1), 53–83. https://doi.org/10.1163/2031356X-02301006

Di Lorenzo, C., Dell'Agli, M., Badea, M., Dima, L., Colombo, E., Sangiovanni, E., Restani, P., & Bosisio, E. (2013). Plant food supplements with anti-inflammatory properties: a systematic review (II). *Critical Reviews in Food Science and Nutrition, 53*(5), 507–516. https://doi.org/10.1080/10408398.2012.691916

Goodrich, L. R., & Nixon, A. J. (2006). Medical treatment of osteoarthritis in the horse—A review. *The Veterinary Journal, 171*(1), 51–69. https://doi.org/10.1016/j.tvjl.2004.07.008

Hatcher, H., Planalp, R., Cho, J., Torti, F. M., & Torti, S. V. (2008). Curcumin: From ancient medicine to current clinical trials. *Cellular and Molecular Life Sciences: CMLS, 65*(11), 1631–1652. https://doi.org/10.1007/s00018-008-7452-4

Kare, S. K., Vinay, V., Maresz, K., Prisk, V., & Vik, H. (2022). Tamarindus indica Seed extract-based botanical compositions alleviate knee pain and improve joint function in mild-to-moderate Osteoarthritis: A randomized, double-blind, placebo-controlled clinical study. *Evidence-based Complementary and Alternative Medicine: eCAM, 2022,* 2226139. https://doi.org/10.1155/2022/2226139

Kufa, M. (2019). A review on lameness in equine. *International Journal of Advanced Research in Biological Sciences, 6*(10), 101–109.

Liu, X., Machado, G. C., Eyles, J. P., Ravi, V., & Hunter, D. J. (2018). Dietary supplements for treating osteoarthritis: A systematic review and meta-analysis. *British Journal of Sports Medicine, 52*(3), 167–175. https://doi.org/10.1136/bjsports-2016-097333

Ma, T. W., Li, Y., Wang, G. Y., Li, X. R., Jiang, R. L., Song, X. P., Zhang, Z. H., Bai, H., Li, X., & Gao, L. (2017). Changes in synovial fluid biomarkers after experimental equine Osteoarthritis. *Journal of Veterinary Research, 61*(4), 503–508. https://doi.org/10.1515/jvetres-2017-0056

Mike, W. R. (2010). Movement. M. W. Ross & S. J Dyson (Eds.), *Diagnosis and management of lameness in the horse-e-book* (2nd ed., pp. 64–80). Elsevier Health Sciences.

Murray, R. C., Walters, J. M., Snart, H., Dyson, S. J., & Parkin, T. D. (2010). Identification of risk factors for lameness in dressage horses. *Veterinary Journal (London, England: 1997), 184*(1), 27–36. https://doi.org/10.1016/j.tvjl.2009.03.020

Nagy, A., Murray, J. K., & Dyson, S. J. (2014). Descriptive epidemiology and risk factors for eliminations from Fédération Equestre Internationale endurance rides due to lameness and metabolic reasons (2008-2011). *Equine Veterinary Journal, 46*(1), 38–44. https://doi.org/10.1111/evj.12069

Niemelä, T. M., Tulamo, R. M., & Hielm-Björkman, A. K. (2016). A randomised, double-blinded, placebo-controlled clinical study on intra-articular hyaluronan treatment in equine lameness originating from the metatarsophalangeal joint. *BMC Veterinary Research, 12*(60), .https://doi.org/10.1186/s12917-016-0687-7

Rao, C. V. (2007). Regulation of COX and LOX by curcumin. Advances in experimental medicine and biology. *The Molecular Targets and Therapeutic Uses of Curcumin in Health and Disease, 595,* 213–226. https://doi.org/10.1007/978-0-387-46401-5_9

Rao, P. S., Ramanjaneyulu, Y. S., Prisk, V. R., & Schurgers, L. J. (2019). A combination of Tamarindus indica seeds and Curcuma longa rhizome extracts improves knee joint function and alleviates pain in non-arthritic adults following physical activity. *International Journal of Medical Sciences, 16*(6), 845–853. https://doi.org/10.7150/ijms.32505

Reix, C. E., Dikshit, A. K., Hockenhull, J., Parker, R. M., Banerjee, A., Burn, C. C., Pritchard, J. C., & Whay, H. R. (2015). A two-year participatory intervention project with owners to reduce lameness and limb abnormalities in working horses in Jaipur, India. *PLoS One, 10*(4), e0124342. https://doi.org/10.1371/journal.pone.0124342

Ross, M. W., & Dyson, S. J. (2011). *Diagnosis and management of lameness in the horse.* Elsevier/Saunders.

Sundaram, M. S., Hemshekhar, M., Santhosh, M. S., Paul, M., Sunitha, K., Thushara, R. M., NaveenKumar, S. K., Naveen, S., Devaraja, S., Rangappa, K. S., Kemparaju, K., & Girish, K. S. (2015). Tamarind seed (*Tamarindus indica*) extract ameliorates adjuvant-induced arthritis via regulating the mediators of cartilage/bone degeneration. *Inflammation and Oxidative Stress. Scientific Reports, 5,* 11117. https://doi.org/10.1038/srep11117

Vandeweerd, J. M., Coisnon, C., Clegg, P., Cambier, C., Pierson, A., Hontoir, F., Saegerman, C., Gustin, P., & Buczinski, S. (2012). Systematic review of efficacy of nutraceuticals to alleviate clinical signs of osteoarthritis. *Journal of Veterinary Internal Medicine, 26*(3), 448–456. https://doi.org/10.1111/j.1939-1676.2012.00901.x

Yimam, M., Horm, T., Wright, L., Jiao, P., Hong, M., Brownell, L., & Jia, Q. (2019). UP1306: A composition containing standardized extracts of *Acacia catechu* and *Morus alba* for arthritis management. *Nutrients, 11*(2), 272. https://doi.org/10.3390/nu11020272