EVALUATION OF EFFICACY OF POLYHERBAL FORMULATION FOR AUGMENTATION OF MILK PRODUCTION IN BUFFALOES

Thesis

Submitted to Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY PHARMACOLOGY AND TOXICOLOGY
(Minor Subject: Veterinary Physiology)

By

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CERTIFICATE – I

This is to certify that the thesis entitled, **"Evaluation of efficacy of polyherbal formulation for augmentation of milk production in buffaloes"** submitted for the degree of **M.V.Sc.**, in the subject of **Veterinary Pharmacology and Toxicology** (Minor Subject: **Veterinary Physiology**) of Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Gurleen Kaur (L-2019-V-85-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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ABSTRACT

Present study was undertaken to investigate the efficacy of a novel polyherbal formulation on production performance and safety profile of lactating buffaloes. Twenty lactating animals were selected and divided in two groups of ten animals each. Control group was given regular diet whereas treatment group was fed with polyherbal supplementation at dose rate of 20 grams per 100 kg body weight twice a day along with their scheduled diet, for 45 days and animals were observed for another 30 days post-treatment. There was no significant difference observed in body weight of both groups. No significant change was observed in milk yield (kgs) between control and treatment group. Milk protein% and total solids% in treatment group showed significant improvement ($p \leq 0.05$) over time. Somatic cell count and electrical conductivity significantly declined ($p \leq 0.05$) in milk of buffaloes in treated group as compared to control group. There was no significant change observed in flavor, colour, odour, appearance or overall acceptability among both groups. There was no significant change observed in ALT, AST, GGT, ALKP, BUN, Creatinine, LDH or CK levels of animals in both groups but results indicated significant incline ($p \leq 0.05$) in calcium, phosphorus, albumin and glucose levels of supplemented animals. Significantly lower ($p \leq 0.05$) levels of plasma total cholesterol and LDL were observed. There was no significant change in LPO and GSH levels but significant increment ($p \leq 0.05$) in catalase and SOD levels of buffaloes in supplemented group over time was observed. No change in hematology was observed in both groups except significant decline ($p \leq 0.05$) in TLC levels of buffaloes in treatment group. It can be concluded that polyherbal formulation supplemented is beneficial in maintaining general health of lactating buffaloes by alleviating production stress and preventing various production disorders.

Keywords: Polyherbal, Galactagogue, buffaloes, milk augmentation, safety study, antioxidant parameters, milk parameters

Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVIATIONS

%	:	percentage
µg	:	micrograms
µM	:	micromolar
µmol	:	micromoles
^{0}F	:	Degree fahrenheit
ANOVA	:	Analysis of Variance
b.i.d.	:	twice a day
BBB	:	Blood Brain Barrier
BCSP	:	Black Cumin Seed Powder
CARI	:	Central Ayurveda Research Institute
CAT	:	Catalase
CIN	:	Cinnamon
CLA	:	Conjugated Linoleic Acid
CMT	:	California Mastitis Test
DADF	:	Department of Animal husbandary, Dairying and Fisheries
DLC	:	Differential Leucocyte Count
DLFC	:	Directorate of Livestock Farm Complexes
DMI	:	Dry Matter Intake
DMSO	:	Dimethyl Sulfoxide
DTNB	:	Dithio-bis-(2-nitrobenzoic acid)
EC	:	Electrical Conductivity
EDTA	:	Ethylene Diamine Tetra Acetic Acid
EO	:	Essential Oils
FAO	:	Food and Agriculture Organization
FCM	:	Fat Corrected Milk
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
GAR	:	Garlic
GDP	:	Gross Domestic Product
GIN	:	Ginger
gms	:	grams
GSH	:	Blood Glutathione
GSH-PX/GPX	:	Glutathione Peroxide
GST	:	Glutathione-S-Transerase
Hb	:	Hemoglobin
HDL	:	High Density Lipids
HFA	:	Herbal Feed Additive

HR	:	Heart Rate
IU	:	International Units
kgs	:	kilograms
LDL	:	Low Density Lipids
LPO	:	Lipid Peroxide
MCH	:	Mean Corpuscular Hemoglobin
MCHC	:	Mean Corpuscular Hemoglobin Concentration
MCV	:	Mean Corpuscular Volume
MDA	:	Malondialdehyde
MIC	:	Minimum Inhibitory Concentration
MLF	:	Mulberry Leaf Extracts
MTT	:	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)
NEFA	:	Non Esterified Fatty Acid
NPA	:	Neutrophil Phagocytic Activity
NRC	:	National Research Council
p.o.	:	orally
PCV	:	Packed Cell Volume
PFA	:	Phytogenic Feed Additive
PRHM	:	Phytobiotics Rich Herbal Mixture
RBC	:	Red Blood Cells
RFM	:	Retention of Fetal Membranes
RR	:	Respiration Rate
RT	:	Rectal Temperature
SCC	:	Somatic Cell Count
SFA	:	Saturated Fatty Acids
SNF	:	Solid Not Fat
SOD	:	Super Oxide Dismutase
T-AOC	:	Total Antioxidant Capacity
TEC	:	Total Erythrocyte Count
TLC	:	Total Leucocyte Count
UFA	:	Unsaturated Fatty Acids
VFAs	:	Volatile Fatty Acids
WBC	:	White Blood Cells

CHAPTER I

INTRODUCTION

Over time, India has remodelled itself from a milk-deficient country to world's greatest in terms of milk production. Dairy businesses and infrastructure have modernised and expanded to the point where milk now accounts for over a third of rural households' gross income and accounts for around 4% of the country's GDP. India ranked first in milk production in 2018-19, with 187.7 million tonnes produced and 394 grams per capita availability (http://www.dahd.nic.in/ reports/annual-report-2019-20). The livestock sector plays a vital part in India's agro-based economy for a variety of reasons, making its development a priority for nation.

Buffalo encompasses a major contribution to the national milk pool however with the population rise in India as well as all across the globe, demand for milk is still increasing driven by sustained economic growth, rising incomes and urbanization. A total of 97% of the buffalo population in the world is confined to Asia. India has 109 million buffaloes which contribute to 57% of the total buffalo population and its contribution to the total milk production of world is 11 %, making the nation top ranker in both aspects (http://www.fao.org/faostat/en/data/QA/ visualize). Buffaloes as an animal are hardier than cattle and can sustain much more extremities in surroundings (Hegde, 2019). The superiority of the buffalo in obtaining its nitrogen and mineral needs from poor quality forages may make it worth more than a cow for many situations (Gangaraj, 2012). So they are more likely to meet increasing demand for quality milk.

Another reason for exploring new methods to augment milk production is that in our nation demand for livestock feed and fodder is higher than the availability and the demand will be hardly fulfilled in future due to land constraints and increasing population. The average milk yield in Indian animals is below the global average by 20-60% and nearly half (50.2%) of this loss is due to feed and fodder deficiency. Also, being the leading nation in buffalo population, the contribution of individual buffalo to total milk production on an average is still less as compared to average milk production of the animal in some other countries such as Nepal (66.6%) and Pakistan (59.5%) (Siddiky & Faruque, 2017) and hence has room for improvement. So,

observing this, applying novel methods to increase feed conversion efficiency and enhance their productivity directly or indirectly is a necessity that will aid in food security around the world.

Advancement in science and technology has now come up with many methods and techniques for milk augmentation and one of them is the use of Galactogogues. Galactagogues are pharmaceuticals, foods, or herbal supplements that help with milk production commencement, continuation, or amplification (Zapantis et al., 2012). These synthetic, plant-derived, or endogenous compounds could be used in both human clinical circumstances (such as non-infectious agalactias and hypogalactias) and dairy industry massification. The majority of galactogogues work by interacting with dopamine receptors, resulting in higher prolactin levels and thus, incrementing milk production (Gabay, 2002).

Synthetic galactogogues like domperidone, chlorpromazine, etc. may increase the milk production but these may also alter animal metabolism or leave residues that are of health concern. Synthetic medications that are commercially available are thought to have a negative impact on breastfeeding physiology's neuro-endocrine axis. Their long-term use has resulted in toxicity, which poses a risk to human and animal health (Mohanty et al., 2014). Dopamine antagonists have been linked to serious side effects in humans, including anxiety, heart arrhythmia, despair, sleeplessness, intestinal pain, seizures, and sudden death. These effects should be considered in veterinary practice, and their potential negative effects on breastfeeding animals and milk residues need to be thoroughly investigated.

Herbal galactogogues can be an alternate option for achieving the goal of higher milk production in buffaloes. Herbs may directly enhance milk production by increasing prolactin release like synthetic galactogogues or may indirectly augment milk yield as it might be stomachic, immunomodulatory, antimicrobial, anthelmintic, antioxidant in action or contain secondary metabolites possessing such properties i.e. in short plants derived galactogogues have potential to improve overall growth and productivity of animals. Plants containing essential oils (Herbal galactogogues) can be a cost-effective means of improving efficiency of milk production and optimizing milk composition of dairy animals (Tassoul & Shaver, 2008) and some results in

lactating cattle indicated supplementing their diets with particular aromatic herbal oils improved milk yield, udder health, and some immune parameters (Salem et al., 2019). Majority of these herbal preparations that may have potential to be used as galactogogues have yet not been evaluated through scientific means, but their literature and consumption since traditional times suggest that they are safe and effective (Mohanty et al., 2014). Some research that has been done on such ayurvedic preparations conclude that they are readily available, affordable, safe and do not leave residues in body secretions such as milk (Krishana et al., 2005).

Many plants based products are available in the market as galactogogues but the potential of most of these products has not been probed enough in veterinary world. Herbal galactogogues that are available in veterinary market namely Anifeed, Galactin, Ruchamax, Payapro, etc. are known to increase the milk production in animals but even their mechanism of action is not fully explored. Polyherbal formulation that we tend to use for our study contains following ingredients: *Asparagus racemosus, Cuminum cyminum, Carum carvi, Zingiber officinale, Embelia ribes, Allium sativum, Piper longum, Piper nigrum, Anethum sowa, Swertia chirata, Picrorhiza kurroa, Pistacia integerrima,* Red Ochre.

Though some standalone studies have been done on consumption and effect of some individual ingredients on animals but study of this combined polyherbal formulation, having potential galactogogue effect, action of this particular combination and outcome of these polyherbs not only on milk production or milk quality but general growth and health of animals is rather novel. The phyto-pharmacological research on this new polyherbal formulation may pave a pathway for successful milk augmentation without any health hazard to animal. So, keeping in mind the research gaps, current study was aimed to explore the impact of novel polyherbal formulation on buffaloes with following objectives:

1. Evaluation of efficacy of a polyherbal formulation for production performance in buffaloes.
2. To assess the safety profile of polyherbal formulation through biochemical, hematological and antioxidant parameters.

CHAPTER II

REVIEW OF LITERATURE

2.1. Importance and current scenario of buffalo milk production in India

India, like many other south Asian countries, is primarily an agricultural country, and livestock or animal husbandry has been a crucial sub-sector of agriculture since the dawn of civilization, as the two are fundamentally intertwined. Currently, the livestock sector contributes a significant amount to India's gross domestic product (GDP). Livestock husbandry and dairy sector play a vital role in our country's rural economy, notably among the landless, marginal farmers, and women, along with supplying nutrition and food security to the general public.

Asian buffaloes are regarded to have the most promise and potential for output of any domestic animal (Cockrill, 1994). Buffaloes are an essential component of our livestock, which is understandable given that most Asian countries are agrarian, with 60–80 percent of the population involved in agriculture and its operations in some manner (Nanda & Nakao, 2003). Buffalo is the backbone of India's farmer economy and a key source of milk in several south Asian and European countries, benefiting approximately half of humankind in over 40 countries (Ingawale & Dhoble, 2004) and contributing significantly to overall social development through milk contribution.

India has emerged as largest milk producer in the world since last two decades with rapid development in dairy sector and is presently responsible for 22% global milk production. Milk is a part of staple diet in most South Asian countries, especially predominantly vegetarian nation like India. Out of total milk production in India, maximum contribution is by indigenous buffaloes (35%) followed by crossbred cows (26%) (DADF, 2019-20). But despite the progress over the time, productivity of Indian dairy animals is still on lower side which might be due to number of reasons such as lack of proper nutrition, diseases, stress, etc. which puts constraint on dairy industry’s economy in our nation (Choudhary et al., 2020).

Furthermore, despite the fact that milk and milk products are produced in large quantities, global demand continues to grow at a rate of 2% per year, owing to expanding population, urbanisation, and changes in lifestyles. Milk consumption is

quickly increasing as people become more aware of the nutritional importance of milk and its products, necessitating milch animals to become more productive without jeopardising their well-being and health. Buffalo has evolved into a powerful and hardy animal that can thrive on relatively low-quality feed and forage as a result of natural and human selection, making it the focus of the dairy sector for boosting production efficiency. Therefore, augmenting the milk production in buffaloes through innovative changes in management and nutrition are vital for taking care of ever rising demand of quality milk and milk products all over the world keeping in mind that supplementation used for bringing out such a change should significantly improve production performance of buffaloes thereby increasing the profit of the farmers without causing any health concerns.

2.2. Need for herbal formulations to amplify dairy industry

Certain combinations of herbs have proved useful in different functions to stimulate metabolism or even as therapeutic agents (Tiwari et al., 1993). Of these, some of the herbs arc having the property of galactogogue and the others possessing stomachic action.

The European Union's 2006 prohibition on the use of antibiotics as animal growth promoters was just the push needed to get producers' attention on alternative feed additives that can boost animal productivity without compromising the animals' overall health. Plant-derived compounds such as flavonoids, tannins, saponins, and essential oils, probiotics or enzymes, etc. are among the most prevalent non-antibiotic feed additives currently used or potentially employed in ruminant nutrition, as per Jouany & Morgavi (2007). The additives listed above have been shown to improve rumen feed digestion and ruminant production. These additives generally act in different way than antibiotics as plant derived compounds are more subtle in action and less specific as compared.

The need for massification of dairy industry has been recognized and there are various methods that are being explored for serving the purpose. Feed supplements, hormones, minerals and feed additives are some of the ingredients used in dairy sector to augment the milk production of animals. For example, Bovine Somatotropin is the hormonal therapy administered to dairy animals and is reported to increase milk production, but such a treatment is very expensive and the milk from treated cattle may not be safe for human consumption (El-Ghousein, 2010).

Organic dairy farming entails feeding organic feed to dairy animals and limiting or eliminating the use of antibiotic feed additives and hormones. It is a relatively new concept that sprang onto the scene in the mid-1990s and quickly established itself as an important category, owing to consumer concerns about the traditional dairy farming paradigm and its reliance on synthetic inputs to enhance productivity and profits. Synthetic feed additives have an effect on both the environment and animal health. As a result, herbal feed additives could be a viable option for increasing milk supply without causing serious issues while keeping organic integrity.of dairy farming (Oruganti, 2011).

Due to various reasons, antibiotic growth promoters once used extensively and successfully to influence animal health and production are now raising concerns all over the world. One of the reasons for this change is reducing the unnecessary use of antibiotics to tackle microbial resistance which is a grave health hazard to population. Another acceptable reason is to address customer concerns about the use of non-natural xenobiotics in animal diets, so natural medicine resources, such as phytomedicines, can assist smallholders in rural regions in managing their only source of revenue and protecting it from animal diseases and mortality (Mirzaei, 2012).The economic status of farmers also have to be kept in mind and synthetic formulations might prove costly in long run unlike providing an alternative using medicinal herbs available in abundance in our herb-rich nation.

Due to rising concerns about the potential development of antibiotic-resistant bacteria in case of antibiotic feed additives that are supplemented in animal feed to improve growth and production as well as at the same time to prevent economic losses of the farmers because of unavailability of wholesome nutrition from basic feed and fodder throughout the year has created an urgent need for the alternatives (Papatsiros et al., 2013). This gap for alternative nutrition supplement can be fulfilled by phyto-additives as there are many herbal preparations and formulations available in Indian landscape.

Oxytocin is a hormone involved in milk ejection reflex and is very commonly used in many species when lactating mother has inadequate milk supply but due to its misuse or excessive use in dairy sector, it has reported side effects. Consumption of oxytocin as a galactagogue is banned in Indian dairy industry and some other

countries because of its effects on animal welfare on continuous usage. Recombinant Bovine Somatotropin is the major galactogogue which is being used in cattle. But to its synthetic origin, it has created a doubt in the minds of consumer regarding ingestion and secretion of a synthetic xenobiotic. Contraindications in cattle include low conception rates, increased open days, retained placentas, mastitis, laminitis, digestive disturbances, decreased apetite, allergies, etc. and hence, have been banned in many countries (Tabares et al., 2014).

Most common galactogogues of non-natural origin used in breastfeeding humans are metoclopramide, domperidone, sulpiride, chlorpromazine, oxytocin, etc. But in humans, many deleterious effects including dry mouth, gastrointestinal disturbances, cardiac arrhythmia, lethargy, sedation and extrapyramidal symptoms such as hypertension, sudden demise, etc. have been reported in mothers (Tabares et al., 2014). Despite domperidone being considered safe among them all because of lesser penetration into blood brain barrier (BBB), it still has serious side effects when used for long duration and hence, it might not be beneficial to use it long term as a galactogogue in dairy animals to enhance milk production.

So, when contemplating these drugs for usage in dairy animals, all these aftermath effects should be taken into consideration and supplements of natural origin should be added in animal's diet for fulfilling any purpose or treating any anamoly. The increased awareness towards phytomedicines and a trend towards organic production is also motivated, mainly due to growing evidence of its efficacy as well as safety.

An eco-friendly alternative to antibiotic growth promoters and synthetic or hormonal preparations to avoid and rectify disease condition of animals along with improving production is a great challenge. Herbal feed additive may be utilized as a drug, herbal extracts or herbal isolates such as essential oils. Herbs can enhance secretions of digestive tract and improve feed intake of animals, changing their feeding patterns because they add as well as improve flavour in animal. One plant can have numerous active principles and therefore, adding herbal supplement in animal feed may affect different systems in animal body all at the same time. Various experiments have now proved that different herbs may exhibit anti-inflammatory, anti-microbial, antioxidant properties. Also, they improve the quality as well as

stability of animal products and can ameliorate animal production as well as growth (Bhatt, 2015).

Inclusion of herbs should be encouraged in animal diet as it can have a positive influence on feed conversion efficiency, production performance, general health and it can help alleviating environmental stress (Patel et al., 2017). Phyto-additives offer a rich, comprehensive outlook to a healthy life. The present-day approach to phytomedicine therapy is based on traditional knowledge of herb properties, its active constituents backed by scientific research and the evidence of influence of herbal active substances on animals.

The active principles or secondary compounds present in plants influence the digestibility, dry matter intake and absorption of nutrients in ruminant's nutrition. Major herbal secondary compounds such as tannins, saponins and essential oils, in a right dose, may improve animal's health, average live weight gain and production performance (Ebrahim & Negussie 2020).

2.3. Effect of polyherbal supplementation on milk yield of animals

An experiment was undertaken by Tiwari et al. (1993) to see how a polyherbal formulation named Anifeed affected milk output in Jersey crossbred cows. Preparation consisted of *Zingiber officinale* (Ginger), *Lepidium sativum* (Garden cress), *Trigonella* (Fenugreek), *Ptychotis ajowan* (Ajwain), *Allium sativum* (Garlic), *Ficus religiosa* (Sacred Fig), *Myristica officiale fragrans* (Nutmeg), and other herbs. Some of the herbs were thought to have galactogogue properties, while others were thought to have stomachic properties. The results reported that the cows in supplemented group showed an increment in milk yield of total lactation period by 74 kg than animals in control group. The increase in yield was attributed to positive change in feed conversion efficiency when the polyherbal preparation was added to the animals' meals, as feed efficiency for milk production was 1.41 and 1.37 kg DM/kg milk produced, respectively.

Galactin, a multiherbal commercial product, was evaluated in dairy cows in an investigation (Ramesh et al., 2000). The increase in milk production was observed three to four days after the animals were fed Galactin. Galactin treatment enhanced milk supply when compared to the control group during the declining phase after peak milk production.

Thakur et al. (2006) investigated the effects of 10 different herbal feed supplements on lactating cow performance. Animals in the treatment group were given different herbs at a rate of 10 grammes per cow per day for 90 days, and the results showed that while the herbal feed supplement had no effect on DM intake, nutrition digestibility, or live weight, milk and Fat Corrected Milk (FCM) yield was improved without affecting its composition. It was deduced that dietary supplementation of an herbal feed additive @10grams/day to lactating crossbred cow, improved milk yield.

The effect of feeding two herbal remedies, Ruchamax and Payapro, on milk yield and rumen parameters in lactating crossbred cows was studied by Bhatt et al. (2009). Ruchamax is a herbal-mineral supplement that comprises 28 different herbs, including *Allium sativum*, *Embelica ribes*, *Picorrhiza kurora*, *Zingiber officinale*, and *Piper longum*, among others. Payapro is a galactagogue made up of *Leptadenia reticulata*, *Nigella sativa*, *Glycerriza globra*, *Cuminum cyminum*, and *Asparagus racemosus*, among other plants. For three months, Ruchamax was administered at 30 grams per day and Payapro was given at dose rate of 4 tablets per day for 15 days in a month. The study found that using herbal preparations enhanced average milk production in cows, with the highest (11.8 litres/day) in Ruchamax supplemented cows, moderate (9.3 litres/day) in Payapro supplemented and the lowest (7.1 litres/day) in non-supplemented cows. The researchers concluded that the supplements enhanced milk production in lactating cows by improving their rumen environment, as beneficial rumen microflora and total VFAs were found to be highest in the ruchamax supplemented group, moderate in the payapro supplemented group, and lowest in the control animals.

A lactogenic polyherbal preparation named Lactovedic contains Shatavari, Jivanti, Vidarikanda, etc. herbs which are known to have galactogoguic effect in traditional ayurvedic texts. A study was performed by Sumanth & Narasimharaju (2011) to evaluate the effect of this preparation on lactating rats and nursing pups. Lactovedic incremented the milk yield as well as body weight of lactating female and nursing offspring indicating that the formulation has significant galactogogic effect. This was also evident from serum cortisol and prolactin levels. Lactovedic-treated rats' mammary glands revealed proliferation of acini and a significant increase in milk production in the ducts in a transverse section.

Mirzaei & Venkatesh (2012) documented the use of 5 herbs namely, *Asparagus racemosus* (Shatavari), *Leptadenia reticulate* (Jivanti), *Cuminum cyminum* (Jeeraka), *Nigella sativa* (Kalaunji) and *Pueraria tuberosa* (Vidarikanda) that are known to increase production performance of livestock in an eco-friendly way. They also reviewed the polyherbal mixtures that have been researched, studied or experimented for their galactogogue potential. Leptaden, galog and ricalex are commercial preparations available in market that are proved to enhance milk production in domestic animals without causing any potential hazard to animal health. The review further mentioned the polyherbal preparations that increased animal production by improving the ruminal digestion or general health of animal such as Livol and Hbstrong. Payapro can increase milk production upto 30% and hence, can be considered as good natural origin alternative for hormones used for inducing milk production and enhancing it in lactating animal.

Patel et al. (2013) investigated the impact of a polyherbal galactagogue formulation on Surti buffalo milk output, quality, and overall health. The formulation was composed of *Anethum graveolens, Aspargus racemosus, Cumium cymium, Leptadenia reticulate, Tinospora cordifolia, Acacia catechu* and 10 other herbal ingredients. Buffaloes in the treatment group were given two polyherbal galactogogue biscuits (19 g each) every day during the first ten days of the month throughout a three-month period, and daily milk output was recorded. As a result, animal fed with herbal supplement revealed significantly higher total milk yield (14.24%) and dry matter intake percentage (DMI %) was significantly higher for animals in treatment group. When the cost-benefit ratio was calculated, it was concluded that the herbal galactagogue formulation fed to buffaloes safely enhanced the production and benefitted the dairy economics for farmers.

Ghafari et al. (2015) conducted another study to examine the effects of cumin seed (*Cuminum cyminum*) supplementation on nutritional intake, milk production, and blood metabolites in lactating Holstein dairy cattle. Total dry matter intake was increased proportional to addition of cumin in diet and milk yield saw a curvilinear increase. When 0, 100, 200, and 300 g/d cumin seed were fed, the DMI improved curvilinearly from 22.8 to 25.2, 26.0, and 25.8 kg/day, respectively. In addition, milk yield increased in a curvilinear pattern with cumin seed concentration (averages 47.9,

52.5, 55.1, and 53.6 kg/d for the four levels, respectively). Although there was no significant change in milk fat percentage or most blood metabolites when cumin seeds extract was included in the diet, cholesterol did seem to decrease. As a result, it was established that feeding lactation dairy cows' meals with up to 200 g/day of cumin seed could boost performance, but that increasing the amount of supplementation could lower efficiency.

Jingar et al. (2018) conducted on farm trial by feeding powder of Shatavari (*Asparagus racemosus*) roots to lactating buffaloes and observed increase in milk yield. For a period of 180 days, 20 lactating buffaloes were chosen and fed 50 gm powder of shatavari roots mixed in concentrates, once a day. In comparison to their prior production, milk production grew by 0.60±2.27 kg (9.67 percent) each day. When the increased milk production was compared to the control group, the gain in income from feeding Shatavari was determined to be Rs. 12.00 per day per animal. As a result, it was determined that shatavari is useful in increasing milk production economically.

2.4. Effect of polyherbal supplementation on milk composition and quality

Chandra et al. (2017) conducted research to see how dietary supplementation with poly-herbal mixture and butyric acid affected postpartum milk supply, milk quality, and somatic cell counts in Murrah buffaloes. The herbo-mineral preparation given was composed of equal amounts of 6 herbs namely, *Trigonella foenumgraecum* (Methi), *Zingiber officinale* (Sundh), *Foeniculum vulgare* (Saunf), *Trachy spermumammi* (Ajwain), *Anethum graveolens* (Sowa) and *Elettaria cardamomum* (Cardamom). Results were analysed after supplementing feed of buffaloes in one group (T-1) with polyherbal mixture for 7 days post-partum and another group (T-2) with butyric acid for 30 days post-partum along with polyherbal mixture for 7 days post-partum. Control group (T-0) was not supplemented but given daily ration. It concluded that buffaloes fed with polyherbal mixture showed significantly higher milk yield (T-1- 9.91±1.10 kg/day, T-2- 9.72±1.18 kg/day) and total milk solids (T-1- 17.34±0.3, T-2- 17.80±0.40) than the control group milk yield (T-0- 8.62±0.97 kg/day) and total solids (T-0- 6.74±0.25) respectively. Although there was no significant difference in milk protein, lactose, or SNF of both groups, the treatment group's results were on the upper side as compared to control. In addition, in the

polyherbal mixture fed group, the Somatic Cell Count (SCC) was significantly lower. Therefore, polyherbal mixtures are effective in enhancing milk production in dairy animals with benefit of improvement in general health.

Morsy et al. (2018) conducted an experiment on lactating Damascus goats to investigate the effects of including cumin and mustard seeds in animals' diet on feed utilisation, milk production, milk composition, and milk fatty acid profile. Control group was fed their daily ration without any supplement whereas one group was supplemented with mustard seeds @10 grams/day and another group supplemented with cumin seeds @10 grams/day. Treatments had no influence on feed intake, but they did improve the digestibility of the DM, organic matter, non-structural carbohydrates, and fibre fractions. When comparing the cumin and mustard treatments, the cumin treatment had a higher digestibility. Mustard and cumin seeds raised serum total proteins, globulin, and glucose levels while lowering serum cholesterol levels. Mustard and cumin seeds boosted milk production in animals (by 6.8% and 11.1 percent, respectively), while cumin significantly raised fat and lactose content in milk, resulting in improved milk composition. Additionally, cumin treatment decreased milk saturated fatty acids (SFA) while increasing total unsaturated fatty acids (UFA) and total conjugated linoleic acid (CLA). Overall, it can be concluded that supplementing goat ration with herbal seed or seed extracts not only has a positive impact on digestibility of feed and lactational performace of animals, but it also positively enhanced milk fatty acid profile.

Harjanti et al. (2019) investigated the milk production and quality of ruminants with sub-clinical mastitis after they were offered several herbal supplements in their diet. The goal of the study was to see how a mixture of five different herbs with therapeutic qualities affected milk output and bacterial count in cows suffering from sub-clinical mastitis. In comparison to the control group, the bacterial count in the milk of treatment group animals was decreased. In a dose-dependent fashion, the treatment group produced more milk, lactose, and milk fat percentage than the control group. To conclude, for ensuring milk productivity as well as milk safety, herbal feed additive could be used for mastitis treatment.

Hendawy et al. (2019) observed the effect of some plants with medicinal properties on colostrum, haematological indices, and milk composition of lactating ewes. Ewes were fed with ginger fine powder for 3 months after parturition at dose

rate of 5gm/day/ewe and results revealed that the percentage of milk fat increased in animals fed black seed (5.24%) and ginger fine powder (5.04%) than the control group (4.39%) and same pattern was followed by total solids which increased significantly in ewes that received herbal supplementation(15.61% and 15.37% with black seed and ginger powder respectively) compared with the control (14.42%) whereas there was no significant change in milk production, SNF, ash or lactose content of milk but the values were on higher side in supplemented groups than the control group animals. RBC count was also higher in ewes of treatment group.

Hassan et al. (2021) performed a study for evaluation of herbal mixture containing equal quantities of black pepper, ginger, cinnamon bark, peppermint leaves, ajwain seeds and garlic bulbs on milk production in buffaloes and results revealed that supplementing the mixture did not cause any significant increase in daily milk yield but significant increment in milk fat percentage (8.91%) was observed in buffaloes group supplemented with 20 grams/day herbal mixture as compared to milk fat percentage (7.28%) in control group buffaloes. Mixture also increased saturated fatty acid content of milk in treatment animals.

2.5. Effect of polyherbal supplementation on sensory attributes of milk

Randby et al. (1999) indicated that organoleptic properties of milk are affected by diets of animals. He conducted an investigation by adding pure ethanol in diets of lactating cows in same amount that could be found in well-fermented silages. The supplemented animals showed off flavour in milk due to effect of tainted feed. Additionally, there was increment in proportion of palmitic acid and decline in the proportion of unsaturated fatty acids which might be one of the reasons in change of sensory attributes of milk due to oxidation of lipids in milk.

A study was conducted by Mendieta-Araica et al. (2011) testing the effect of feeding dairy cows with fresh or ensilated moringa leaves on milk yield and milk flavour along with feed digestibility, milk composition and overall organoleptic properties of milk. Milk yield or milk composition did not differ among control and treatment groups but supplemented animals had a grassy flavour and different aroma in their milk. Milk color and appearance was not affected indicating that addition of herbal supplements to diets of lactating animals may change flavour and aroma of milk.

In an experiment conducted by Vasta & Luciano (2011), effect of supplementing animals' diet with secondary metabolites and compounds present in herbal supplements on milk and meat quality was studied. It was concluded that some secondary metabolites such as tannins manipulate fatty acid profile of milk as well as meat by modifying ruminal biohydrogenation of dietary polyunsaturated fatty acids i.e. changing rumen ecology when added to animal diet. This in turn reduces the accumulation of skatole- a component giving off-odour to milk and meat. So, they can enhance the flavour and improve fatty acid profile.

Mirzaei (2010) investigated the effects of a polyherbal mixture in lactating crossbred goats. The goats were divided into three groups, each with ten goats. Every day, a polyherbal supplementation containing five herbs in the same proportion was manually combined with the concentrate combination @ 0.125g/kg BW for one group and @ 0.250 g/kg BW for the other group. One group was used as a control group, and they were given no supplements but solely regular diet. The organoleptic qualities of raw milk were assessed. The results showed that the milk from the treatment groups had no undesirable odour or flavour and was safe for human consumption.

Lejonklev et al. (2013) investigated transfer of volatile phyto-components of essential oils into milk of lactating animal. Lactating Danish Holstein cows were exposed to two different essential oils through respiratory as well as oral route for nine hours in different groups. Milk samples were collected before and after the exposure as well as on next day. Study concluded that almost all terpenes were found in milk just some time after the exposure in groups supplemented orally indicating that volatile compounds present in phyto-additives are transferred to milk in very less time but the action is temporary and hence, effect on sensory attributes does not last after the supplementation is discontinued. Results stated that compounds present in milk were dependent on amount of supplementation in the feed. Therefore, effect of herbal additives on sensory properties of milk can be controlled with proper dosage

2.6. Effect of polyherbal supplementation on rumen environment

The study was undertaken to evaluate the impact of herbal feed additives on the changes in the biochemistry of rumen in buffaloes by Wadhwa & Bakshi (2006). Experiment was performed on six fistulated male buffaloes in each group and nine herbs namely, *Asparagus racemosus* (Shatavari), *Eclipta alba* (Bhringraj), *Leptidenia*

reticulata (Jiwanti), *Kutaki picorrhiza* (Kutki), *Anethum sowa* (Sowa), *Foehieulim vulgare* (Saunf), *Plumbago zeylanica* (Chitrak), *Cyperus scariosus* (Mustak) and *Cuminum cyminum* (Jeera), were mixed with daily roughage @0.4 percent per animal. All the supplemented additives improved rumen activity but among them, kutaki had maximum positive impact on ruminal activity. The net gas production, true organic matter digestibility and metabolizable energy availability were enhanced significantly when either bhringraj or kutaki was supplemented with wheat straw as compared to addition of other additives. When compared to other feed additives or a control diet, the significantly higher activity of most microbial enzymes was observed in the rumen of animals fed a diet supplemented with *Kutaki picorrhiza* which further resulted in significantly higher availability of the end products of digestion and significantly higher microbial protein synthesis in the rumen. It may be concluded that this herb, out of all the phytochemical feed supplements examined, had the most beneficial effect on the rumen environment in buffaloes and should be investigated further for its effects on other parameters.

Bunglavan et al. (2010) studied the consequence of feeding particular herbal supplements along with basic diet on methanogenesis in ruminants. Methane emission or methanogenesis reduces the efficiency of nutrient utilization. So, reduction in methane emission from ruminants will enhance the efficiency of nutrient utilization and will augment animal productivity alongside providing the benefit of reducing methane impact on global warming. Eight different herbal supplements were added to basal diet of various groups of ruminant animals. Among these 8 herbs, all were able to significantly reduce methane production as compared to the levels in control group animals but *Acacia concinna* pods extract ranked highest in reducing methane emissions with methane production at just 13.3% whereas *Allium sativum* bulb water residue kept methane production at 15%. *Zingiber officinale* rhizome water residue was close to *Allium sativum* with methane production at 15.02%. So, the study concluded that the active principles in various herbs have capacity to decrease methane production indirectly increasing animal productivity.

Giannenas et al. (2011) conducted an experiment to evaluate the effectiveness of supplementing essential oils in rations of lactating ewes on milk production, milk composition, and rumen microbiota. Individual milk yield was recorded daily during the first 5 months of lactation. Milk samples were analyzed for chemical

composition, somatic cell count, and urea content whereas rumen samples were analysed for protozoa, cellulolytic, hyper-ammonia-producing and total viable bacterial counts along with pH, NH_3-N content. Results showed that inclusion of Essential Oils (EO) increased milk production per ewe, the effect dependent on dose and hence, milk yield was highest in the group with highest amount of supplemented essential oils i.e. 150mg/kg concentrate diet. Feed utilization tendency was improved. Although the addition of EO had no effect on milk composition, it did reduce the concentration of urea and somatic cell count in milk samples. Total counts of viable and cellulolytic bacteria and protozoa did not change significantly, but counts of hyper-ammonia-producing bacteria were lower in treatment groups compared to the control group, suggesting that herbal essential oils can reduce ammonia production in ruminants. However, the underlying mechanism for all of the above findings needs to be investigated further.

Jain et al. (2011) conducted a similar experiment on crossbred cows to determine the effect of supplementing herbal mixture on methane Emission and milk production. A polyherbal preparation containing *Trigonella foenum, Ptychotis ajowan, Asparagus racemosus, Lepidium sativum, Anacyclus pyrethrum, Ficus religiosa, Myristica officinalis Zingiber officinale, Anethum sowa,* and *Anaqua sodichlordon* was supplemented in the feed of treatment group animals for 90 days. The methane emission was estimated using SF6 tracer technique and milk production was observed for the experimental period. Although there was no significant improvement in milk yield, the treatment group's methane emission/kg DMI was dropped by 20% when compared to the control group animals following the inclusion of the herbal mixture. Methane emission is seen as loss of ingestible feed energy and is one of the reasons for production losses. So, reduction in methane gas production or release might improve productivity of animal in long run. Furthermore, body weight was maintained in the experimental group, but body weight was reduced in the control group. It was discovered that the herbal mixture had a beneficial influence on the body wellness. Even when the differences were not significant, the digestibility of nutrients in experimental animals was higher than in control animals.

Hassan et al. (2020) did a study to see how a mixed phytogenic preparation affects the composition of the Ruminal Bacteria and the Milk Fatty Acid Profile in Water Buffaloes. The phytogenic formulation containing ginger (tubers), fennel

(seeds), ajwain (seeds), *Swertia chirata* (leaves*), Citrullus colocynthis* (fruit), turmeric, fenugreek (seeds), licorice (roots), *Terminalia chebula* (fruit) and *Phyllanthus emblica* (fruit) in equal quantities was fed to animals for 6 weeks. The results indicated that the polyherbal supplementation improved ruminal microflora that is positively associated with milk yield and milk fat percentage. General fatty acid content was increased in buffalo milk, while specifically reducing the stearic acid percentage which is desirable from human consumption point of view as stearic acid is the major saturated fatty acid.

Kholif et al. conducted an assay to determine the effect of supplementing Phytogenic Feed Additives (PFA) mixture in the diets of lactating holstein friesian cows (2020). The phytogenic mixture was fed to cows at a dose of 3 grams/cow, which boosted feed intake and nutrient digestibility. The treatment group's ruminal pH, total volatile fatty acids, propionate, and acetate levels were all significantly higher. PFA at 3grams/cow and 6grams/cow enhanced serum total protein and antioxidant capacity while decreasing serum urea-N, triglycerides, total lipids, cholesterol, and malondialdehyde (MDA) concentrations, demonstrating that herbal feed additives can have a positive impact on animal health. PHA enhanced milk production as well as total solids, protein, lactose, and fat content in milk. The introduction of 3 g/cow/day of feed additives mixture in the diet of lactating Friesian cows increased milk output and feed utilisation, with some negative effects detected when the quantity of supplements was increased to 6 g/cow daily. This indicates that the main limitation of PFA is defining the optimal doses that enhance animal performance and the adaptation of ruminal microflora to their presence in diets, resulting in inconsistent and inconclusive results and hence, despite having numerous beneficial effects, there needs to be a standard for the dose rates so that production performance can be improved without causing any harm to animal health. (Kholif and Olafadehan 2021)

2.7. Effect of polyherbal supplementation on immunity and antioxidant status of animals

Kumar et al. (2008) stated that Shatavari (*Asparagus racemosus* was not only galactopoetic herb that can be used in cases of lactational inadequacy by but it also increases reproductive efficiency of animal. Shatavari is therapeutic in many cases as

it can also boost the immune system and consequently prevent the infection of the udder. It can also be effectively used to reduce the stress of dairy animals as it has antioxidant properties.

The ethanol and aqueous extracts from galls of *Pistacia integerrima* were evaluated for antibacterial activity *in-vitro* by Ramachandra et al. (2010). Results offered scientific evidence that *Pistacia integerrima* galls have considerable antimicrobial activity which is better against gram positive bacteria as compared to gram negative bacteria. These galls are used as a source for preparation of herbal drug karkatasringi which is part of many polyherbal formulations. The zone of inhibition in MIC was tested with Ciprofloxacin acting as positive control and in dose dependent manner, some extract preparations of herb produced zone of inhibitions comparable to ciprofloxacin. This phyto preparation needs to be tested *in-vivo* to test if the antimicrobial activity sustains and the tissues to which active principles are able to reach in the animal body.

Wang et al. (2011) observed the effect of three various polyherbal supplements on beef cattle growth, digestive enzymes activity and antioxidant activity. Cattle were weighed every fortnight till the end of experiment and it was revealed that growth rate was more in herb supplemented group than the animals in control group. Activity of digestive enzymes such as trypsin, chymotrypsin and lipase was enhanced in animals supplemented with polyherbal formulations. Blood samples were collected and serum was analysed for different antioxidant enzymes namely SOD, MDA and GSH-PX. Results indicated that herbal mixtures when supplemented at a right dose, can significantly reduce MDA levels and significantly raise SOD levels though there was no change in levels of (Glutathione peroxidase) GPX observed.

Hashemzadeh et al. (2014) investigated the effects of a phytobiotics-rich herbal mixture (PRHM) supplementation on feed intake, performance, udder health, ruminal fermentation, and plasma metabolites in Holstein dairy cows with moderate to high somatic cell counts (SCC) in the milk.. Results revealed that the herbal mixture led to improvement in milk production of treatment group animals as compared to control group animals. The DM intake was also increased in supplemented animals but these animals also lost back fat thickness. Milk fat

percentage showed a slight decrease in the treatment group. The somatic cell count reduced in the animals supplemented with herbal mixture. Overall, supplementing ruminants with phytobiotic nutrients appears to be an efficient method for improving productivity and lowering SCC, particularly in animals with high SCC levels in their milk. So, even if herbal compounds do not have galactogogue effect, they might increase productivity of an animal indirectly by increasing their resistance to diseases and improving health status.

Mahipal et al. investigated the effects of dietary supplementation of a novel phytogenic feed supplement on nutritional metabolism, antioxidant status, and immunological response in goats (2016). A polyherbal preparation containing three herbs namely, *Woodfordia fruticosa*, *Solanum nigrum* and *Trigonella foenum-graecum* was given to fifteen Jamunapari goats for a period of 60 days. Animals were divided into four different groups where three groups were fed with polyherbal formulation at different dose rates respectively in addition to their daily feed and the control group was fed with only basal standard diet. Results revealed that herbal supplementation increased the digestibility of nutrients in treatment groups. At larger doses, PFA supplementation improved the activity of antioxidant enzymes such as glutathione peroxidase, catalase, and glutathione-S-transferase. The cell-mediated and humoral immune responses were unaffected by PFA.

Kumari et al. (2018) conducted a study to evaluate efficacy of supplementating polyherbal-potash alum mixture on immune status of cattle during transition period. *Ocimum sanctum*, *Trachyspermum ammi*, *Cinnamomum zeylancium*, *Asparagus racemosus*, *Emblica officinalis* and potash alum were used for preparation of a mixture which was fed to seven crossbred cows for 60 days during transition period (30 days ante-partum and 30 days post-partum). Although no significant difference was observed in DLC levels, on the day of parturition, the supplemented mixture significantly lowered cortisol levels and TLC levels, while increasing phagocytic activity. Mastitis, metritis, and retention of foetal membrane (RFM) incidences were 28.57 percent, 28.57 percent, and 14.28 percent respectively for animals in control group while no such cases were found in supplemented group which indicates that herbal supplementation containing immunity enhancing properties can avoid production losses by reducing these clinical complications during and after parturition.

Japheth et al. (2019) conducted an experiment to investigate the effects of a polyherbal formulation on occurrence of mastitis and production efficiency in postpartum buffaloes. 30 Murrah buffaloes were fed supplementation along with their standard diets (NRC schedule). From the day of calving until day 10 of postpartum, buffaloes in the treatment group were supplemented with a finely grounded powder of polyherbal mixture containing 25 grams each of Ajwain, Jeera, Haldi, Saunf, Sowa, Methi and Sundh, as well as 25 grams of kala namak and 250 grams jaggery. Milk samples were taken on days 7, 14, 21, and 28 after parturition and tested for somatic cell burden using the California Mastitis Test (CMT). The lactation total milk yield of both supplemented and non-supplemented groups were also recorded. In supplemented group, the average total milk yield was 2642.87±75.45 kg, while it was 2292.27±77.65 kg in control group. Results inferred that supplementing polyherbal mixture in postpartum Murrah buffaloes reduced the ocurrence of mastitis in buffaloes and also improved the milk production.

Li et al. (2020) conducted a study in Murrah buffaloes to assess the effects of mulberry leaf flavonoids (MLF) on oxidative stress, metabolic hormones, and milk production. The trial contained one control group and four treatment groups (10 buffaloes each) with varied doses of MLF: control (0 g/d), MLF15 (15 g/d), MLF30 (30 g/d), and MLF45 (45 g/d), with the trial lasting five weeks. Daily milk yield was recorded, and physiological parameters (respiratory rate, rectal, and body surface temperature) as well as milk composition were examined on a monthly basis. Malondialdehyde (MDA), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) among antioxidant enzymes, and also blood biochemical markers, were investigated. Treatment group animals showed higher milk yields than the control group. MDA, T-AOC, and CAT levels all decreased significantly, however GSH-Px levels increased and SOD levels remained same.

One way to increase total milk production in dairy industry is by decreasing the production losses incurred by various mammary diseases. Sub-clinical mastitis is a great challenge and cause of huge economic losses to dairy sector and focus can be given to treat it in a holistic way. Patel & Gupta (2020) assessed a non-antibiotic therapy for treating subclinical mastitis in dairy cows. The therapy comprised of a

commercial herbo-vitamin preparation namely MAGIC-3 comprising of *Glycorrhiza glabra, Curcuma longa, Tinospora cordifolia, Psoralea Corylifolia, Argemone Mexicana, Asparagus racemosus, Vermonia anthemintica, Emblica officinalis,* neem extract, citric acid, Vitamin A and Vitamin D3. Results indicated therapeutic and protective nature of herbal medicine as the values of Electrical Conductivity, California Mastitis Test, Somatic Cell Count, NAGase (N-Acetyl-beta-D-Glucosaminidase) enzyme activity and phagocytic activity declined significantly in animals of the treatment group and the values remained in normal range after that.

Walkenhorst et al. (2020) undertook a two-stage, multicenter, placebo-controlled long-term on-farm experiment to assess the influence of a multicomponent herbal feed additive (HFA) on general health, production performance, and some fertility parameters in dairy cows. From 14 days pre-partum to 300 days post-partum, groups of cows were fed two different doses of herbal feed additive along with their regular diets. Calving intervals in HFA animals were 15 days shorter than in control animals. HFA-50 significantly reduced lameness incidence. Somatic cell count was significantly lower in treatment groups and animals fed with herbal supplementation also showed lesser culling rates that occur due to fertility diseases. Hence, study concluded that phytochemically rich and diverse feed additives create a positive impact on dairy cows' nutrition and physiology.

2.8. Effect of polyherbal supplementation on biochemical parameters of animals

Kholif et al. (2012) conducted a study on lactating Damascus goats where Cinnamon oil (*Cinnamomum cassia*) (CIN), Garlic oil (*Allium sativum*) (GAR) or Ginger oil (*Zingiber officinale*) (GIN) were added as a dietary supplement to their ration in various groups respectively for a period of 90 days. All the additives raised ruminal volatile fatty acids and propionate proportions while decreasing ruminal acetate proportion and ammonia nitrogen concentration. With EOs supplementation, blood albumin levels increased while serum urea nitrogen levels dropped. In addition, CIN and GAR supplementation increased blood globulin values while GIN supplementation decreased them when compared to control. CIN and GAR supplementation improved serum glucose levels. Cinnamon essential oil treatment increased healthy fatty acids in milk namely, omega-3 and omega-6. In comparison to the control group, animals in the treatment groups had significantly higher milk

output, protein, and SNF contents; however, fat % and milk non protein nitrogen were lower in the essential oil supplemented animals.

In an experimental study by Singh et al. (2012), supplementing *Asparagus racemosus* as an individual supplementation given to buffaloes @ 150 g/day/animal after mixing with 200 g of concentrate mixture before parturition and @ 300 g/day/animal following parturition, revealed that Shatavari not only is galactopoietic in buffaloes but did so by increasing plasma prolactin and cortisol which were present in levels significantly higher in animals of treatment group than that of control group. While mean plasma triglycerides, LDL cholesterol, HDL cholesterol, glucose, and non-esterified fatty acid (NEFA) levels varied non-significantly between groups, Shatavari supplementation increased milk fat cholesterol without affecting total plasma cholesterol levels.

Sahoo et al. (2017) provided preclinical evidence for galactogogue property of *Carum carvi* (Caraway) by studying the impact of *Carum carvi* on serum prolactin and cortisol in nursing rats. Albino winstar nursing rats were fed with methanolic extract of caraway seeds and results reported significantly increased milk production in treatment group compared to control group. The study indicated increase in serum prolactin levels which might have resulted in proliferation of mammary gland cells. Body weight of lactating mothers was significantly higher than that of animals in control group. Along with that positive significant difference was seen in body weight of nursing neonates.

El-Tarabany et al. (2018) investigated the impact of dietary fenugreek seeds on lactational performance as well as blood biochemical, haematological, and antioxidant parameters in Baladi dairy goats during stressful summer conditions. They discovered that supplementing dietary fenugreek seeds significantly enhanced milk output and antioxidant status in treatment group goats as compared to the control group. The amounts of serum glucose, triglycerides, cholesterol, and tri-iodothyronine were all significantly lowered after fenugreek seeds were supplemented in the diet. Baladi goats fed with 50 grams and 100 grams fenugreek seeds/day increased serum total protein at a rate of 7.7 % and 12.6 % respectively. Although albumin levels were not significantly different, globulin levels were significantly higher in treatment groups. So, the results revealed that fenugreek supplementation in animal diet may

improve production parameters, biochemical parameters, and antioxidant capacity of heat-stressed goats.

Hendawy et al. (2019) examined as to how several medicinal plants affected colostrum, haematological indices, and milk composition in lactating ewes. Ewes were fed ginger fine powder at a dose rate of 5gm/day/ewe for 3 months following parturition, and the results showed that red blood cell and white blood cell counts were significantly greater in the supplemented group than in the control group. MCH levels were significantly higher in the black seed and control groups than in the ginger group ($p \leq 0.05$). Meanwhile, there were no significant variations in Hb, PCV, MCV, MCHC, or platelet values.

CHAPTER III

MATERIALS AND METHODS

The present study was designed to evaluate the effect of a novel polyherbal formulation on production and blood parameters along with general health of buffaloes.

3.1. Location of the experiment

The experiment was conducted on buffaloes at livestock farm of GADVASU (fig 1) whereas the analysis of samples was done in Department of Veterinary Pharmacology and Toxicology, Department of Medicine and Department of Livestock Products & Technology at Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (Punjab, India).

3.2. Duration of the experiment

Polyherbal formulation containing 13 ingredients was supplemented for 45 days. The animals were observed during the treatment period (45 days) and one month post feeding for various production and blood parameters which were analysed fortnightly and monthly respectively.

Fig.1. Experimental animals at livestock farm, GADVASU

3.2. Selection and grouping of Experimental animals

Twenty lactating buffaloes were selected from buffalo section at GADVASU dairy farm on the basis of their daily milk yield record and also animals in same parity and same stage of lactation were preferred. For conduction of clinical trial, buffaloes were divided into two groups of ten animals each and attempt was made to divide the animals in such a way as to create groups that are homogenous. Group I served as

control group in which no supplement was given and animals were fed normal diet based on feeding schedule followed at GADVASU dairy farm only, whereas animals of group II were treated with the prepared polyherbal formulation at a suitable dose rate mixed with their scheduled diet for 45 consecutive days orally.

G1: Control: Standard feeding schedule + No supplementation

G2: Treatment: Standard feeding schedule + Polyherbal formulation supplementation

3.3. Preparation and dosing of Polyherbal supplementation

Polyherbo-mineral formulation used for this study contained 13 ingredients mentioned in Table 1 and Fig. 2.

Table 1: Ingredients of Polyherbal formulation used in the study are as follows:

S. No.	Sanskrit Name	Scientific Name	Part used	Quantity
1	Śatāvarī	*Asparagus racemosus* willd.	Root	1 Part
2	Śveta jīraka	*Cuminum cyminum* Linn.	Fruit	1 Part
3	Kṛṣṇa Jīraka	*Carum carvi* Linn.	Fruit	1 Part
4	Śatahvā	*Anethum sowa* Roxb.ex Flem.	Fruit	1 Part
5	Kirātikita	*Swertia chirata* Buch.-Ham. ex Wall.	Plant	1 Part
6	Kaṭukā	*Picrorhiza kurroa* Royle ex Benth.	Rhizome	1 Part
7	Śuṇṭhī	*Zingiber officinale* Rosc.	Rhizome	1 Part
8	Pippalī	*Piper longum* Linn.	Fruit	1 Part
9	Marica	*Piper nigrum* Linn.	Fruit	1 Part
10	Viḍaṅga	*Embelia ribes* Burm.f.	Fruit	1 Part
11	Laśuna	*Allium sativum* Linn.	Bulb	1 Part
12	Karkaṭaśṛṅgī	*Pistacia integerrima* Stewart ex Brandis	Gall	1 Part
13	Gairika	Red Ochre	Powder	1 Part

The formulation was prepared based on standard operating procedures and all the ingredients were mixed in equal proportions.

The said formulation was supplemented at the dose rate of 20gms/100 kg body weight b.i.d. p.o. mixed with their daily portion of concentrate mixture fed to animal for consecutive 45 days (fig 3 to 6). Green fodder was fed to both the groups according to their standard feeding schedule.

Shatavari roots
(*Asparagus racemosus*)

Safed Jeera
(*Cuminum cyminum*)

Kaala Jeera
(*Carum carvi*)

Sowa
(*Anethum sowa*)

Chirata
(*Swertia chirata)*

Kutaki
(*Picrorhiza kurroa*)

Sunthi
(*Zingiber officinale*)

Pippali
(*Piper longum*)

Miracha
(*Piper nigrum*)

Vidanga
(*Embelia ribes*)

Lasuna
(*Allium sativum*)

Karkatasringi
(*Pistachia integerrima*)

Gairika (Red Ochre)

Fig. 2: Ingredients of Polyherbal formulation used in the study

Fig. 3: Packing of polyherbal formulation according to calculated dosage

Fig. 4: Packed formulation according to animal number

Fig. 5: Polyherbal formulation mixed with concentrate mixture

Fig. 6: Animal of treatment group feeding on polyherbal formulation

3.4. Body weight measurement

Fasting body weight of all animals was recorded early in the morning one day before the beginning of the experiment for calculating dose of polyherbal formulation to be supplemented in animal diet and every month i.e. 30 and 60 after the start of the experiment using mechanical weighing balance available at the buffalo section of livestock farm, GADVASU.

3.5. Physiological parameters

The following parameters were recorded for lactating buffaloes at start of the experiment and on day 30 and day 60.

3.5.1. Rectal Temperature (RT)

Clinical thermometer was used to record the rectal temperature.

3.5.2. Respiration Rate (RR)

The movement of flank i.e., one outward and inward movement as one respiration was measured as respiration rate per minute.

3.5.3. Heart Rate (HR)

The heart rate per minute was measured by placing a stethoscope over the heart with minimum disturbance to the animal (fig 7).

Fig. 7: Measurement of heart rate in animals

SAMPLE COLLECTION AND ANALYSIS

3.6. Milk recording, sample collection and analysis of production parameters

3.6.1. Milk yield

Milk yield was recorded twice a day as per the GADVASU Dairy farm milking practice (fig 8) (morning at 5:00 am and afternoon at 3:00 pm) using a digital balance.

Fig. 8: Milking of experimental animals

3.6.2. Milk composition analysis

Milk samples from every animal of both groups was taken for analysis of milk components viz. Fat %, Protein %, Lactose %, Solid not fat %, Total solids %. Each time 100 ml of milk sample was collected in plastic bottles after uniformly mixing of milk of each buffalo. Representative amount of each sample was used for estimation of milk components by Lactoscan milk analyser (fig 9). The analysis was done fortnightly during treatment period and a month after that.

Fig. 9: Milk composition analysis by lactoscan milk analyser

3.6.3. Milk quality parameters

Somatic cell counts (SCC): Somatic Cell Counter (fig 10) was used to estimate the total number of somatic cells secreted per ml of milk. Somatic cell count was expressed as no. of cells ($\times 10^3$) per ml of milk sample. Generally agreement rests that somatic cell count greater than 25,000 cells per ml milk indicates infection in udder of lactating buffaloes.

Milk pH: Milk pH was estimated using pH meter (fig 11). Normally, for buffalo milk physiological range is 6.68 to 6.9.

Electrical conductivity: Conductivity meter was used to measure electrical conductivity of milk samples. Conductivity is expressed in mS/cm.

Fig. 10: Somatic cell counter used to measure SCC in milk samples

Fig. 11: pH meter for measuring milk pH

Milk sensory attributes: A portion of milk collected every fortnight from day 0 to day 75 was evaluated by a panel of two expert and two trained judges for checking the effect of polyherbal formulation on organoleptic features of milk including color & appearance, odour, flavour and overall acceptability (fig 12 and 13).

Color and appearance were judged based on the presence of suspended particles, filth, foreign matter or blood. Defects in odour could be milk being stale, acidic or any other abnormal smell. Cooked, oxidized, rancid, metallic, neutralizer, barny, etc. flavour defects due to adulterants and other additives could deteriorate quality of milk and hence, scored less. After observing all the factors, overall acceptability was scored according to the score card given in table no. 2 using 10 point hedonic scale method.

Table 2: Score card for milk sensory analysis

Characteristic	Max. score	Sample score		
		Sample 1	Sample 2	So on…
Color and appearance	10			
Odour	10			
Flavour	10			
Overall acceptability	10			

Fig. 12: Expert panel analyzing organoleptic properties of milk samples

Fig. 13: Score card filled for every sensory parameter of milk samples

3.7. Collection of Blood samples and Plasma biochemical analysis

The blood samples were collected from jugular vein of experimental animals (fig 14) on day 0, day 30 and day 60 of the trial. Heparinized vials (20 IU heparin/ml blood) were used to collect samples for the estimation of various biochemical and antioxidant parameters. EDTA containing vials were used for collecting blood for analysis of hematological parameters. Immediately after sampling the blood was placed in ice box and taken to the laboratory where the samples were centrifuged at 3000 rpm for 15-20 minutes to collect plasma which was then stored at -20^0 C until further analysis.

Fig. 14: Blood collection in experimental animals from jugular vein

3.8. Assessment of biochemical parameters

3.8.1. Processing of samples

The samples stored in deep freezer are taken out and thawed at room temperature (fig 15) and then analysed using kits procured from ERBA diagnostics Mannheim GmbH, Germany (fig 16).

Fig. 15: Thawing of plasma samples kept in deep freezer before analyzing

3.8.2. Parameters assayed

Aspartate aminotransferase (AST)

The amount of aspartate aminotransferase (AST) in plasma was determined using an ERBA autopak kit and an ELISA reader according to the procedure of the International Federation of Clinical Chemistry (IFCC, 1986). AST catalyses the amino group transfer from L-aspartate to -ketoglutarate, resulting in formation of oxaloacetate and glutamate. In the presence of malate dehydrogenase, the produced oxaloacetate interacts with NADH to form NAD. The rate of oxidation of NADH to NAD is determined as a reduction in absorbance proportional to the sample's AST activity. The absorbance was measured at 340 nm. AST levels were expressed as U/L.

$$\text{L-Aspartate} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^{+} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}$$

$$\text{Sample pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}$$

AST = Aspartate aminotransferase

MDH = Malate dehydrogenase

LDH = Lactate dehydrogenase

Alanine aminotransferase (ALT)

The amount of alanine aminotransferase (ALT) in plasma was determined using an ERBA autopak kit and an ELISA reader according to the international federation of clinical chemistry's (IFCC) 1972 technique. ALT catalyses the amino group transfer from L-alanine to -ketoglutarate, resulting in formation of pyruvate and glutamate. In the presence of lactate dehydrogenase, the produced pyruvate interacts with NADH to generate NAD. The rate of NADH oxidation is determined as a fall in absorbance that is proportional to the amount of ALT in the sample. The absorbance was measured at 340 nm. ALT level was expressed as U/L.

$$\text{L-Alanine} + \alpha\text{-Ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyurvate} + \text{L-glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}$$

ALT= Alanine aminotransferase

LDH= Lactate dehydrogenase

Lactate dehydrogenase (LDH)

The optimized kinetic approach of the Deutsche Gesellschaft für Klinische Chemie (DGKC, 1972) was used to estimate lactate dehydrogenase in plasma using an ERBA Autopak lit on an ELISA reader. Lactate dehydrogenase catalyses the conversion of pyruvate to lactate and NADH to NAD. LDH activity is expressed as U/L and is directly proportional to the rate of decrease in NADH absorbance at 340 nm.Pyruvate + NADH + $H^+ \xrightarrow{LDH}$ L-Lactate + NAD^+

Gamma- glutamyl transpeptidase (GGT)

Gamma-glutamyl transpeptidase catalyses the transfer of the gamma-glutamyl group from the gamma-glutamyl para-nitranilide substrate to glycylglycine, producing free p-nitroaniline that absorbs light at 405 nm. ERBA Autopak kits were used to estimate plasma gamma-glutamyl transpepetidase using the kinetic method defined by Kachmar et al. (1976) of the International Federation of Clinical Chemistry. The rate of formation of 5-amino-2-nitrobenzoate is proportional to the activity of GGT present in sample and can be measured kinetically at 405 nm.

L-ϒ-glutamyl-3-carboxy-4-nitroanilide + Glycylglycine

↓

L-ϒ-glutamylglycylglycine + 5-amono-2-nitrobenzoate

Blood urea nitrogen (BUN)

The GLDH-Urease method, originally described by Tietz (1976), was used to estimate blood urea nitrogen using ERBA Autopak kits on an ELISA reader. In the presence of water and urease, urea is hydrolyzed to create ammonia and carbon dioxide. In the presence of glutamate dehydrogenase, the ammonia generated reacts with α-ketoglutarate and NADH to form glutamate and NAD. At 340nm, the rate of drop in absorbance is measured and is directly proportional to the amount of urea in the sample, given in mg/dl.

Urea + $H_2O \xrightarrow{Urease} 2NH_3 + CO_2$

NH_3 + α-Kg + NADH $\xrightarrow{GLDH}$ Glutamate + NAD

α-KG = α- Ketoglutarate

GLDH= Glutamate dehydrogenase

Alkaline phosphatase (ALP)

The kinetic method recommended by the International Federation of Clinical Chemistry (1999) was used to quantify plasma alkaline phosphatase. The substrate for this technique is 4-nitrophenyl phosphate. The following reaction is catalysed by ALP present in the sample under optimal circumstances.

$$AMP + 4\text{-}NPP + H_2O \xrightarrow{ALP,\ Mg^{2+}/Alkaline\ pH} 4\text{-nitrophenol} + Phosphate$$

4-nitrophenol exhibits a bright yellow colour at the reaction's optimal pH. A metal ion buffer system is also included in the reagent to ensure that the optimal concentrations of Zinc and Magnesium are maintained. Other potentially inhibiting ions that may be present can be chelated using the metal ion buffer. The rate of increase in absorbance at 405 or 415 nm, which is proportional to the activity of ALP in the plasma and represented as IU/L, was monitored.

Creatine kinase (CK)

Creatine kinase in the plasma was estimated by the method recommended by International Federation of Clinical Chemistry (IFCC). Creatine kinase in the sample catalyses the below given reaction.

$$\text{Creatine phosphate} + ADP \xrightarrow{\text{Creatine kinase}} \text{Creatine} + ATP$$

$$\text{D-Glucose} + ATP \xrightarrow{\text{Hexokinase}} ADP + \text{Glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow{G6PDH} \text{6-Phospho gluconate} + NADPH + H^+$$

G6PDH= Glucose -6- Phosphate dehydrogenase

The rate of absorbance change at 340nm is directly proportional to creatine kinase activity and expressed as IU/L.

Creatinine Level

Using ERBA Autopak kits and an ELISA reader, plasma creatine was estimated with Jaffe's reaction. The Jaffe reaction occurs when creatinine combines with alkaline picrate to generate an orange-yellow colour. The adoption of an initial rate method increased the assay's specificity. The absorbance of the orange-yellow colour produced, which is measured at 500-520nm, is directly proportional to creatinine content which is represented in mg/dl.

Total plasma protein (TPP)

Total plasma protein estimation was done using ERBA Autopak kits by Biuret End Point method on Auto analyzer. In an alkaline solution, protein peptide bonds react with copper || ions to generate a blue-violet complex (Biuret reaction). With 5 or 6 pepetide linkages, each copper ion forms a complex. Iodine is employed to avoid auto-reduction of the alkaline copper complex, and tartrate is added as a stabiliser. The colour produced is proportional to the protein concentration and is measured at 546 nm (520-560 nm) in g/dL units.

Calcium

Moorehead and Briggs' modified O- Cresolphthalein Complexone (OCPC) technique was used to calculate plasma calcium. In an alkaline solution, OCPC interacts with calcium to generate a purple-colored complex. The intensity of the purple colour produced is proportional to the calcium concentration and is photometrically measured at 575nm for maximum absorption.

Phosphorus

The ammonium molybdate method, in which phosphorus reacts with ammonium molybdate in the presence of strong acids to create phosphomolybdate, is used to detect inorganic phosphorus in plasma. This formation is measured at 340nm and is proportional to the amount of inorganic phosphorus present.

Albumin (ALB)

Albumin is a significant plasma protein that was tested photometrically with maximum absorbance at 625nm using the BCG dye method, in which albumin interacts with BCG at pH 4.2 to produce a blue green colour proportionate to the amount of albumin present in plasma.

BCG = Bromocresol green

Glucose

Glucose was measured in plasma sample using Trinder's method in which glucose undergoes below given reactions:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + 4\text{HBA} + 4\text{AAP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 2H_2O$$

4AAP: 4-Aminoantipyrine

4HBA: 4-Hydroxy benzoic acid

The intensity of pink colored quinoneimine complex is proportional to concentration of glucose present in sample and can be measured at 505nm.

HDL-Cholesterol

The Phosphotungstic Acid End Point Method was used to estimate plasma HDL-cholesterol using ERBA Autopak kits on an ELISA reader. In this approach, phosphotungstate is used to precipitate chylomicrons, LDL, and VLDL (low and very low density lipoproteins) from plasma in the presence of divalent cations like magnesium. The HDL cholesterol in the supernatant is unaffected and measured with the ERBA cholesterol reagent. The absorbance was calculated as mg/dl and measured at 505 nm or 505/670 nm.

Serum/plasma

$$\xrightarrow{\text{Phototungstate, } Mg2^{+}} \text{HDL(supernatant)} + \{(\text{LDL} + \text{VLDL} + \text{Chylomicrons})(\text{precipitate})\}$$

LDL- Cholesterol

ERBA Autopak kits were used on an ELISA reader at a wavelength of 600 nm to estimate plasma LDL-Cholesterol. The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene – glycol methyl ether (PEGME) coupled classic precipitation method, with the addition of optimum PVS/PEGME amounts and selected detergents. PVS and PEGME react with LDL, VLDL, and chylomicrones (CM), rendering LDL, VLDL, and CM inaccessible to cholesterol oxidase (CHOD) and cholesterol esterase (CHER), whereas HDL reacts with the enzymes. LDL is released from the PVS/PEGME complex when R2 containing a particular detergent is added. The released LDL reacts with enzymes to produce H2O2 which is quantified by the Trinder reaction.

$$\text{HDL} + \text{LDL} + \text{VLDL} + \text{CM} \xrightarrow{\text{PVS, PEGME}} \text{HDL} + (\text{LDL} + \text{VLDL} + \text{CM}).\ \text{PVS/PEGME}$$

$$\text{HDL} \xrightarrow{\text{CHOD, CHER}} \text{fatty acid} + H_2O_2$$

$$(\text{LDL} + \text{VLDL} + \text{CM}).\text{PVS/PEGME} \xrightarrow{\text{Detergent}} \text{LDL} + (\text{VLDL} + \text{CM}).\text{PVS/PEGME}$$

$$\text{LDL} \xrightarrow{\text{CHOD, CHER}} \text{fatty acid} + H_2O_2$$

$$2H_2O_2 + 4\text{-AA} + \text{TODB} \xrightarrow{\text{Peroxidase}} \text{quinone} + 5H_2O$$

Total cholesterol

Total amount of cholesterol in plasma was measured by modified Roeschlau's technique. The estimation involves the following enzyme catalyzed reactions:

$$\text{Cholesterol ester} \xrightarrow{\text{CE}} \text{Cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

CE: Cholesterol esterase

CHOD: Cholesterol oxidase

4AAP: 4-Aminoantipyrine

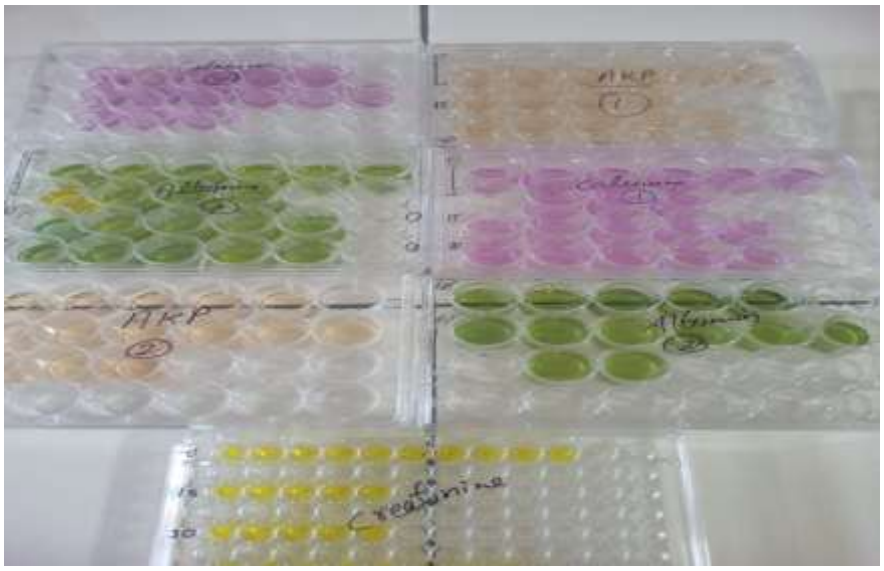

Fig. 16: Estimation of Biochemical parameters

3.9. Assessment of Antioxidant parameters

3.9.1. Preparation of RBC lysate

The blood was collected in heparinised vials and gently mixed to avoid any clot formation. The collected samples were centrifuged for 15 min at 3000 rpm. Plasma as well as buffy coat were removed. The resulting erythrocyte pellet was washed thrice with PBS (pH 7.4). The washed erythrocyte pellet was resuspended in distilled water and kept at -20°C until further analysis. The 1:10 dilution of erythrocyte lysate (10%) was used for estimation of oxidative stress indicators and

membrane enzymes. PBS (pH 7.4) was formed by dissolving KCl (0.2g), NaCl (8g), KH_2PO_4 (0.2g) and Na_2HPO_4 (0.94g) in about 800 ml of distilled water and the volume was made to 1 litre with distilled water.

Levels of Catalase (CAT), Superoxide dismutase (SOD), Blood glutathione (GSH) and Lipid peroxidase (LPO) were measured.

3.9.2. Estimation of SOD

Madesh and Balsubramaniam described a method for estimating superoxide dismutase (SOD) (1998). It entails the production of superoxide via pyrogallol autooxidation, as well as the suppression of superoxide-dependent reduction of the tetrazolium dye MTT (3-(4-5 dimethyl thiozol 2-yl) 2, 5- diphenyl tetrazolium bromide) to its formazan at 570 nm. The reaction was stopped by adding dimethyl sulfoxide (DMSO), which aids in the solubilization of the produced formazan. The colour developed remained steady for several hours and was measured in SOD Units (one unit of SOD equals the quantity of (g) protein required to prevent MTT decrease by 50%).

Reagents:

1. Phosphate buffer saline (PBS) solution (pH= 7.4)
2. MTT solution: 1.25 mM in disteilled water. 2.58 MTT was dissolved in distilled water to make 5 ml solution.
3. Pyrogallol solution: 100µM in distilled water. 12.6 mg pyrogallol was dissolved in 10 ml of distilled water. One ml of this solution was diluted to 100 ml of distilled water.
4. Dimethyl sulfoxide (DMSO).

Procedure:

0.65 ml of PBS and 30µl of MTT solution were taken in sample, control and blank tubes. Then 10 µl of RBC lysate / tissue homogenate were added in sample test tube. Then 75 µl of pyrogallol solution was added to sample, control and blank tubes. The tubes were incubated for 5 min at room temperature. Then after 5 min. 0.75 ml of DMSO was added in all the three tubes. Finally 10 µl of RBC lysate / tissue homogenate was added to control tube. These solutions were thoroughly mixed and

read absorbance at 570 nm. The whole procedure is summarized in the form of protocol given below:

Superoxide dismutase estimation protocol

	Sample	**Control**	**Blank**
PBS	0.65 ml	0.65 ml	0.65 ml
MTT	30µl	30µl	30µl
RBC lysate/ tissue homogenate	10 µl	-	-
Pyrogallol	75 µl	75 µl	75 µl
The sample, control and blank tubes were incubated for 5 min at room temp.			
DMSO	0.75 ml	0.75 ml	0.75 ml
RBC lysate/ tissue homogenate	-	10 µl	-

The reagents were added in the sample, control and blank as shown above. Mixed properly and absorbance was read at 570 nm.

Y % = (OD of test/ OD of control)× 100

SOD (units /mg of protein) = Mg protein in 0.01 ml homogenate / Y% × 50 × DF

Where,

Y % = % Inhibition of MTT reduction by SOD protein.

DF = Dilution factor

For RBC lysate: EU/mg Hb

3.9.3. Estimation of Blood glutathione (GSH)

The activity of GSH was determined using the Beutler & Kelly method (1963). The reaction of reduced glutathione with 5, 5-dithiobis, 2-nitrobenoic acid, which can be detected spectrophotometrically, provides the basis for this approach. The produced yellow colour was detected at 412nm in comparison to a blank reagent.

Reagents:

1. m-phosphoric acid precipitation solution : Dissolve 1.67 g glacial metaphosphoric acid, 0.2 g disodium EDTA (Ethylene di-amine tetra-acetic acid) and 30 g sodium chloride in 100 ml of distilled water.
2. Phosphate solution: 0.3 M disodium hydrogen phosphate (Na2HPO4) prepared in distilled water.

3. DTNB reagent: Dissolve 40 mg 5, 5'-dithiobis (2-nitrobenzoic acid) in 100 ml of 1% aqueous sodium citrate solution.

Procedure

0.2ml of 10 tissue homogenate (prepared in phosphate buffer pH 7.4 containing 0.02 M EDTA) or RBC lysate was added to 1.8 ml of distilled water. 3.0 ml of precipitating solution was mixed. It was then allowed to stand for approximately 5 min and then centrifuged. Two ml of filtrate was added to 8.0ml of the phosphate solution. A blank was prepared with 8.0ml of the phosphate solution, 2.0ml of the diluted precipitated solution (3 parts precipitated solution + 2 parts distilled water. 1.0 ml of the DTNB reagent was added to sample and blank tubes. Standard solutions of different concentration of GSH were prepared in phosphate buffer containing 0.02 M EDTA solution. The optical density was measured photometrically at 412 nm.

The whole procedure is summarized in the form of protocol as follows:

	Sample	**Standard**	**Blank**
10% sample	0.2 ml	-	-
Reduced glutathione standard	-	-	-
Dilute precipitating solution	-	Diff. conc. of reduced glutathione (0.2ml)	2.0ml
Distilled water	1.8 ml	1.8 ml	-
Precipitating solution	3.0 ml	3.0 ml	-
5 min incubation then centrifuged and supernatent was pipette out			
Supernatent	2.0 ml	2.0 ml	-
Phosphate solution	8.0 ml	8.0 ml	8.0 ml
DTNB reagent	1.0 ml	1.0 ml	1.0 ml
Read absorbance at 412 nm			

Formula from standard curve of GSH (fig 17)

y=0.0005+0.1105, where Y= absorbance, X= Conc. in micromole/ml

Conc. of GSH in micromole/g tissue = micromole/ml ×DF where DF is the dilution factor

Unit for RBC lysate: micromole/ml

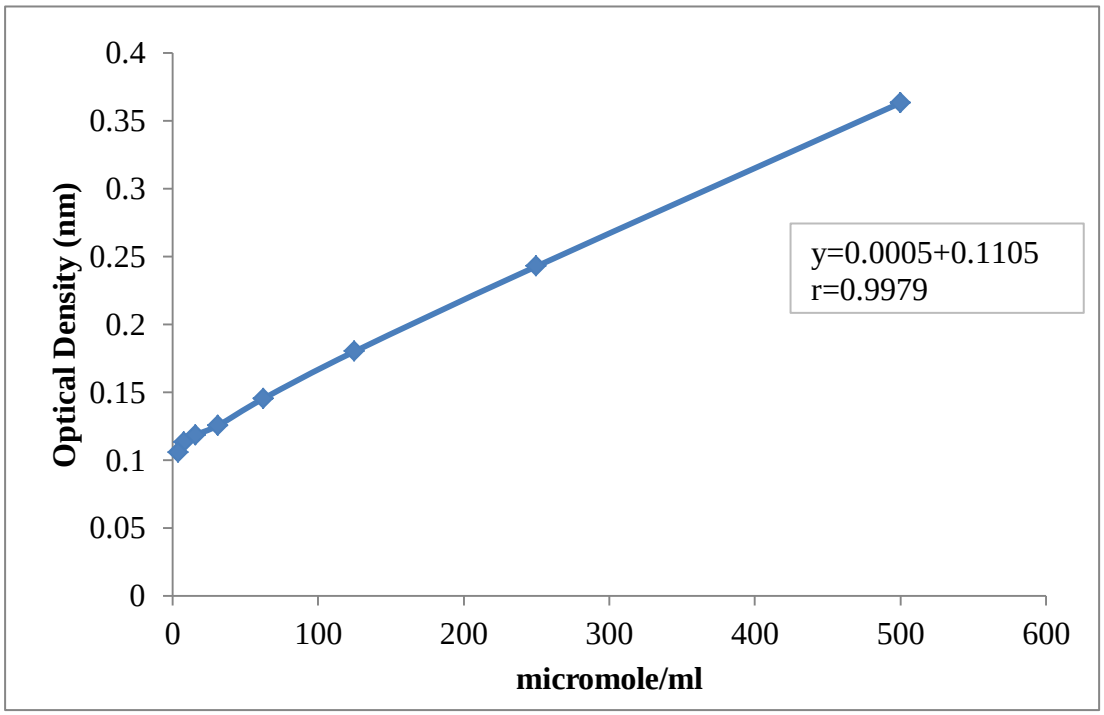

Fig. 17: Standard curve of reduced glutathione (GSH)

3.9.4. Estimation of Catalase (CAT)

Catalase (CAT) was determined by a spectrophotometric method as given by Aebi (1983). 10 % homogenate was used for measurement of catalase.

Reagents

1) Phosphate buffer (50 mM pH 7.0)

   (a) 50 mM KH_2PO_4 - 1.37 g/ 200 ml

   (b) 50 mM Na_2HPO_4 - 1.42 g/ 200 ml

   The solutions (a) and (b) were mixed in a ratio of 1:1.5 (v/v) and the pH was adjusted to 7.

2) H_2O_2 (30 mM): 0.34 ml of 30% H_2O_2 was diluted to 100 ml in water. The solution was checked at 240 nm and the concentration was adjusted to 1.5 OD using the molar extinction coefficient of H_2O_2 (freshly prepared).

Procedure

1.99 mL phosphate buffer and 10 µL homogenate were combined in a test tube, and the contents were transferred to a cuvette. The reaction was started by directly injecting 1 ml of H_2O_2 into the cuvette. At 240 nm, the optical density was measured every 30 seconds for 3 minutes against distilled water (blank).

Calculation

The activity of catalase was expressed as mM H_2O_2 utilized/min/mg protein and calculated using the formula:

$$= \frac{\Delta OD}{0.067} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times \frac{1}{\text{mg protein} \times \text{time}}$$

Unit for RBC lysate: µmole decomposed H_2O_2 min/mg Hb

3.9.5. Estimation of lipid peroxidation (LPO)

The Stocks and Dormandy (1971) method was used to perform lipid peroxidation in erythrocyte lysate and tissue homogenate.

Principle: The end product of lipid peroxidation, malondialdehyde (MDA), interacts with thiobarbituric acid (TBA) to produce a pink-colored trimethine complex with a maximum absorption wavelength of 532 nm.

Reagents:

(a) Phosphate buffered saline (0.1 M, pH 7.4).

(b) 40 mM sodium azide.

(c) 28 percent trichloroacetic acid (TCA).

(d) 1 percent thiobarbituric acid (TBA).

(e) 40 mM hydrogen peroxide.

Procedure:

In the test tube, 1 ml of 10% RBC/tissue homogenate, 1 ml of 40 mM hydrogen peroxide, and 0.1 ml of sodium azide were added and incubated for 1 hour at 37ºC. After incubation, the total volume in each tube was increased to 4 ml with PBS, and the reaction was stopped with 2 ml of ice cold TCA. The tubes were centrifuged for 15 minutes at 3000 rpm. 1 ml TBA was added to 4 ml supernatant, and tubes were placed in a boiling water bath for 15 minutes. After cooling the contents of the tubes to ambient temperature, the optical density was measured at 532 nm against a blank (no H2O2 was supplied). 1 mL PBS was used to make the blank.

The values were expressed for lysates as nmoles of MDA produced/g Hb/h using a molar extinction coefficient of pure MDA as 1.56 X 10^5 (Esterbauer et al., 1982).

For tissues the amount of LPO was expressed as nanomoles of MDA produced/ g protein of tissue and for RBC lysate, LPO quantity was expressed as nanomoles of MDA produced/grams Hb/hour

$$\text{LPO (nm MDA gm}^{-1}) = \frac{\text{OD}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Amount of sample taken}} \times 10^9$$

Where OD: Optical Density, EC: Extinction coefficient.

3.10. Assessment of haematological parameters

Table 3 shows haematological parameters that were analysed from the blood collected in EDTA vials at day 0, day 30 and day 60 of experiment. About 3 ml blood was taken in EDTA vials to avoid coagulation.

Table 3: Haematological parameters assayed with units and method used

Parameters	**Units**	**Reference / Method**
Haemoglobin (Hb)	g/dl	Benjamin (1985)
Packed cell volume (PCV)	%	Benjamin (1985)
Total leucocyte count (TLC)	X 10^3/mm^3	Benjamin (1985)
Differential leucocyte count (DLC)	%	Benjamin (1985)
Total erythrocyte count (TEC)	X 10^6/mm^3	Benjamin (1985)
Mean corpuscular volume (MCV)	Fl	Benjamin (1985)
Mean corpuscular haemoglobin (MCH)	Pg/dl	Benjamin (1985)
Mean corpuscular haemoglobin concentration (MCHC)	g/dl	Benjamin (1985)

The erythrocyte indices

a) Mean Corpuscular Volume (MCV) expresses the average volume of individual erythrocyte and is calculated from the formula:

$$\text{MCV (fl)} = \frac{\text{Packed Cell Volume (\%)}}{\text{Total erythrocyte count (million /µl)}} \times 10$$

b) Mean Corpuscular Haemoglobin (MCH) is the amount of haemoglobin in the average erythrocyte and is calculated from the formula:

$$\text{MCH (pg/dL)} = \frac{\text{Hemoglobin (g/dL)}}{\text{Total erythrocyte count (million /µl)}} \times 10$$

c) Mean Corpuscular Haemoglobin Concentration (MCHC) is the concentration of haemoglobin in the average erythrocyte or ratio of weight of haemoglobin to volume in which it is contained and is calculated from the formula:

$$\text{MCHC (pg/dL)} = \frac{\text{Hemoglobin (g/dL)}}{\text{Packed Cell volume (\%)}} \times 100$$

3.11. Statistical analysis

The data was evaluated statistically using analysis of variance (one way ANOVA) within group with time and independent sample t-test for analyzing between the group parameters, both at 5% significance ($p \leq 0.05$) with SPSS 26 software.

CHAPTER IV

RESULT AND DISCUSSION

BACKGROUND

The ban on use of antibiotics as growth promoters and feed additives around world and increased awareness of consumers regarding antibiotic resistance and residue in animal products sparked a demand for natural and safe feed additives to improve farm animal productivity (Frankic et al., 2009).

Plant-derived products have demonstrated to be natural, less toxic, and residue-free when compared to synthetic antibiotics, synthetic growth promoters, chemical feed additives, or synthetic galactagogues, and so can be considered as an alternative additive choice in animal feed. Phytoadditives, a new class of growth promoters, have gotten a lot of attention in the feed business in recent years. Although multiple papers have established the antioxidative, antibacterial, and immunity-stimulating efficiency of plant-derived compounds *in-vitro*, experimental evidence *in-vivo*, particularly in domestic animals, is still lacking (Hashemi & Davoodi, 2011).

Buffaloes (*Bubalus bubalis*) are among the domesticated livestock that provide milk to millions of people throughout the world, including in underdeveloped countries like India. Buffalo milk production is crucial for economic development, rural livelihood, and poverty alleviation in our country because it is the backbone of the farmer's economy. Qualities like better disease resistance due to hardy nature, better adaptability to climatic conditions, greater sustainability on poor quality pasture due to better digestibility, faster growth and body weight gain in buffaloes make them more versatile and hence, make them best choice to focus on for augmentation of milk production using natural resources for better food security all over the world (Naveena & Kiran, 2014).

Keeping in mind the research gaps, present clinical trial was designed and implemented to investigate the efficacy of a novel polyherbal formulation containing thirteen ingredients on production performance of lactating buffaloes. Twenty lactating buffaloes were classified into two groups of ten buffaloes each, on the basis of milk production, stage of lactation, parity and body weight for their homogeneity. Feeding in both the groups was as per the feeding schedule of GADVASU dairy farm.

Buffaloes without any supplemention served as control group or Group 1. Group 2 was supplemented with herbal formulation @20 grams/100 kg body weight, two times in a day (BID) mixed with concentrate feed for 45 days. The animals were also observed for one month of post feeding to find out the duration for which the treatment was effective.

Formulation was evaluated for its effect on production performance of animal by estimating various production parameters and it was also evaluated for its safety on general health of animal by estimating various biochemical, antioxidant and haematological parameters. Along with that physiological status of animal was observed during study period for any change in heart rate, respiratory rate, temperature, and body weight.

4.1. Effect of polyherbal formulation on body weight

Table 4 and Fig. 18 represent body weight of experimental buffaloes on day 0, 30 and 60 from start of the experiment in control and treatment groups respectively. Buffaloes in control group showed decrease in body weight which is a physiological response to production stress causing negative energy balance. The animals in treatment group showed maintenance of body weight (623±24.3 kg to 638.6±9.63 kg) as compared to animals in control group that showed a slight decrease (636.5±24.56 to 631.2±18.75 kg) on day 60 but change between both groups was non-significant. In line of similar observations, Thakur et al. (2006) reported that feeding polyherbal formulation to crossbred cows for 90 days @210 grams/day revealed no changes in live weights of animals. The findings are also consistent with those of Jain et al. (2011), who found that after feeding crossbred cattle with polyherbal supplement for 90 days, body weight was sustained in the test group but declined in the control group. The current study's findings could be attributed to the good impact of supplementation on animal health and nutrient utilisation. Kumar et al. (2011) found that although there was no statistically significant difference in body weight between the treatment and control groups in lactating buffaloes but the treated animals gained more weight over time as compared.There are no more clinical reports on effect for herbal supplements on weight gain of lactating buffaloes

Sumanth & Narasimharaju (2011) and Sahoo et al. (2017) observed that after feeding polyherbal supplementation to lactating rats, their body weight had increased

by the end of experiment as compared to control group. Patel et al. (2013) recorded significant increment in the mean dry matter intake (DMI) (kg/day) and DMI % in buffaloes fed a polyherbal formulation which had some ingredients common with the novel preparation used in present study such as Shatavari, Jeera and Sowa. Difference in results might be due to different ingredients, species, dose rates and environment factors like stress.

Table 4: Body weight of experimental animals in different groups

Group	Body Weight (kgs)		
	0 DAY	30 DAYS	60 DAYS
Control	636.5 ± 24.56^{Aa}	635.3 ± 12.46^{Aa}	631.2 ± 18.75^{Aa}
Treatment	623.0 ± 24.30^{Aa}	630.0 ± 25.84^{Aa}	638.6 ± 9.63^{Aa}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

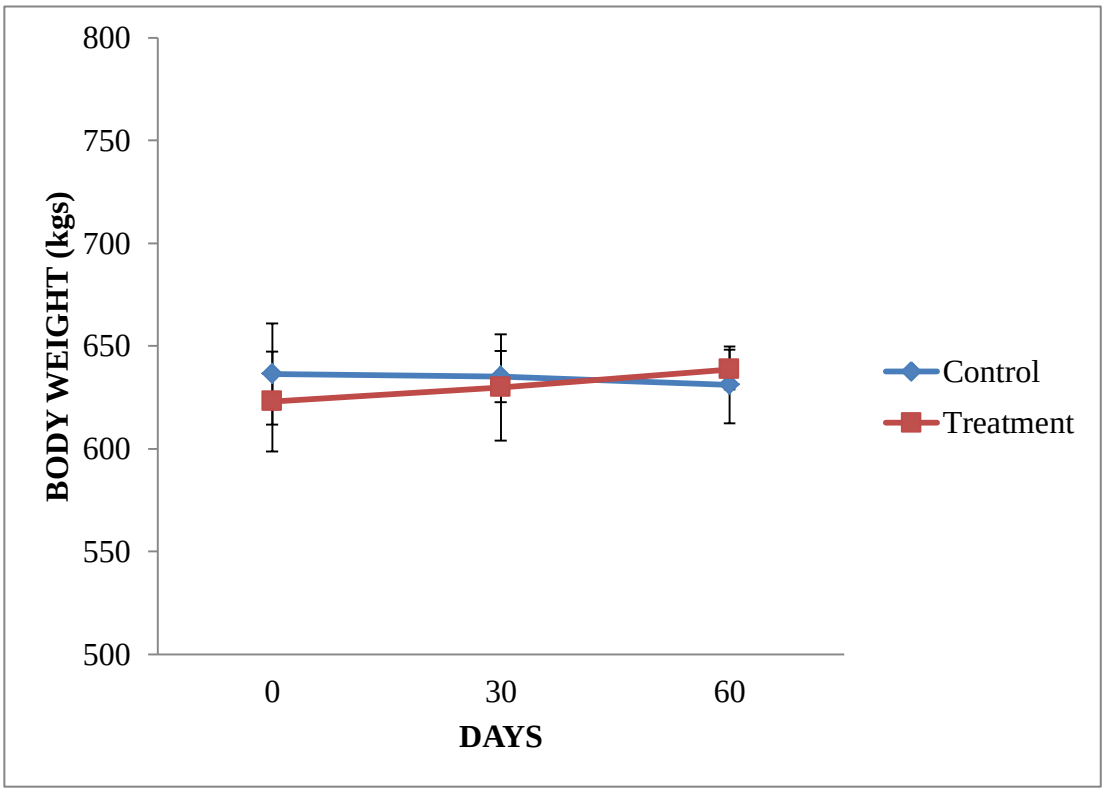

Fig. 18: Average body weight of animals in different groups

4.2. Effect of polyherbal formulation on physiological parameters in animals

Table 5 represents physiological parameters of animals in control and treatment groups measured on day 0, 30 and 60 from start of the trial. Respiration rate did not show any significant difference between the groups or within groups over time. Same results were followed by temperature and heart rate of animals in both the groups which did not show any significant difference amongst the groups or with time. These results indicate that polyherbal formulation supplemented to animals in the treatment group do not cause any harm to physiological status or general health of animals and did not cause any stress to animals. Results match with those reported by Dash et al. (1972) who concluded that after feeding polyherbal supplements, respiratory rates, rectal temperatures, and heart rates of all cows were within normal physiological ranges throughout the experiment. In contrast to findings of current study, Mirzaei (2010) found rise in rectal temperature as well as respiration rate in goats supplemented with a polyherbal formulation containing mixture of five different herbs. Li et al. (2020), after supplementing feed of Murrah buffaloes with mulberry leaf flavonoids, observed no significant change in respiration rate or heart rate but found higher rectal temperature in case of supplemented groups.

Table 5: Physiological parameters of animals in different groups

Group	0 Day	30 Days	60 Days
Respiration Rate (per minute)			
Control	12.60 ± 0.62^{Aa}	12.10 ± 0.35^{Aa}	12.30 ± 0.50^{Aa}
Treatment	12.80 ± 0.73^{Aa}	12.10 ± 0.43^{Aa}	12.00 ± 0.33^{Aa}
Temperature(^{0}F)			
Control	100.76 ± 0.14^{Aa}	101.08 ± 0.20^{Aa}	101.40 ± 0.29^{Aa}
Treatment	101.08 ± 0.14^{Aa}	101.00 ± 0.19^{Aa}	101.06 ± 0.16^{Aa}
Heart rate (beats per minute)			
Control	57.80 ± 2.12^{Aa}	58.60 ± 1.63^{Aa}	58.00 ± 2.37^{Aa}
Treatment	55.20 ± 1.07^{Aa}	56.60 ± 0.94^{Aa}	56.00 ± 2.13^{Aa}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

4.3. Effect of polyherbal formulation on milk yield

Average milk yield record of buffaloes in control as well as treatment group on day 0, 15, 30, 45, 60 and 75 are represented in Table 6 and Fig.19. After the completion of experimental time period i.e. 75 days, it was observed that buffaloes in control group saw a loss in average milk yield by 14.25% (9.47±1.01 on day 0 to 8.12±1.11 on day 75) whereas decrease in average milk yield among animals fed with polyherbal supplementation was 10.20% (11.47±1.17 on day 0 to 10.3±0.49 on day 75) Decrease in milk yield with advancing lactation length is a natural phenomenon. Results showed that there was no significant change seen in milk yield due to the supplementation given to animals in treatment group.

On the other hand, Ramesh et al. (2000) reported that total milk yield of lactating animals was incremented as compared to control animals in declining phase of their lactation time after feeding Galactin, a commercial polyherbal formulation. Increase in milk yield was also reported by Thakur et al. (2006) who recorded at least 10% increment in the milk yield of lactating cows in treatment group fed with polyherbal supplement at dose rate of 10 grams per animal per day for 90 days whereas results were in line to those reported by Jain et al. (2011) who stated that feeding herbal supplement at 100 parts per million of daily ration to crossbred cows for 90 days did not affect their milk yield at all but only improved ruminal digestion. Lee et al. (2020) reported improvement in milk yield of Holstein Friesian cattle throughout experimental period after feeding them with concoction of herb extracts. 19.67 percent improvement in average milk yield among treatment animals as compared to animals in control was reported by Meena et al. (2020) after feeding 50 grams Shatavari root powder per animal per day for 60 days. Significant increase in milk yield of lactating cattle after feeding Shatavari roots was reported by Muwal et al. (2020) as well. Alvarez et al. (2020) reported that adding phyto-additives did not directly increase milk yield but resulted in higher income by reducing animal health costs which might have occurred due to metabolic disturbances in animal body because of production stress. Difference in results of this study can be attributed to animals in different stages of lactation during the trials and different dose rates for individual ingredients.

Table 6: Average fortnightly milk yield of animals in different groups

Group	Milk yield (kgs)					
	0 Day	15 Days	30 Days	45 Days	60 Days	75 Days
Control	9.47± 1.01^{Aa}	9.58± 1.51^{Aa}	9.15± 1.45^{Aa}	8.88± 1.1^{Aa}	8.62± 0.98^{Aa}	8.12± 1.11^{Aa}
Treatment	11.47± 1.17^{Aa}	10.65± 0.75^{Aa}	10.80± 0.62^{Aa}	10.14± 0.49^{Aa}	10.42± 0.48^{Aa}	10.30± 0.49^{Aa}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

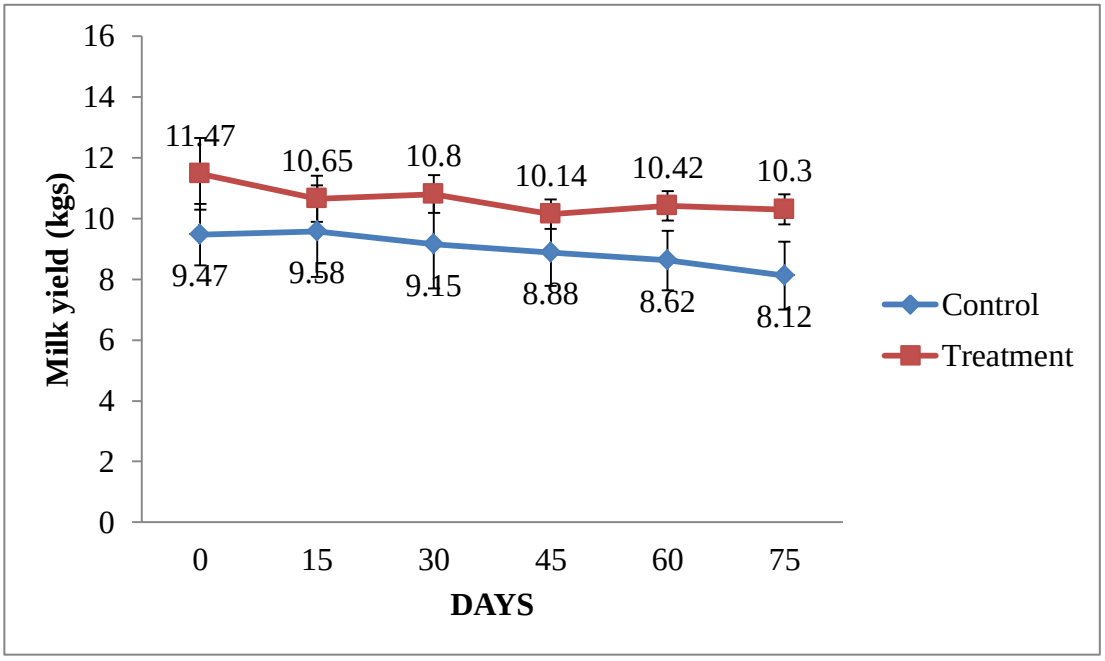

Fig. 19: Average fortnightly milk yield of animals in different groups

4.4. Effect of polyherbal formulation on milk composition

Milk composition (fortnightly average) of animals in control and treatment groups is given in Table 7. Different milk constituents over period of time in buffaloes of control and treatment groups are exhibited in fig.20 to fig.22. There was no significant difference seen in any of the milk composition parameters except milk protein and total solids that showed significant increment within treatment group over time. The highest value was seen on day 60 for both milk protein (3.77±0.04 %) as well as total solids (18.09±0.51 %).

Table 7: Average milk constituents values of animals in different groups

Group	0 Day	15 Days	30 Days	45 Days	60 Days	75 Days
Fat (%)						
Control	7.81±0.43^{Aa}	7.75±0.29^{Aa}	7.91±0.37^{Aa}	7.97±0.10^{Aa}	7.65±0.24^{Aa}	7.45±0.40^{Aa}
Treatment	7.69±0.17^{Aa}	7.59±0.29^{Aa}	7.95±0.11^{Aa}	8.00±0.19^{Aa}	7.90±0.44^{Aa}	7.81±0.19^{Aa}
Solid not fat (SNF) (%)						
Control	10.04±0.11^{Aa}	10.11±0.15^{Aa}	10.11±0.10^{Aa}	10.24±0.15^{Aa}	10.30±0.12^{Aa}	10.17±0.05^{Aa}
Treatment	9.91±0.11^{Aa}	10.01±0.09^{Aa}	10.00±0.09^{Aa}	10.03±0.12^{Aa}	10.19±0.10^{Aa}	10.15±0.07^{Aa}
Specific gravity						
Control	3302.65±19.35^{Aa}	3335.65±33.79^{Aa}	3356.40±42.44^{Aa}	3400.65±37.98^{Aa}	3370.40±45.79^{Aa}	3323.35±28.34^{Aa}
Treatment	3316.35±36.39^{Aa}	3325.25±21.87^{Aa}	3369.20±16.34^{Aa}	3324.90±30.56^{Aa}	3371.10±43.73^{Aa}	3339.75±36.42^{Aa}
Protein (%)						
Control	3.63±0.06^{Aa}	3.70±0.04^{Aa}	3.75±0.07^{Aa}	3.81±0.08^{Aa}	3.76±0.06^{Aa}	3.65±0.06^{Aa}
Treatment	3.65±0.03^{Aa}	3.69±0.02^{ABa}	3.72±0.02^{ABa}	3.69±0.03^{ABa}	3.77±0.04^{Ba}	3.69±0.04^{ABa}
Lactose (%)						
Control	5.54±0.06^{Aa}	5.59±0.08^{Aa}	5.56±0.08^{Aa}	5.64±0.05^{Aa}	5.62±0.03 ^{Aa}	5.58±0.04^{Aa}
Treatment	5.55±0.07^{Aa}	5.61±0.05^{Aa}	5.62±0.03^{Aa}	5.58±0.06^{Aa}	5.64±0.05 ^{Aa}	5.64±0.07^{Aa}
Total solids (%)						
Control	17.85±0.50^{Aa}	17.85±0.40^{Aa}	18.18±0.45^{Aa}	17.17±0.34^{Aa}	18.35±0.59^{Aa}	17.63±0.43^{Aa}
Treatment	16.87±0.37^{Aa}	17.59±0.36^{ABa}	17.15±0.23^{ABa}	17.43±0.37^{ABa}	18.09±0.51^{aB}	17.31±0.35^{ABa}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

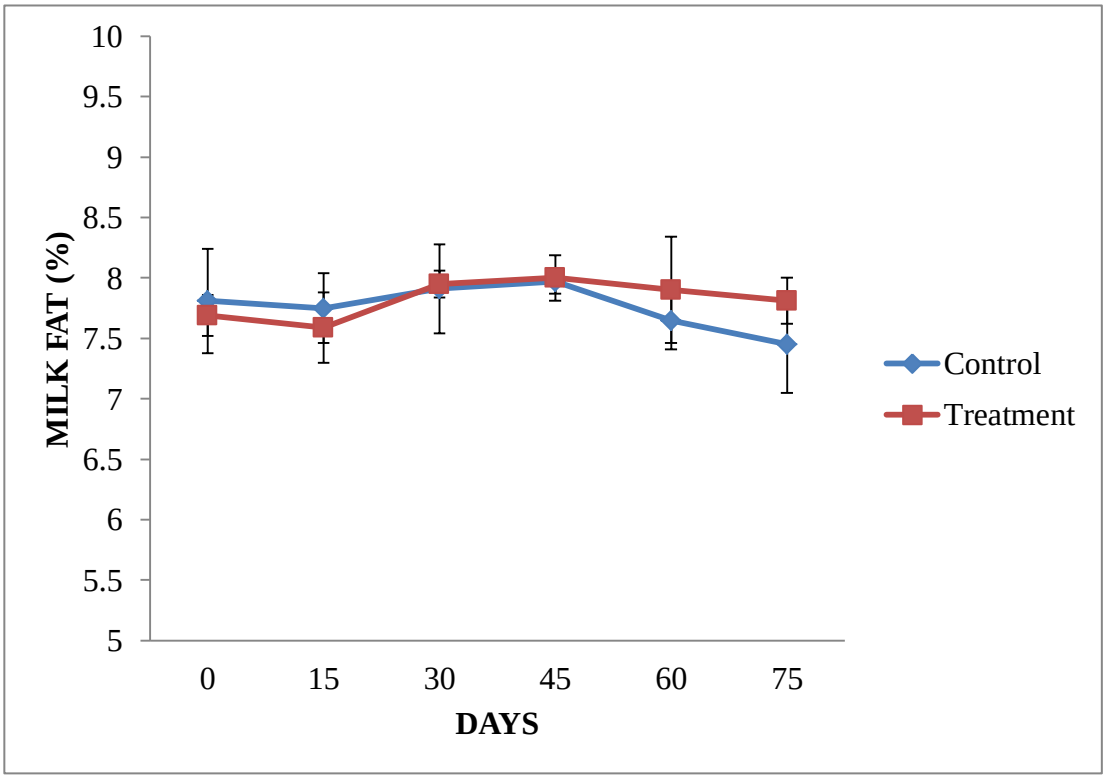

Fig. 20: Average fortnightly milk fat of animals in different groups

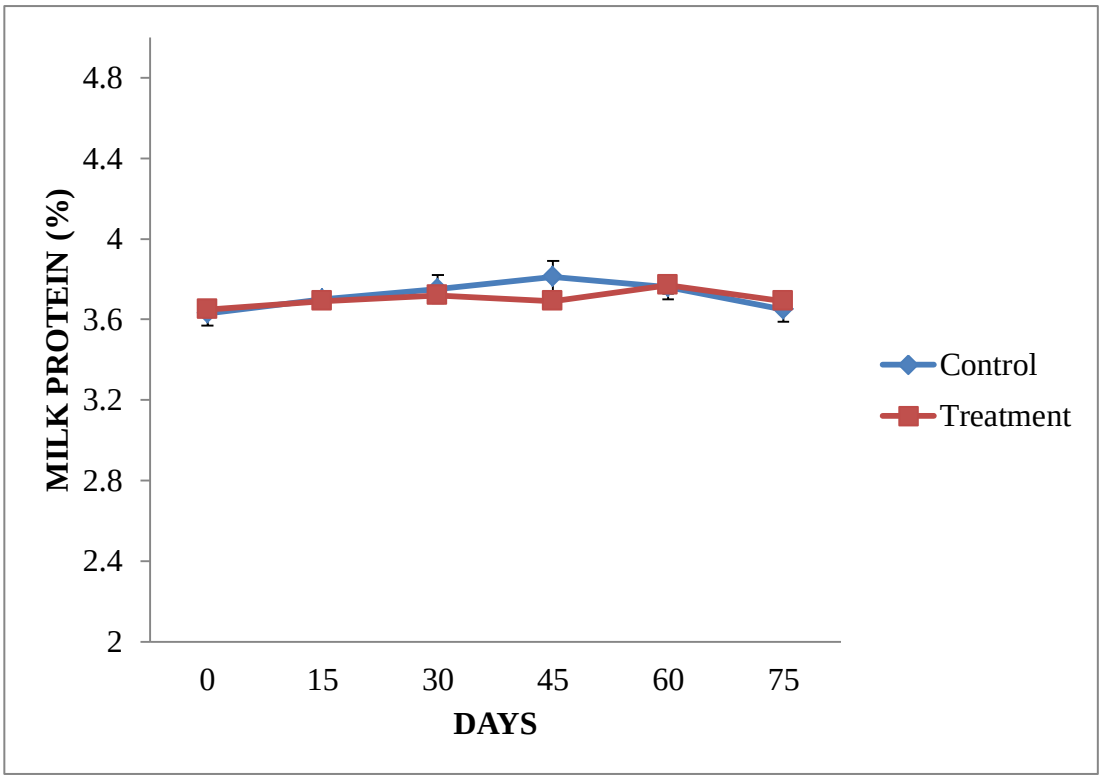

Fig. 21: Average fortnightly milk protein of animals in different groups

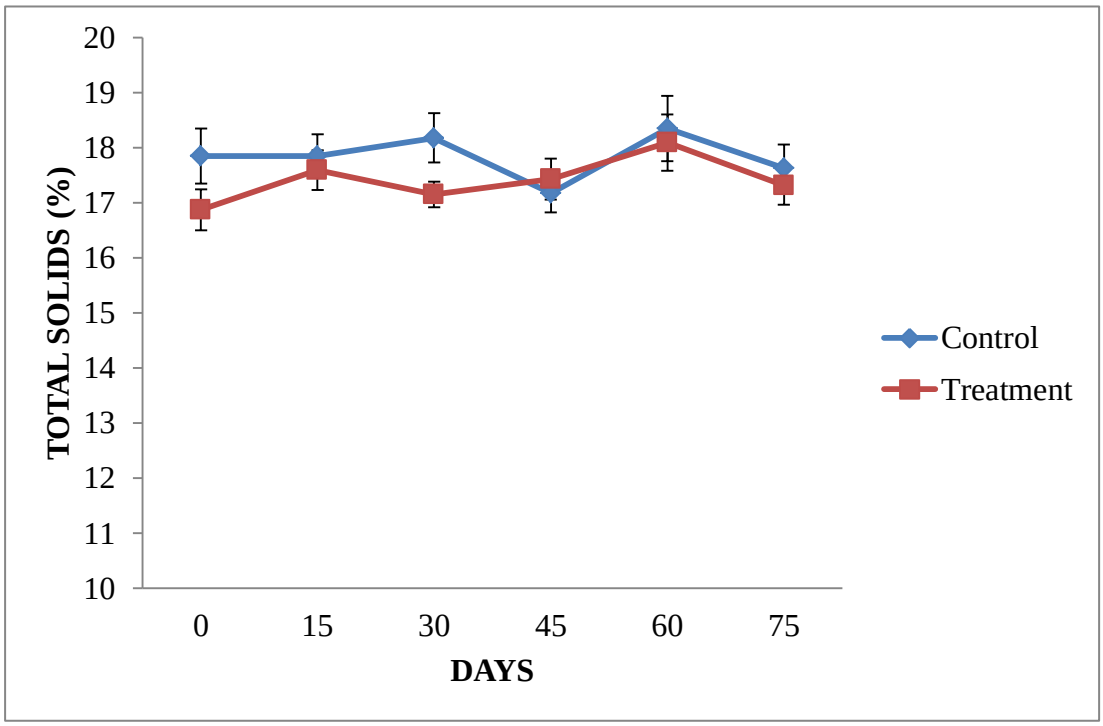

Fig. 22: Average fortnightly total solids of milk in animals of different groups

Khattab et al. (2011) recorded higher milk fat, SNF, milk protein and total ash but saw no effect on milk lactose content with herbal supplementation in diets of dairy buffaloes. Results of this study are in line with those stated by Jain et al. (2011) and Patel et al. (2017) where feeding a polyherbal supplement to dairy animals did not show any significant change in total solids, fat, SNF, milk protein or lactose content of milk between control and treatment groups.

Chandra et al. (2017) also reported that herbal additive did not change milk composition significantly but the values for all components were on higher side for treatment group animals as compared to that of control. However, Pathak (2017) reported significance increase in milk fat% and SNF% in milk of animals supplemented with herbal mixture containing Shatavari and Methi. Results are contradicting to those of Saini et al. (2018) who reported increase in all milk constituents with Shatavari supplementation for 60 days in lactating crossbred cattle.

Chandrasekar et al. (2019) reported no effect on fat and SNF after feeding a polyherbal supplementation to Karan Fries cattle for 60 days but there was significant increase in lactose and milk protein on day 60 in treatment group as compared to control group.

4.4. Effect of polyherbal formulation on milk quality parameters

Table 8 represents average fortnightly changes over time in somatic cell count (SCC), electrical conductivity (EC) and milk pH of buffaloes in control and treatment groups respectively. Somatic cell count ($\times 10^3$ cells/ml milk) saw an increase in animals of control group from 132.9±8.24 on day 0 to 161.3±5.43 on day 75 (Table 8) whereas there was a significant decrease in SCC within treatment group as well as significant decrease in SCC of treatment group as compared to that of control group from 45 days onwards. SCC in total saw a significant decrease from 155.1±13.77 on day 0 to 79.8±6.81 on day 75. Fig. 23 presents the trend in SCC over time in both the groups graphically.

Present results indicate that the tested herbal preparation possess therapeutic and protective properties for mammary glands and hence, can be used to decrease incidence of mammary diseases in high producing dairy animals. Results are in line with those stated by Sharma et al. (2014) where lactating Karan Fries crossbred cows were supplemented with a polyherbal supplementation at the dose rate of 200–250 mg/kg body weight and as a result, it reduced stress during periparturient phase and improved health of udder, indicated by decreased somatic cell count in treatment groups as compared to control. Similar findings presented by Hashemzadeh et al. (2014), stating that supplementing dairy animals having moderate to high SCC with a phytobiotic rich herbal mixture resulted in decrease in somatic cell score of animals

Increased somatic cell score (SCS) is generally considered as a sign of udder inflammation and generally linked to bacterial infestation in mammary glands. Udder infections are one of the most important health problems in lactating animals and cause for the most antibiotic treatments in dairy animals. Walkenhorst et al. (2016) reported that supplementing dairy cows with herbal feed additive for around 300 days resulted in significant decrease of SCS from >3 to <3 i.e. it helped increase udder immunity.

Pandey et al. (2020) also reported significant decrease in somatic cell count ($\times 10^5$ cells/ml) in cattle suffering from sub-clinical mastitis after feeding with various doses of *Embilica officinalis* (Amla) for two weeks. Animals fed with 250 grams amla reported decrease in SCC ($\times 10^5$ cells/ml) from 5.78± 0.89 to 2.86 ± 0.95 after the treatment, group fed with 200 grams amla reported decrease from 6.25± 0.78 to 4.24 ± 0.54 and group fed 150 grams of amla showed decrease in SCC from 6.24± 1.84 to 5.16 ± 1.88 after treatment.

Table 8: Average fortnightly SCC, EC and milk pH values of animals in different groups

Group	0 Day	15 Days	30 Days	45 Days	60 Days	75 Days
SCC ($\times 10^3$ cells/ml)						
Control	132.9 ± 8.24^{Aa}	138.2 ± 7.36^{Aa}	136.4 ± 8.81^{Aa}	148.8 ± 14.78^{aA}	156.7 ± 7.79^{aA}	161.3 ± 5.43^{aA}
Treatment	155.1 ± 13.77^{Ca}	141.9 ± 4.8^{BCa}	127.5 ± 4.58^{Ba}	102.7 ± 4.99^{bA}	93.7 ± 7.71^{bA}	79.8 ± 6.81^{bA}
EC (mS/cm)						
Control	1.66 ± 0.05^{Aa}	1.91 ± 0.07^{Ba}	2.18 ± 0.05^{aC}	2.19 ± 0.06^{aC}	2.23 ± 0.07^{aC}	2.05 ± 0.09^{aBC}
Treatment	1.68 ± 0.04^{Ba}	1.72 ± 0.08^{Ba}	1.55 ± 0.12^{bBC}	1.49 ± 0.10^{bAB}	1.29 ± 0.10^{bA}	1.32 ± 0.07^{bA}
Milk pH						
Control	6.76 ± 0.01^{aA}	6.72 ± 0.02^{Aa}	6.71 ± 0.01^{Aa}	6.72 ± 0.02^{Aa}	6.68 ± 0.06^{Aa}	6.73 ± 0.02^{Aa}
Treatment	6.75 ± 0.01^{bA}	6.70 ± 0.01^{Aa}	6.70 ± 0.02^{Aa}	6.74 ± 0.02^{Aa}	6.74 ± 0.01^{Aa}	6.70 ± 0.02^{Aa}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

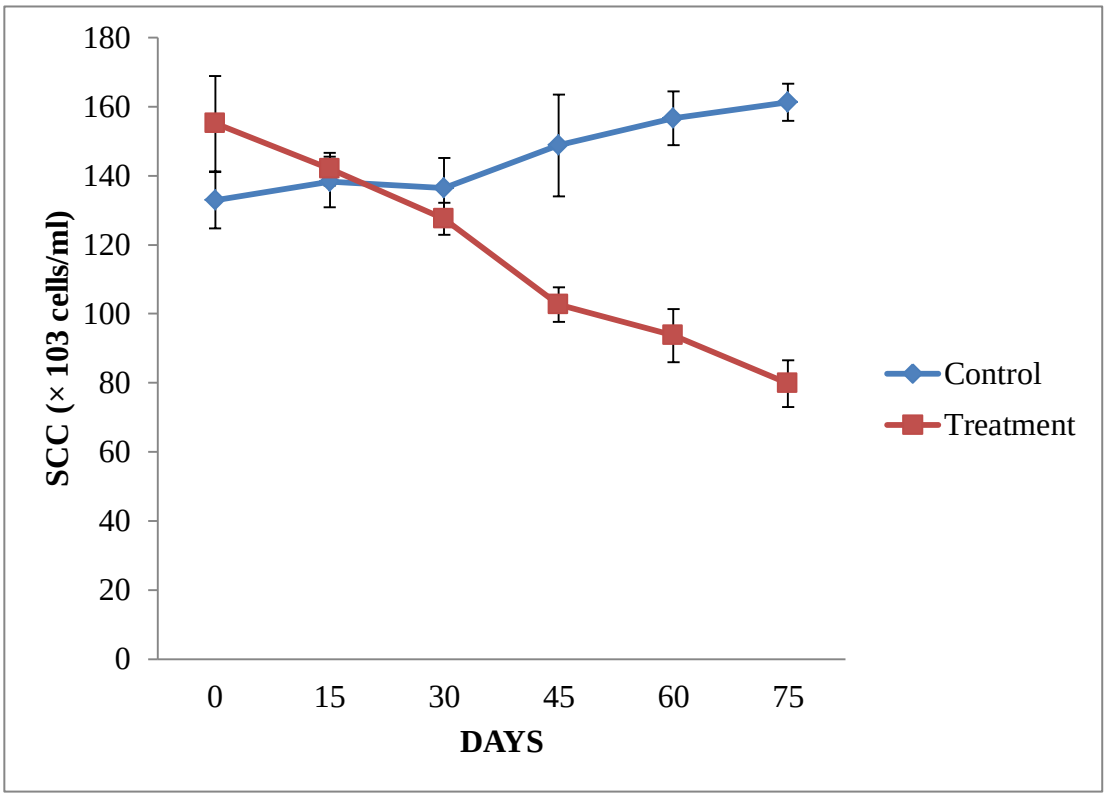

Fig. 23: Average Somatic Cell Count values of animals in different groups

Fig. 24. represents the trend in Electrical conductivity (EC) (mS/cm) over time in both the groups graphically. Kaşikçi et al. (2012) revealed electrical conductivity as a reliable method to diagnose sub-clinical mastitis after reporting a positive correlation between EC and SCC along with CMT test. Findings of present study disclose that EC of animals in treatment group shows significant decline as compared to that of animals in control group. While EC of animals in control group increase over time, EC of treatment group buffaloes show a significant decrease with advancement in time indicating improvement in udder health. EC of animals in control group incremented from 1.66±0.05 (mS/cm) on day 0 to 2.05±0.09 (mS/cm) on day 75 whereas in animals of treatment group, it declined from 1.68±0.04 (mS/cm) on day 0 to 1.32±0.07 (mS/cm) on day 75 (Table 8).

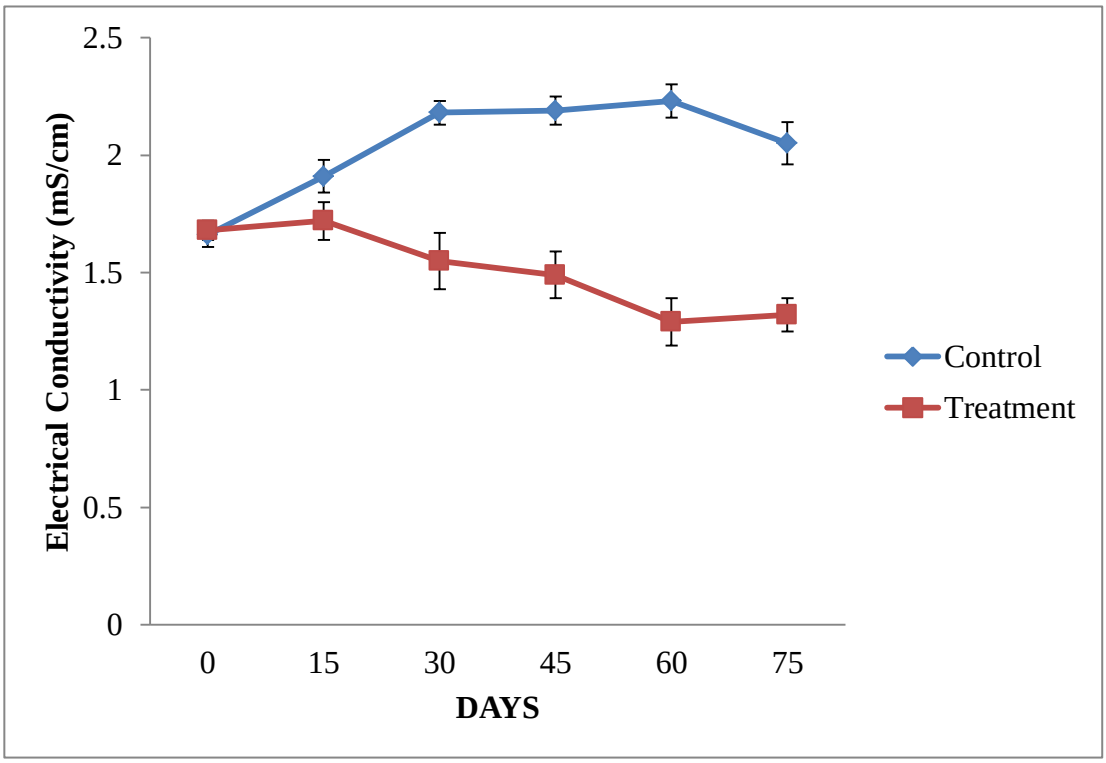

Fig. 24: Average Electrical Conductivity values of animals in different groups

Borys & Jarzynowska (2016) reported similar findings of decrease in electrical conductance or increase in electrical resistance of milk in lactating ewes after supplementing their feeds with herbal mixture containing 9 herbs. Difference in EC of milk was 3.3% between group-I (control) and group-II (supplementation @10grams/day/animal) whereas it was 8.1% between group-I and group-III (supplementation @ 20 grams/day/animal).

Findings also match to those of Salem et al. (2019) who recorded that although the values were within normal physiological range, herbal supplements significantly decreased EC and SCC values in treated animals. EC of control group was highest (5.59±0.02) whereas it was lowest in group fed with Black seed oil (5.21±0.11). SCC ($\times 10^3$ cells/ml)) results were proportional to EC as it was highest in control group (393±3.65) and lowest in black seed oil supplemented group (162±5.96). Results of current study are also in line with reports of Patel et al. (2020) where SCC of milk in treatment group showed significant decline on day 7 (156.92±34.19 x 10^3 cells/ml) i.e. 7 days after feeding a commercial herbal preparation to dairy cows having sub-clinical mastitis, in comparison to SCC in their milk on day 0 (589.33±65.85× 10^3 cells/ml) and EC significantly reduced on day 15 in comparison to day 0.

Fig.25. represents milk pH values of animals in control and treatment groups over time. pH of milk in control group ranged from 6.68 to 6.76 and it varied between 6.7 to 6.75 for treatment group. All the values were within normal physiological range. It indicates that polyherbal formulation used does not cause any acidic/alkaline change in milk of animals and is hence, safe to use.

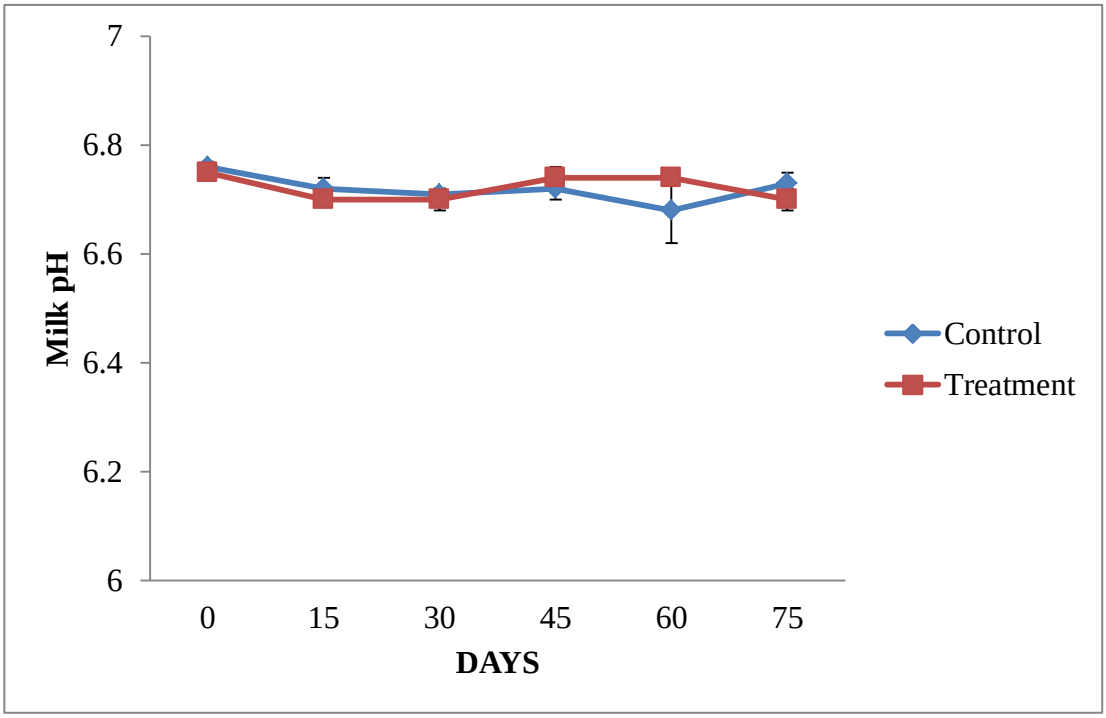

Fig. 25: Average milk pH values of animals in different groups

4.5. Effect of polyherbal formulation on sensory attributes of milk

Sensory evaluation was performed on milk samples of both groups for evaluating organoleptic properties of polyherbal formulation using 10 point hedonic scale method. Scores were given by two expert and two trained judges on fortnightly basis and their average was calculated which is given in table 9 and fig 26. When scores were compared, it was clear that polyherbal formulation did not affect any of the sensory parameters in significant amount and hence, milk supplemented is completely fit for human consumption. Though milk of buffaloes in treatment group got lesser score than the control group animals in terms of odour, the difference was not significant enough to make milk objectionable for use. The change in odour might be due to accumulation of some phyto-components of formulation in fat or water-fraction of milk.

Table 9: Average sensory evaluation scores for milk of animals in different groups

Group	0 Day	15 Days	30 Days	45 Days	60 Days	75 Days
Color						
Control	7.3 ± 0.21^{Aa}	7.1 ± 0.18^{aA}	7.3 ± 0.21^{Aa}	7.1 ± 0.23^{Aa}	7.3 ± 0.21^{Aa}	7.0 ± 0.15^{Aa}
Treatment	7.0 ± 0.3^{aA}	7.1 ± 0.18^{Aa}	6.9 ± 0.18^{aA}	6.9 ± 0.23^{Aa}	7.0 ± 0.15^{Aa}	7.0 ± 0.21^{Aa}
Appearance						
Control	7.0 ± 0.21^{Aa}	7.0 ± 0.26^{Aa}	7.2 ± 0.25^{Aa}	7.1 ± 0.18^{Aa}	7.0 ± 0.26^{Aa}	7.3 ± 0.21^{Aa}
Treatment	7.0 ± 0.21^{Aa}	7.0 ± 0.21^{Aa}	6.9 ± 0.31^{Aa}	6.8 ± 0.13^{Aa}	6.8 ± 0.25^{Aa}	6.7 ± 0.21^{Aa}
Odour						
Control	7.2 ± 0.20^{Aa}	6.9 ± 0.28^{Aa}	7 ± 0.21^{Aa}	7.1 ± 0.18^{Aa}	7.0 ± 0.26^{Aa}	7.0 ± 0.21^{Aa}
Treatment	7.0 ± 0.15^{Aa}	7.1 ± 0.23^{Aa}	7.1 ± 0.28^{Aa}	6.8 ± 0.25^{Aa}	6.6 ± 0.22^{Aa}	6.6 ± 0.27^{Aa}
Flavour						
Control	7.3 ± 0.15^{Aa}	7.1 ± 0.18^{Aa}	7.1 ± 0.23^{Aa}	7.0 ± 0.21^{Aa}	7.2 ± 0.20^{Aa}	7.3 ± 0.26^{Aa}
Treatment	7.2 ± 0.25^{Aa}	7.1 ± 0.18^{Aa}	7.0 ± 0.21^{Aa}	6.9 ± 0.18^{Aa}	6.6 ± 0.22^{Aa}	6.7 ± 0.21^{Aa}
Overall Acceptability						
Control	7.3 ± 0.15^{Aa}	7.0 ± 0.21^{Aa}	7.4 ± 0.22^{Aa}	7.1 ± 0.23^{Aa}	7.2 ± 0.20^{Aa}	7.2 ± 0.20^{Aa}
Treatment	7.2 ± 0.13^{Aa}	7.1 ± 0.23^{Aa}	7.3 ± 0.26^{Aa}	6.8 ± 0.20^{Aa}	6.6 ± 0.22^{Aa}	7.0 ± 0.26^{Aa}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

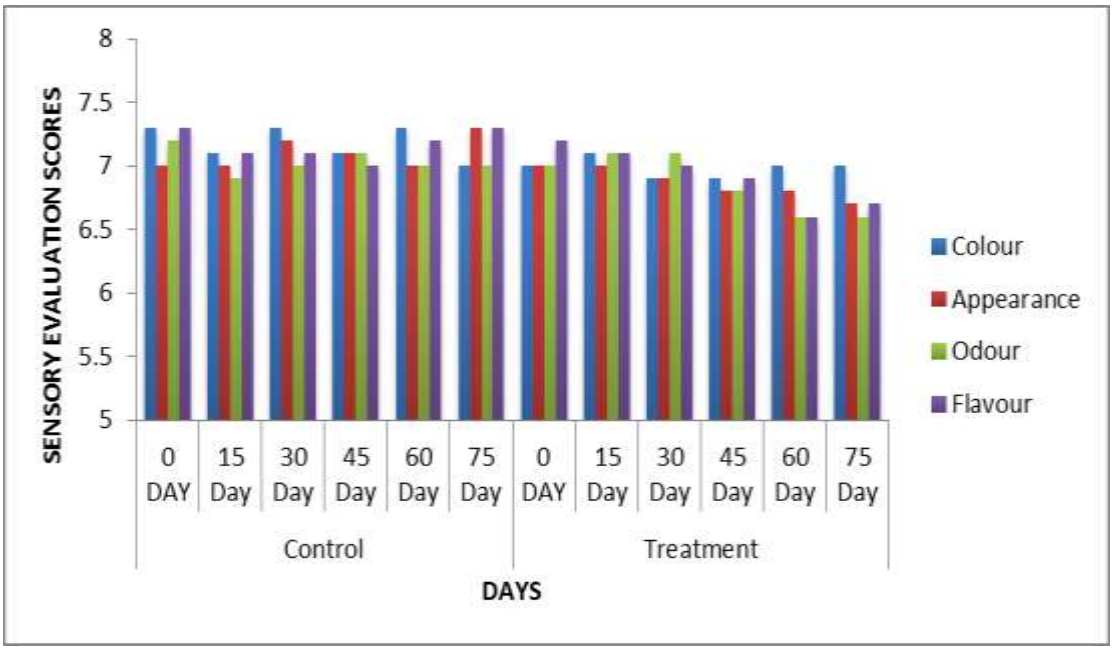

Fig. 26: Average sensory evaluation scores in different groups

Ando et al. (2001) found phyto-components of herbal mixtures in milk of lactating Holstein cows and stated that milk flavour and odour can be influenced by feeding herbs in diets of animals. The results are in agreement with Beyan (2009) & Mirzaei (2012) who reported that there was no significant change in colour, odour, flavour or texture of milk in dairy goats supplemented with herbal supplementation. Similar findings were also reported by Boutoial et al. (2013) who found that feeding lactating goats with herbal extracts (distilled and non-distilled thymus leaves) did not cause changes in sensory profile of milk as well as milk products. In contrast, Choubey et al. (2018) reported positive trend in flavour as well as palatability of buffalo milk after supplementing fenugreek seeds in their rations for 10 weeks.

4.5. Effect of polyherbal formulation on blood parameters

4.5.1. Effect of polyherbal formulation on biochemical parameters in buffaloes

Plasma biochemical parameters are a reflection of body metabolism and can give us an accurate indication towards effect of exogenous substances added in diet on body of animal. So, various biochemical parameters were estimated to find out effect of polyherbal formulation used on general health and safety of buffaloes. Values for different biochemical parameters are presented as Mean±S.E. in Table 10 and Table 11.

Calcium and Phosphorus

Plasma calcium level for animals in control group was 10.55±0.87 (mg/dL) at start of clinical trial and 10.17±0.35 (mg/dL) at the end of trial while for animals in supplemented group; it was 10.69±0.83 (mg/dL) and 11.45±0.44 (mg/dL) at start and end of trial respectively. The results indicated that in treatment group, there was significant improvement in calcium levels on day 30 and 60 ($p \leq 0.05$) when compared to buffaloes in control group (Table 10 and fig 27). Phosphorus level in plasma was 6.15±0.4 (mg/dL) and 6.62±0.42 (mg/dL) at start and end of experiment respectively for control group whereas it was 6.63±0.42 (mg/dL) and 7.28±0.42 (mg/dL) at start and end of experiment for animals of treatment group. Phosphorus level in plasma of treatment animals was significantly more (7.17±0.29 mg/dL) than those of control animals (6.15±0.37 mg/dL) on day 30 ($p \leq 0.05$) (Table 10 and fig 28). On day 60, though the difference was not significant, phosphorus was higher in treatment buffaloes than those in control group. These results are noteworthy because it is reported that post-partum calcium and phosphorus levels decline significantly as compared to their pre-partum levels (Hussain et al., 2001). There was no significant change among animals of same group over time. Similar results were mentioned by Behera et al. (2013) stating that supplementing lactating crossbred cattle with herbal root powder at the dose rate of 5 mg% daily for 60 days proved to be hypercalcemic as well as hyperphosphotemic. Roy et al. (2013) reported that supplementing different herbal galactagogues as neutraceuticals in diets of hypogalactic crossbred cattle in different groups resulted in significant increase in calcium levels of animals in all groups and there was non-significant increment in phosphorus levels as well.

Significant positive change in levels of calcium was also reported by Sanghai et al. (2017) in lactating cattle supplemented with a polyherbal galactagogue for 15 days at dose rate of 100 grams per day per animal. It was also reported that phosphorus levels were slightly improved though not by a significant amount which was attributed to better absorption of mineral from diet due to supplemented herbs. Results are also in line with those recorded by Sharma et al. (2017) who found that supplementing hypogalactic dairy cattle with an herbal mixture along with their basal diet once per day for 15 days produced a significant increment in calcium levels on 60^{th} day post-treatment and phosphorus levels on day 15^{th} and 60^{th} post-treatment.

Glucose

Glucose level (mg/dL) in buffaloes of control group was 78.18±4.83, 76.73±2.73 and 72.73±1.05 and in buffaloes of treatment group, it was 77.27±1.38, 79.55±3.54 and 87.82±4.23 on 0, 30 and 60 days from start of experiment. There was significant increment in amount of glucose in plasma on day 30 and 60 within treatment group with time (Table 10 and Fig 29). This indicated towards glucogenic properties of novel polyherbal formulation used in supplemented group.

Table 10: Values of various biochemical parameters for animals in different groups

Group	0 Day	30 Days	60 Days
Calcium (mg/dL)			
Control	10.55 ± 0.87^{Aa}	10.25 ± 0.32^{aA}	10.17 ± 0.35^{aA}
Treatment	10.69 ± 0.83^{Aa}	11.23 ± 0.27^{bA}	11.45 ± 0.44^{bA}
Phosphorus (mg/dL)			
Control	6.15 ± 0.4^{Aa}	6.15 ± 0.37^{aA}	6.62 ± 0.42^{Aa}
Treatment	6.63 ± 0.42^{Aa}	7.17 ± 0.29^{bA}	7.28 ± 0.42^{Aa}
Glucose (mg/dL)			
Control	78.18 ± 4.83^{Aa}	76.73 ± 2.73^{Aa}	72.73 ± 1.05^{aA}
Treatment	77.27 ± 1.38^{Aa}	79.55 ± 3.54^{ABa}	87.82 ± 4.23^{bB}
Total Protein (g/dL)			
Control	6.45 ± 0.16^{Aa}	6.20 ± 0.28^{Aa}	6.13 ± 0.37^{Aa}
Treatment	6.68 ± 0.14^{Aa}	6.90 ± 0.23^{Aa}	6.97 ± 0.50^{Aa}
Albumin (g/dL)			
Control	2.47 ± 0.03^{Aa}	2.49 ± 0.03^{aA}	2.61 ± 0.03^{aB}
Treatment	2.45 ± 0.03^{Aa}	2.62 ± 0.04^{bB}	2.82 ± 0.05^{bC}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

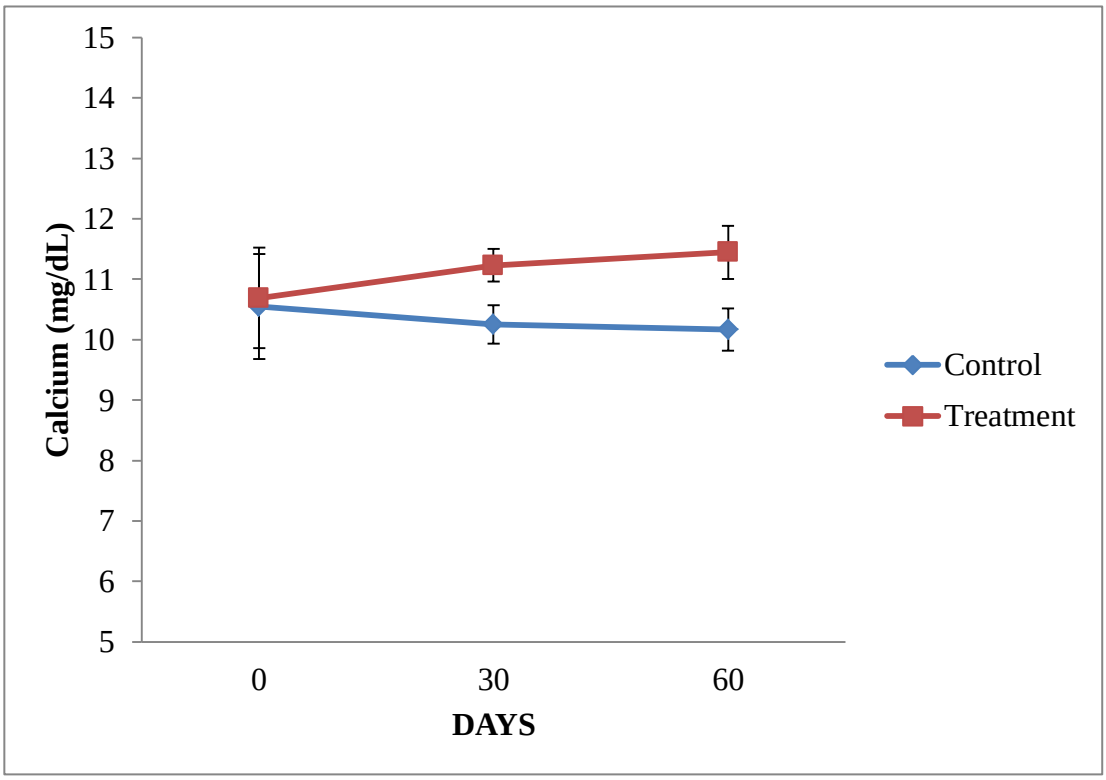

Fig. 27: Plasma calcium values in animals of different groups

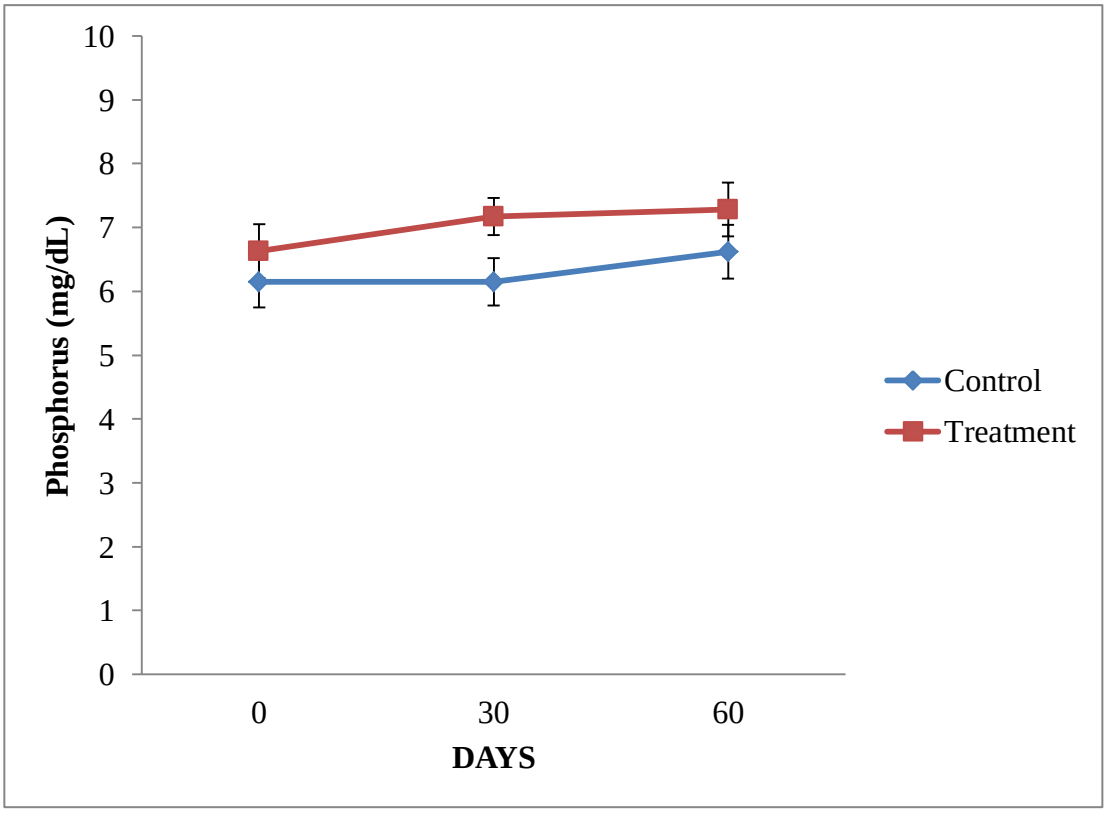

Fig. 28: Plasma phosphorus values in animals of different groups

Results are consistent with those published by El-nor et al. (2007) who stated significant ($p \leq 0.05$) increase in plasma glucose of lactating cows after supplementing their diet with herbs containing medicinal properties and the increment was attributed to better utilization of nutrients by treatment animals (increased apparent digestibility). On contrary, El-ghousein (2010) recorded no effect of various medicinal plants on glucose levels in lactating ewes and Singh et al. (2012) did not observe any noteworthy change in plasma glucose level among Shatavari supplemented group of lactating buffaloes as compared to non-supplemented group but stated that increment in glucose seen after feeding ruminants with herbal formulation containing Shatavari might be due to positive influence of supplemented herbs on propionate production in rumen due to alteration in rumen microflora.

Roy et al. (2013) also reported similar results in lactating crossbred cattle supplemented with an herbal mixture once a day (OD) for 15 days where glucose concentration in plasma of supplemented animals was significantly more than that of control animals. It is also noteworthy due to the fact that low glucose levels after parturition indicate negative energy balance which make animal susceptible to production disorders. Hence, increment in glucose level of lactating buffaloes indicated towards restoration of net energy balance. But in contradiction, Al-dain & Jarjeis. (2015) reported that supplementing lactating dairy cows with ginger root powder at dose rate of 75 grams or 150 grams per day for 4 weeks resulted in significant decrease in glucose levels as compared to non-supplemented group.

Das et al. (2016) stated that glucose level is lowest in early lactation among buffaloes as large amount is transported to mammary gland for lactose synthesis and also observed that milk yield had positive correlation with glucose levels in plasma. Rising trend of glucose in supplemented buffaloes (Table 10) when reflected alongside milk yield values (Table 6) in same group confirm this statement.

Ikyume et al. (2020) supplemented herbal mixture containing a combination of ginger and garlic to mixed breed rams for 91 days and observed that the combination increases propionate concentration in rumen which further will increment glucogenic potential of diet fed to animals.

Khamisabadi (2021) reported no significant change in level of plasma glucose after supplementing diets of lactating ewes with different herbs at 10 grams per day

per ewe for duration of two weeks pre-partum to end of lactation. The variation in results might be due to interaction of different secondary metabolites present in different herbal mixture, difference of species, dose rates, time and duration of experimental trials.

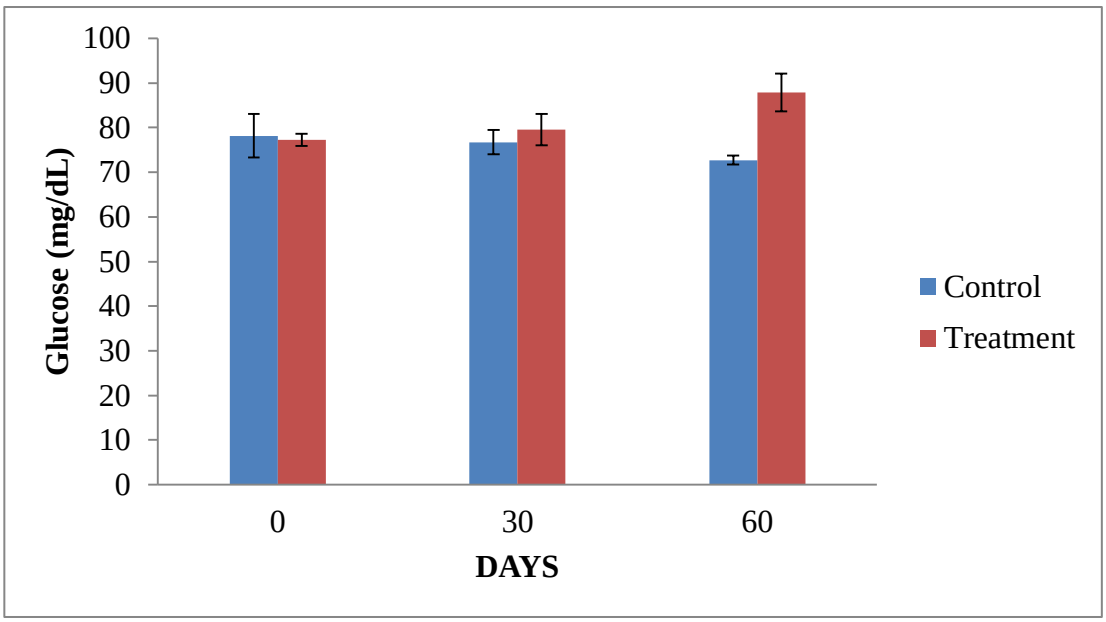

Fig. 29: Plasma glucose values in animals of different groups

Total protein and Albumin

Mean value of total protein for buffaloes in control group and treatment group was 6.45±0.16 (g/dL) and 6.68±0.14 (g/dL) respectively at start of experiment. It varied to 6.20±0.28 (g/dL) and 6.13±0.37 (g/dL) on day 30 and day 60 respectively for control group whereas it came out to be 6.90±0.23 and 6.97±0.5 g/dL on day 30 and 60 respectively for treatment group. Though the variation in values throughout observation period was not significant but mean total protein value in control group showed a slight decrease whereas it incremented in supplemented group with advancement of time (fig 30). On the other hand, albumin values in both groups showed a slight noteworthy rise among individual groups over time. However albumin of treatment group animals showed a positive significant difference on day 30 (2.62±0.04 g/dL) and day 60 (2.82±0.05 g/dL) as compared to those of control (2.49±0.03 and 2.61±0.03 g/dL) in same manner (fig.31). This positive change might be attributable to better digestion and assimilation of dietary proteins or stimulatory effect on biosynthetic activity of hepatocytes for production of plasma proteins.

Results are consistent with those reported by El-nor et al. (2007) where total protein as well as albumin increased significantly ($p \leq 0.05$) in lactating buffaloes supplemented with various medicinal plants as compared to control group and the rise was attributed to improved health along with better digestibility of nutrients in feed. Khattab et al. (2011) & Zanouny et al. (2013) observed positive influence on total protein and albumin levels after supplementing *Nigella sativa* in diets of lambs and dairy cows respectively. Results of current study are in contrast with Hashemzadeh et al. (2014) who reported no change in total protein or albumin levels with phytobiotics rich herbal mixture supplementation in Holstein cows and results were also contradicted by Ikyume et al. (2017) where there was significant decrease ($p \leq 0.05$) in serum total protein and albumin levels after feeding West African dwarf (WAD) bucks with garlic powder as herbal supplement in their diets but this effect was seen at higher dose rates. So, clearly dose of herbal supplements play a vital role in their effect on biochemical constituents in ruminant body. Khamisabadi & Fazaeli (2021) reported that supplementing lactating ewes with peppermint or thyme resulted in significant increase of serum total protein levels but there was no significant change in albumin concentrations.

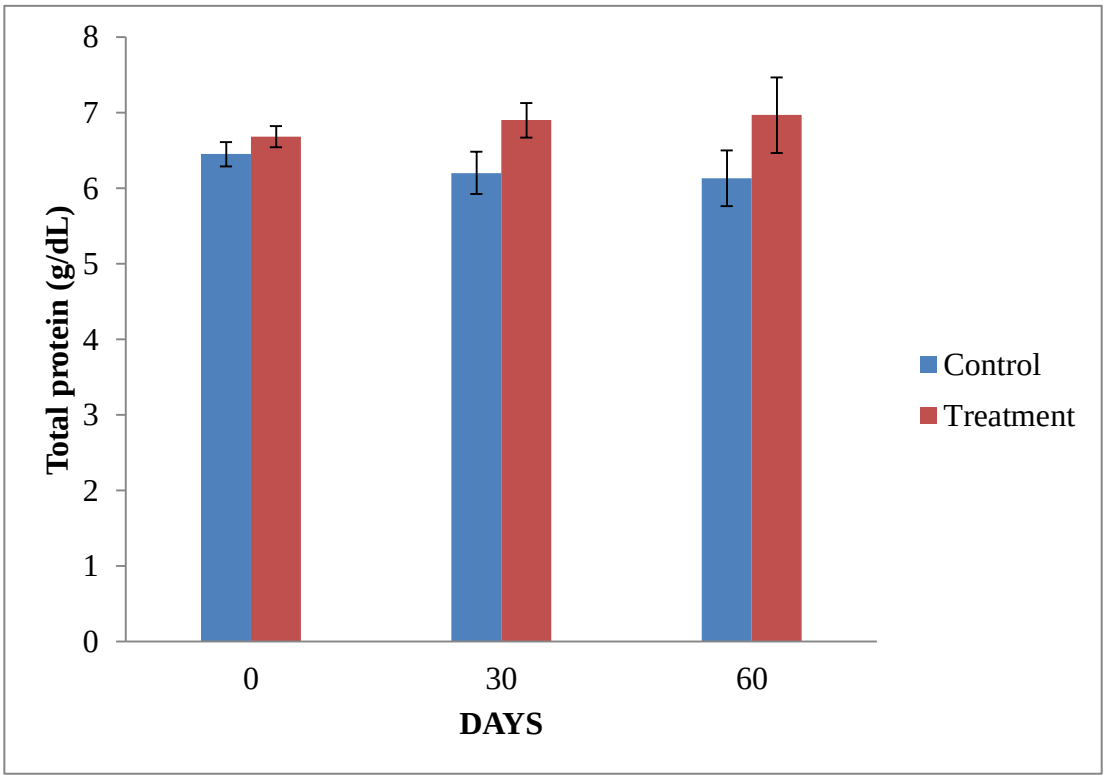

Fig. 30: Plasma total protein values in animals of different groups

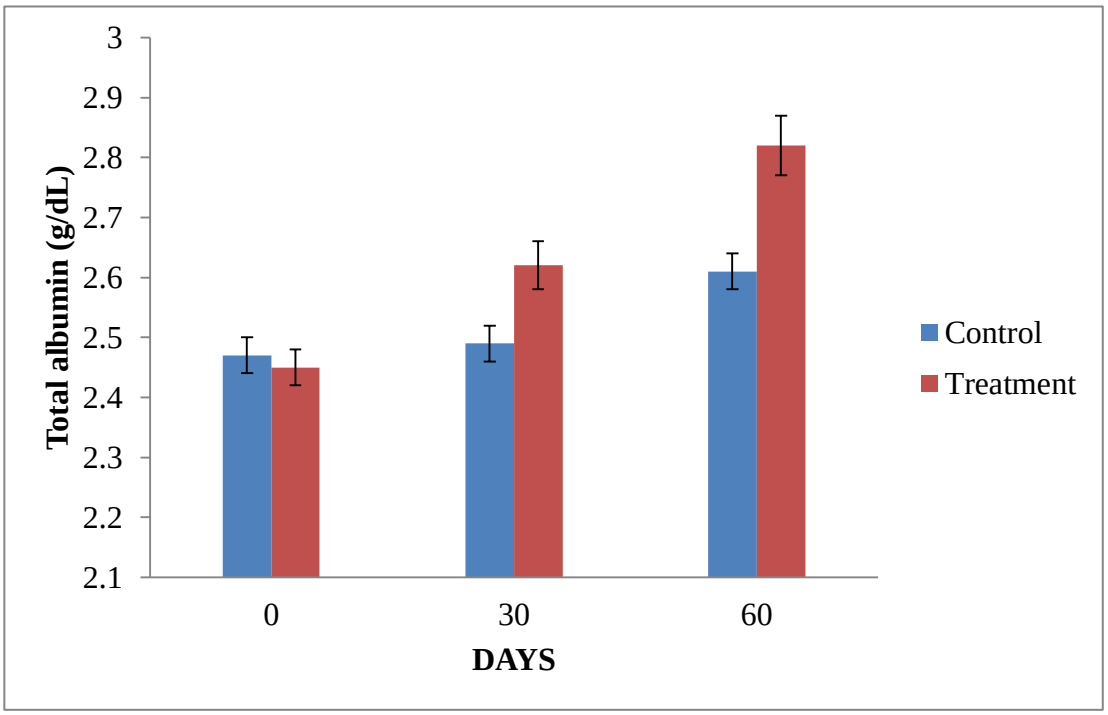

Fig. 31: Plasma albumin values in animals of different groups

Other biochemical parameters

Serum glutamic puruvic transaminase (SGPT) values (U/L) for control group were 29.06±5.48, 30.14±3.9 and 31.23±4.86 and for treatment group animals, the values were 30.83±3.88, 31.8±3.06 and 31.61±3.27 on day 0, 30 and 60 respectively. Serum glutamic oxaloacetic transaminase (SGOT) values (U/L) were 41.68±6.2, 39.23±5.38 and 44.34±4.46 for control group and 42.71±3.49, 44.24±1.78 and 45.51±1.4 for treatment group on day 0, 30 and 60 respectively (Table 11). Alkaline phosphatse (ALKP) (U/L) at start of experiment was 129.21±16.27 and 149.84±14.35 for control and treatment group respectively while it was 144.45±10.86 and 139.15±11.8 at end of observation period. GGT (U/L) and LDH (U/L) values were within normal physiological range (Table 11) for all animals in both groups throughout the research period. There was no significant change in any of the mentioned enzymes between or within groups and all were within normal range. Rise in circulatory levels of these enzymes indicate any cellular damage or altered metabolism in hepatocytes.

Absence of any significant change in enzymes clearly indicates that polyherbal formulation did not cause any potential damage to liver. This might be because many ingredients included in supplementation used in this study have previously reported

hepatoprotective action such as Garlic (Rana et al., 2011), Ginger (Haniadka et al., 2013), Katuka (Shukla 2013), Cumin (Abbas & Naz, 2017) and Shatavari (Selvaraj et al., 2019).

Blood Urea Nitrogen (BUN) (mg/dL), Creatinine (mg/dL) and Creatine Kinase (CK) (U/L) were also estimated to know the effect of herbal formulation used on renal functioning as well as to ensure safety on muscle tissue. Values for all three parameters were within normal physiological range (Table 11) which meant that the novel polyherbal mixture supplemented in this study did not have any adverse effects on kidney or did not cause any cellular level injury.

El-nor et al. (2007) supplemented some lactating buffaloes with different medicinal herbs and observed positive significant change ($p \leq 0.05$) in levels of creatinine whereas no change in levels of ALKP. Lal et al. (2007) reported that a polyherbal mixture fed to Swiss albino mice exerted hepatoprotective action against CCl_4 induced hepatic injury which was attributed to fact that polyherbal mixture prompted antioxidant activity in hepatocytes and also had potential of high regeneration initiation.

Roy et al. (2013) reported no alteration in ALT circulatory levels among hypogalactic dairy cows given herbal supplementation orally. Hashemzadeh et al. (2014) reported that phytobiotic rich herbal mixture decreased creatinine levels in supplemented dairy cows. At the same time, they found no effect on other blood metabolites including ALT, AST, BUN and ALKP. Our results were similar to those reported by Ran et al. (2020) where despite fluctuating biochemical values between groups before experiment, there was no significant change observed in levels of ALT, AST, BUN, ALKP, LDH and CK after supplementation of a herbal mixture in diets of peri-parturient crossbred cows.

Another study done by Iroanya et al. (2014) reported that polyherbal supplementation, showed hepatoprotective and nephroprotective action in wistar albino rats suffering from acetaminophen-induced toxicity. Hepatic and renal enzymes significantly reduced from high levels of damaged liver and kidneys. Additionally, it displayed antioxidant activity indicated by increase in hepatic Catalase, Glutathione peroxidase, Glutathione, GlutathioneS-Transferase and Superoxide Dismutase levels. So, hepato-renl protective action might be due to antioxidant properties of secondary metabolites present in polyherbal formulation.

Table 11: Values of various liver and kidney function parameters for animals in different groups

Group	0 Day	30 Days	60 Days
SGPT (U/L)			
Control	29.06±5.48^{Aa}	30.14±3.90^{Aa}	31.23±4.86^{Aa}
Treatment	30.83±3.88^{Aa}	31.8±3.06^{Aa}	31.61±3.27^{Aa}
SGOT (U/L)			
Control	41.68±6.20^{Aa}	39.23±5.38^{Aa}	44.34±4.46^{Aa}
Treatment	42.71±3.49^{Aa}	44.24±1.78^{Aa}	45.51±1.40^{Aa}
ALKP (U/L)			
Control	129.21±16.27^{Aa}	137.52±14.84^{Aa}	144.45±10.86^{Aa}
Treatment	149.84±14.35^{Aa}	144.55±14.23^{Aa}	139.15±11.80^{Aa}
GGT (U/L)			
Control	7.54±1.57^{Aa}	7.65±1.24^{Aa}	7.50±1.32^{Aa}
Treatment	8.19±1.43^{Aa}	7.94±1.2^{Aa}	7.44±0.88^{Aa}
LDH (U/L)			
Control	754.99±50.52^{Aa}	760.80±53.47^{Aa}	776.28±72.30^{Aa}
Treatment	798.21±51.52^{Aa}	789.73±44.22^{Aa}	772.95±37.99^{Aa}
Creatinine (mg/dL)			
Control	1.17±0.09^{Aa}	1.19±0.08^{Aa}	1.18±0.05^{Aa}
Treatment	1.14±0.09^{Aa}	1.12±0.06^{Aa}	1.16±0.07^{Aa}
Creatine kinase (U/L)			
Control	74.13±6.79^{Aa}	80.07±4.48^{Aa}	78.20±5.25^{Aa}
Treatment	75.15±9.97^{Aa}	77.67±10.54^{Aa}	78.44±10.49^{Aa}
BUN (mg/dL)			
Control	35.93±2.61^{Aa}	40.02±1.73^{Aa}	40.47±1.19^{Aa}
Treatment	31.95±1.85^{Aa}	36.72±2.06^{Aa}	32.87±5.90^{Aa}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

Lipid profile

Values of HDL, LDL and total plasma cholesterol are given in table 12. HDL values (mg/dL) showed no significant difference between buffaloes of both groups but within individual animals of treatment group, plasma HDL values saw a decline over time (Table 12 and Fig 32). Both the groups saw a downturn in LDL levels over time but the reduction in control group was by 16.18% whereas it was by 21.47% in treatment group from start to end of experiment (fig.33). Furthermore LDL levels on day 30 and day 60 were significantly lower ($p \leq 0.05$) in treatment group (52.87±0.85 mg/dL and 46.97±0.64 mg/dL respectively) than control group (58.72±0.74 mg/dL and 52.08±0.74 mg/dL respectively) (Table 12). Similar pattern was observed with total cholesterol levels on day 30 (74.19±0.77 mg/dL and 77.26±1.01 mg/dL in control and treatment group respectively) and on day 60 (72.68±0.49 mg/dL and 75.95±0.8 mg/dL in control and treatment group respectively) (Table 12 and Fig. 34). These results indicate that polyherbal formulation supplemented to lactating buffaloes had hypocholesteremic effect.

Table 12: Values of parameters indicating lipid profile in animals of different groups

Group	0 Day	30 Days	60 Days
HDL (mg/dL)			
Control	25.21 ± 0.16^{Aa}	24.89 ± 0.13^{Aa}	24.79 ± 0.63^{Aa}
Treatment	24.97 ± 0.13^{Ca}	24.65 ± 0.13^{Ba}	24.19 ± 0.05^{Aa}
LDL (mg/dL)			
Control	62.14 ± 0.68^{Ca}	58.72 ± 0.74^{aB}	52.08 ± 0.74^{aA}
Treatment	59.81 ± 1.16^{Ca}	52.87 ± 0.85^{bB}	46.97 ± 0.64^{bA}
Cholesterol (mg/dL)			
Control	78.49 ± 1.15^{Aa}	77.26 ± 1.01^{aA}	75.95 ± 0.8^{aA}
Treatment	77.94 ± 1.45^{Ba}	74.19 ± 0.77^{bA}	72.68 ± 0.49^{bA}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

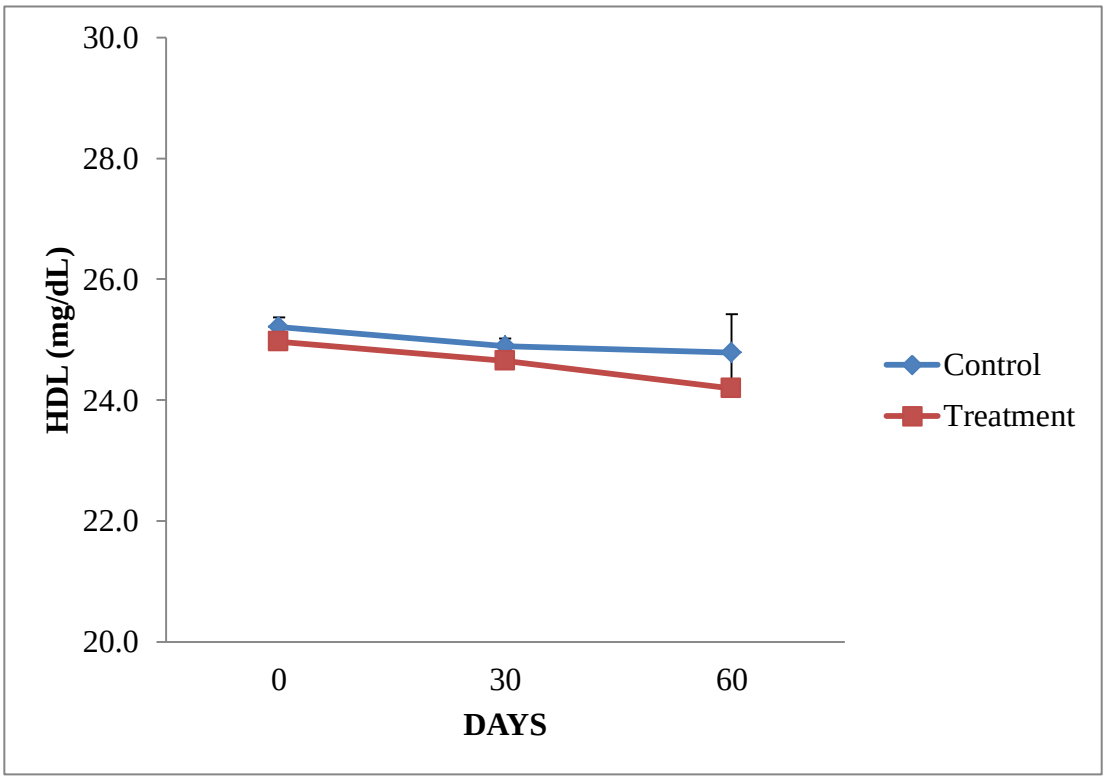

Fig. 32: Plasma HDL values of animals in different groups

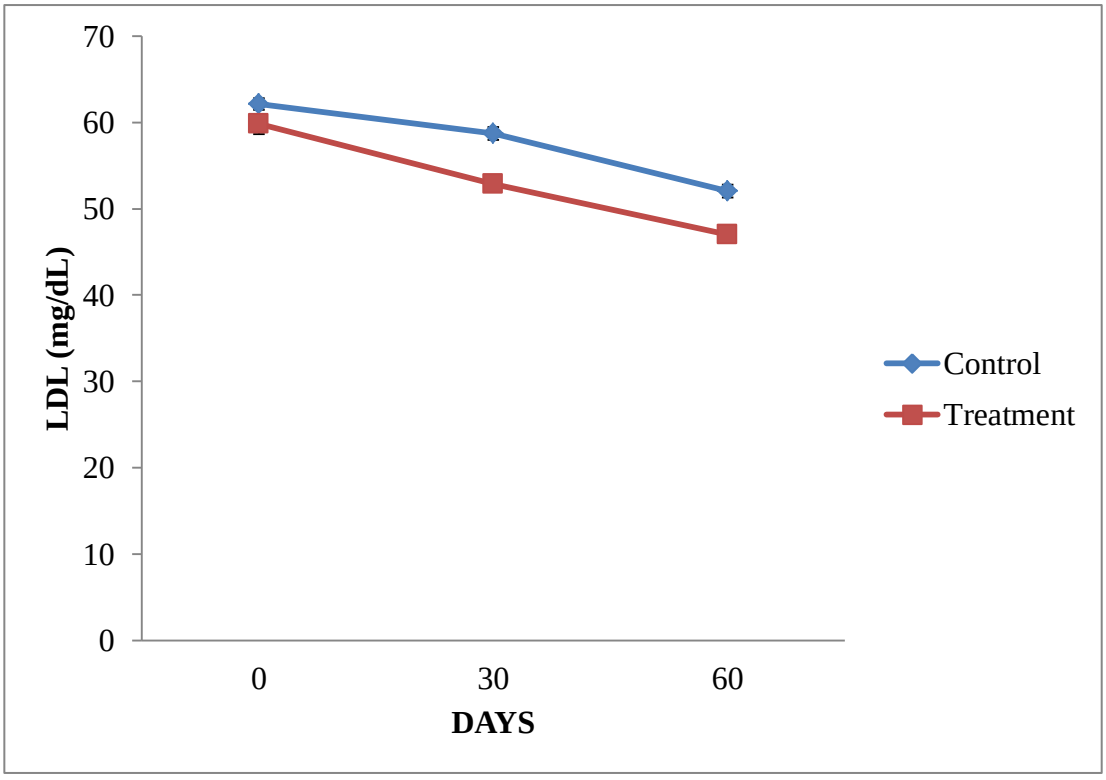

Fig. 33: Plasma LDL values of animals in different groups

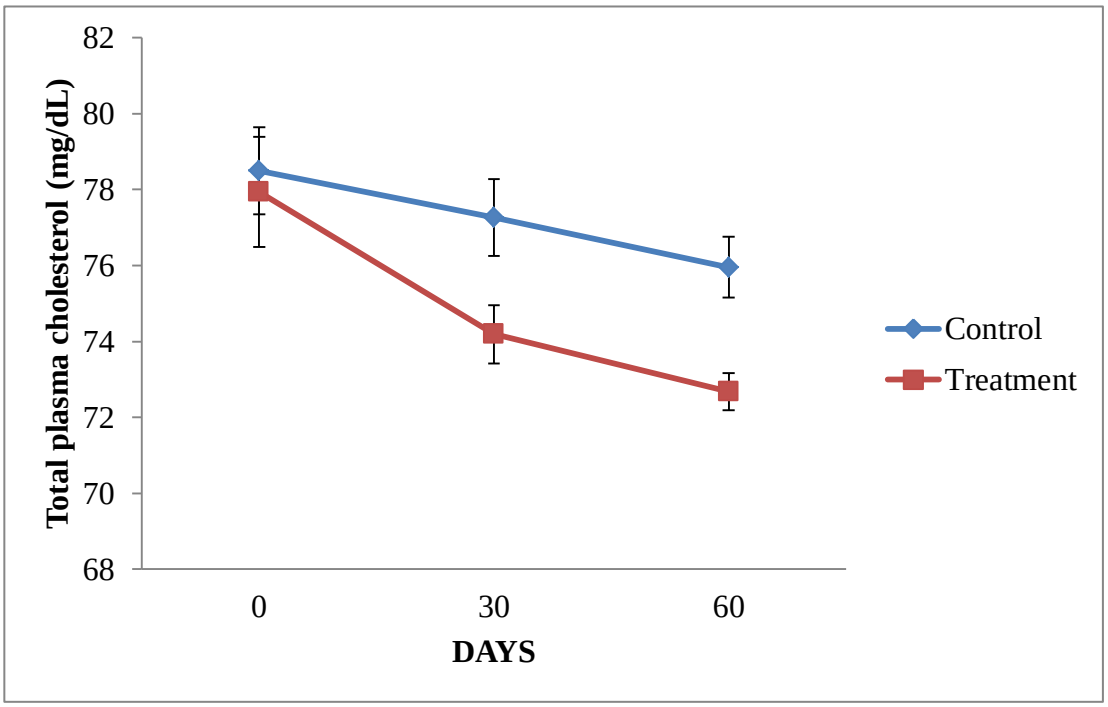

Fig. 34: Plasma Cholesterol values of animals in different groups

Al-shaikh et al. (1999) observed no changes in cholesterol or total lipids levels after supplementing lactating goats with fenugreek seeds. The varied results might just be due to different secondary metabolites in formulation. Such results in our research might be due to presence of *Allium sativum* or *Asparagus racemosus* in formulation which have proved effects on reduction of cholesterol levels. Visavadiya and Narasimhacharya (2005) reported about hypolipidemic properties of *Asparagus racemosus* in hypercholesteremic rats and attributed to increase in bile production. Pirmohammadi et al. (2014) observed significant reduction in total plasma cholesterol levels after supplementing garlic in diets of mahabadi goats.

4.5.2. Effect of polyherbal formulation on antioxidant parameters in buffaloes

To know the extent of oxidative stress in animals due to production, parameters like Lipid peroxidase (LPO), Superoxide dismutase (SOD), blood glutathione (GSH) and Catalase (CAT) were estimated along with above mentioned blood metabolites and the values obtained are presented in table 13. There was no significant effect on LPO levels between both groups which means polyherbal formulation did not cause any oxidative stress in supplemented buffaloes but in control group, LPO values (nmol MDA/g hb/hour) showed a significant rise over time on day 30 (7.51±0.07) and further increased on day 60 (7.78±0.07) as compared to

day 0 (7.22±0.08) which indicated towards lactation stress in dairy animals. Rise in LPO level of treatment group animals can be observed from amount of MDA (malondialdehyde) produced per gram hb represented in fig.35 and the incline was lesser as compared to control which might be due to antioxidative properties of herbal supplementation. Both groups saw a rise in levels of SOD (U/mg hb) among individual animals in same group over time (Table 13 and fig36). But the rise was more in treatment group as compared to control which again indicated that supplementation possesses some antioxidative action. Same pattern is followed by GSH levels (µmol/ml) as both group showed a slight, non-significant increment over time within animals of same group but the treated animals showed a steeper slope of rising GSH levels as compared to that in control group buffaloes (fig 37). In case of CAT (µmol decomposed H_2O_2/min/mg hb), there was again rise in plasma levels of enzyme in both groups over time but difference was not significant in control group whereas it was noteworthy incline in supplemented buffaloes (Table 13 and fig 38). The antioxidative action observed in treatment group animals might be due to the fact that some ingredients of polyherbal formulation used in this study have proven antioxidant properties like Garlic (Rana et al., 2011), Katuka (Shukla 2013), Ginger (Haniadka et al., 2013), Shatavari (Selvaraj et al., 2019), etc.

Table 13: Values of various antioxidant enzymes in animals of different groups

Group	0 Day	30 Days	60 Days
LPO (nmol MDA/g hb/hour)			
Control	7.22 ± 0.08^{Aa}	7.51 ± 0.07^{Ba}	7.78 ± 0.07^{Ca}
Treatment	7.44 ± 0.08^{Aa}	7.49 ± 0.08^{Aa}	7.65 ± 0.05^{Aa}
SOD (U/mg hb)			
Control	15.01 ± 0.21^{Aa}	16.91 ± 0.24^{Ba}	18.24 ± 0.12^{Ca}
Treatment	14.99 ± 0.25^{Aa}	17.36 ± 0.14^{Ba}	18.79 ± 0.26^{Ca}
GSH (µmol/ml)			
Control	420.15 ± 14.53^{Aa}	430.53 ± 11.01^{Aa}	440.72 ± 8.21^{Aa}
Treatment	422.24 ± 10.49^{Aa}	435.2 ± 9.94^{Aa}	447.6 ± 8.25^{Aa}
Catalase (µmol decomposed H_2O_2/min/mg hb)			
Control	14.38 ± 0.22^{Aa}	14.65 ± 0.19^{Aa}	14.95 ± 0.15^{Aa}
Treatment	14.23 ± 0.2^{Aa}	14.93 ± 0.19^{Ba}	15.19 ± 0.16^{Ba}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

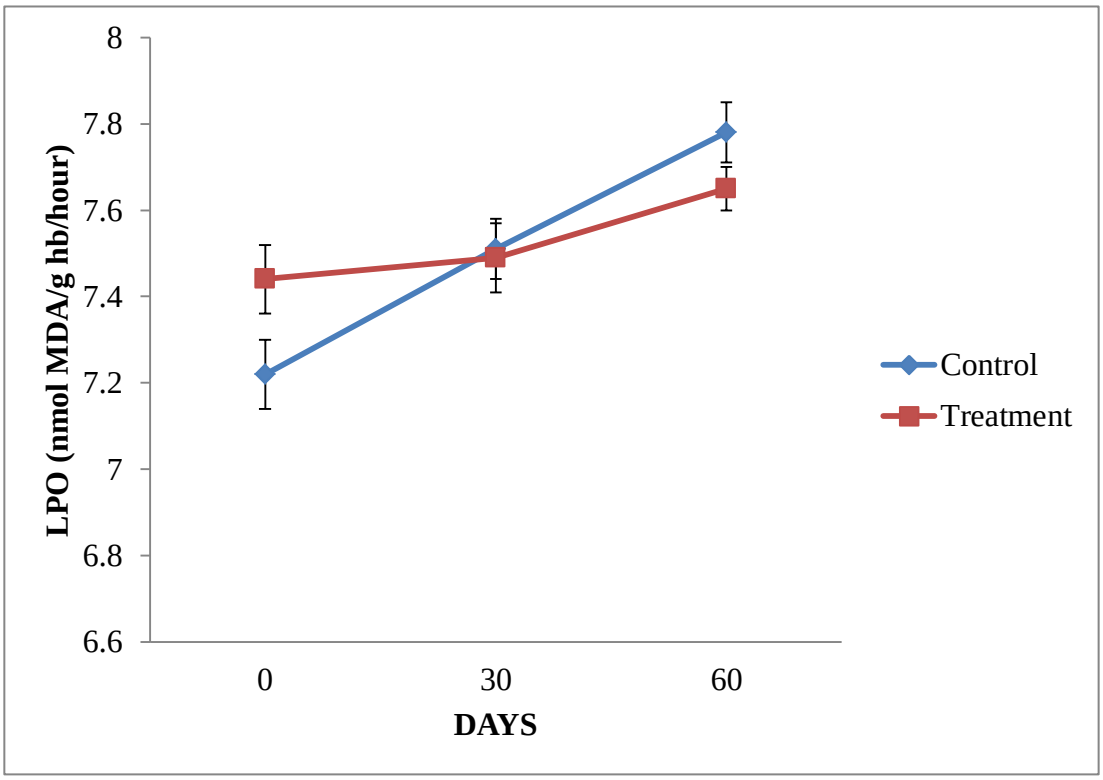

Fig. 35: Lipid peroxidase values of animals in different groups

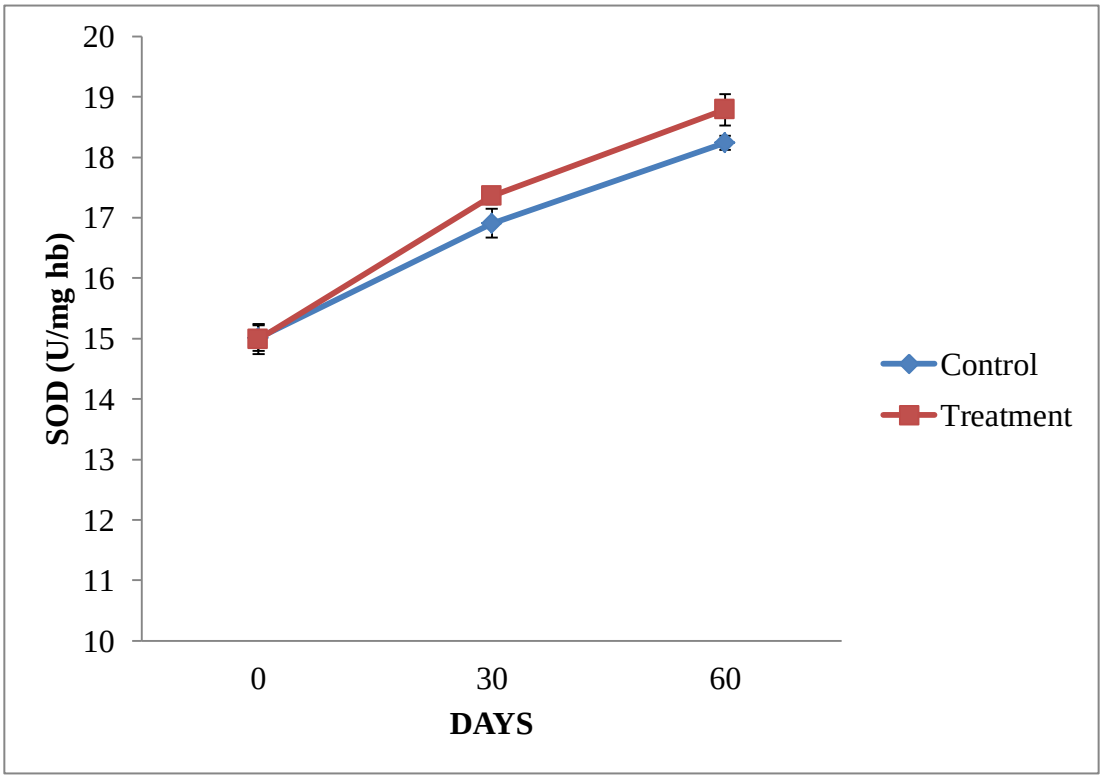

Fig. 36: Superoxide dismutase values of animals in different groups

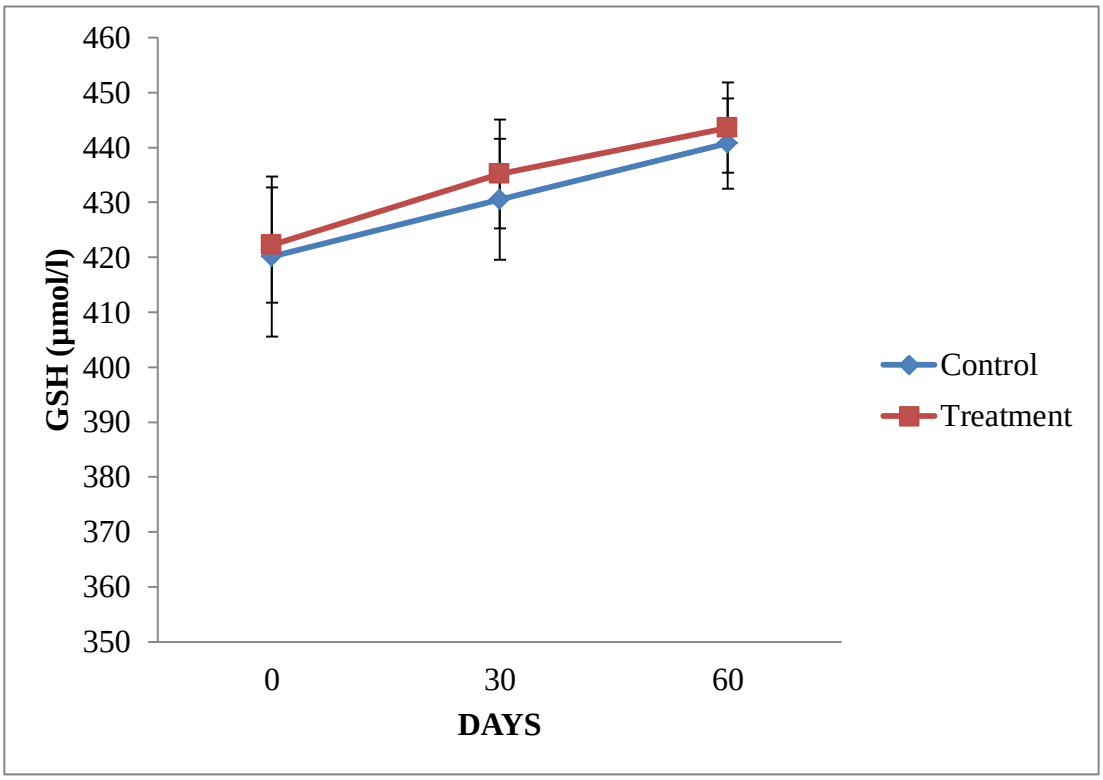

Fig. 37: Blood glutathione values of animals in different groups

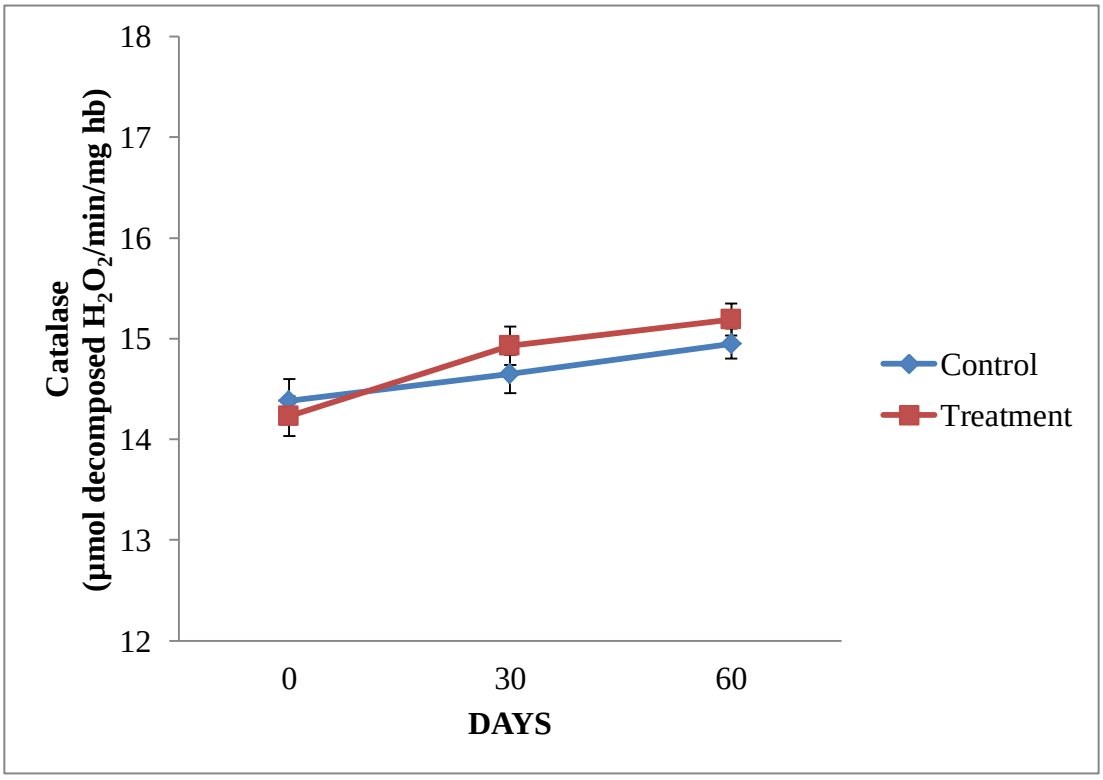

Fig. 38: Catalase enzyme values of animals in different groups

Similar results were reported by Visavadiya & Narasimhacharya (2005) where supplementation of Shatavari root powder at 5% or 10% in diets of hypercholesteremic rats led to significant rise in the levels of catalase, ascorbic acid and SOD whereas significant decline in LPO levels was observed. Positive influence of herbal supplements was also observed by Hosoda et al. (2006) who reported significant increase in plasma antioxidant activity in Holstein steers supplemented with clove. Results were also supported by reports of Wang et al. (2011) who observed that supplementing diets of beef cattle with a Chinese herbal mixture resulted in significantly reduced MDA levels and significantly raised SOD levels though there was no change in levels of (Glutathione peroxidase) GPX or also known as GSH-P_X. Choubey et al. (2018) found no changes in SOD levels but significantly increased levels of CAT, GPX and GST (Glutathione-S-transferase) after supplementing diets of Jamunapuri goats with a high dose (3% of feed intake) of a novel phytogenic feed additive. Li et al. (2020), after supplementing feed of Murrah buffaloes with mulberry leaf flavonoids, observed significant decrease in MDA levels, significant improvement in GPX levels and no change in activity of serum SOD. Increment in the serum concentrations of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT) and total antioxidant capacity (T-AOC) and decline in the malondialdehyde (MDA) concentration was also observed by Ran et al. (2020) in periparturiet dairy cows supplemented with traditional Chinese herbal medicine complex.

4.5.3. Effect of polyherbal formulation on haematological parameters

4.5.3. a) Total leucocyte count (TLC)

Total leucocyte counts in blood ($\times 10^3/mm^3$**)** for control group were 9.68±0.9 on day 0, 13.72±1.89 on day 30 and 14.82±1.15 on day 60 whereas for treatment group, it was 8.59±0.45 on day 0, 8.9±0.43 on day 30 and 7.99±0.2 on day 60 (Table 14 and fig.39). There was numerical increase in average TLC values among individual animals of control group with time. TLC values of control group were still within normal physiological range. So, the increment might be a normal physiological response as Ostensson (1993) reported that total leucocyte count in residual milk show an increment with advancement in lactation. On the other hand, there was significant decrease in TLC of buffaloes in treatment group as compared to control

group buffaloes on day 30 and day 60. Results of present study are in contrast with those reported by Ikyume et al. (2017) who stated no significant change in white blood cell count (WBC) after herbal supplementation of *Allium sativum* powder in diets of lactating goats but the results are in line with reports of Kumar et al. (2018) who observed significant decline in total leucocyte count in cattle suffering from sub-clinical mastitis which were supplemented with a polyherbal formulation at different dose rates. He attributed the decline to immunoprotective capacity of herbs.

Slight increase in TLC levels of non-supplemented group might also be due to rising production stress in animals as Kumari et al. (2018) stated that TLC levels rise with rise in cortisol levels due to increasing stress whereas many secondary metabolites present in herbs are able to reduce stress because of excellent DPPH scavenging activity which could have been the reason for decreasing TLC levels in animals of treatment group in this research. Decline in TLC levels might also have been due to antimicrobial effect of some individual ingredients. As was observed in table 8, SCC also followed same style of variation among individual animals in both groups which is in line with reports of Das and Singh (2020) who observed that somatic cell count and total leucocyte count follow similar pattern as lactation proceeds.

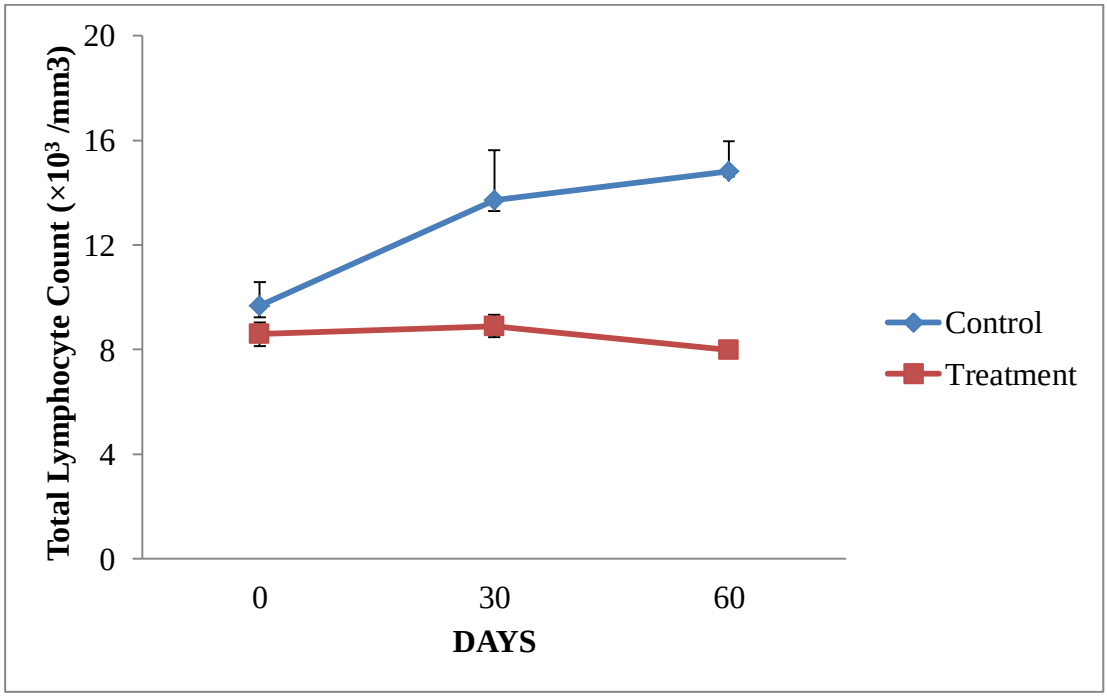

Fig. 39: Total Leucocyte Count values of animals in different groups

4.5.3. b) Other haematological parameters

Table 14 presents values of various haematological parameters over time in both groups. Hemoglobin (Hb) values observed in animals of both the groups were within physiological normal range at all times of experiment (Table 14). The Hb values varied from 10.03±0.46 g/dL on day 0 to 9.77±0.21 g/dL on day 60 in control group and from 9.85±0.45 g/dL on day 0 to 10.18±0.56 g/dL on day 60 in treatment group. TEC values ($\times 10^6$/ml blood) in control group varied from 6.76±0.12 on day 0 to 6.36±0.18 on day 60 and in treatment group, values varied from 6.36±0.22 on day 0 to 6.69±0.34 on day 60 (Table 14). So, there was no significant difference in values within group as well as between the groups for any of the said parameters. PCV values (%) in control group were 33.05±1.08 and 29.32±0.63 whereas in treatment group were 30.14±1.05 and 32.00±1.79 at start and end of observation period respectively (Table 14). There was no significant change between groups but there was slight decrease in PCV % of animals in control group although the values were within physiological range and hence the decrease was just due to individual variation within group. Similarly there was no significant variation in the values of MCV, MCH and MCHC (Table 14) but there was increase in MCHC among animals of treatment group on 60 days (31.87±0.39 g/dL) which might be only due to individual fluctuations and is within normal range.

This indicates that the polyherbal formulation supplemented does not cause any change to erthrocytic indices. Results are in line with those of Mishra (2008) who reported that supplementing Shatavari in lactating crossbred cows did not produce any significant change in haemoglobin or PCV among control and treatment groups despite seeing variation among individual animals but in contrast a significant increase in TEC was seen in supplemented animals as compared to control. Yakubu & Afolayan (2009) reported no significant change in haematological parameters of male wistar rats after feeding them with aqueous herbal extract at dose rate 25, 50 and 100 mg/kg body weight in different group for 14 days. Sanghai et al. (2017) recorded slight but non-significant improvement in haemoglobin of lactating cows after feeding them with a poly-herbal galactogogue consisting of *Lepidium sativum* 50% and *Dioscorea bulbifera* 50% @ 100 grams per day per animal for two weeks. Results match with those reported by Ikyume et al. (2017) who stated no significant variation in haemoglobin, TEC or PCV after supplementing dairy goats with herbal supplement @0.5%, @1% and @1.5% per 100 kg body weight in different groups.

Table 14: Values of various haematological parameters of animals in different groups

Group	0 Day	30 Days	60 Days
Hemoglobin (g/dL)			
Control	10.03 ± 0.46^{Aa}	10.07 ± 0.25^{Aa}	9.77 ± 0.21^{Aa}
Treatment	9.85 ± 0.45^{Aa}	9.74 ± 0.19^{Aa}	10.18 ± 0.56^{Aa}
TEC ($\times10^6$/mm^3)			
Control	6.76 ± 0.12^{Aa}	6.54 ± 0.23^{Aa}	6.36 ± 0.18^{Aa}
Treatment	6.36 ± 0.22^{Aa}	6.45 ± 0.27^{Aa}	6.69 ± 0.34^{Aa}
PCV (%)			
Control	33.05 ± 1.08^{Aa}	33.42 ± 1.39^{Aa}	29.32 ± 0.63^{Ba}
Treatment	30.14 ± 1.05^{Aa}	31.03 ± 0.98^{Aa}	32.00 ± 1.79^{Aa}
MCV (Fl)			
Control	48.87 ± 1.24^{Aa}	51.56 ± 2.54^{Aa}	46.30 ± 1.25^{Aa}
Treatment	47.45 ± 0.72^{Aa}	48.76 ± 2.42^{Aa}	47.81 ± 0.73^{Aa}
MCH (pg/dl)			
Control	14.87 ± 0.73^{Aa}	15.48 ± 0.39^{Aa}	15.43 ± 0.37^{Aa}
Treatment	15.47 ± 0.41^{Aa}	15.27 ± 0.51^{Aa}	15.23 ± 0.27^{Aa}
MCHC (g/dL)			
Control	30.49 ± 1.41^{Aa}	30.52 ± 1.27^{Aa}	33.35 ± 0.21^{aA}
Treatment	32.59 ± 0.64^{Aa}	31.61 ± 0.93^{Aa}	31.87 ± 0.39^{bA}
TLC ($\times10^3$/mm^3)			
Control	9.68 ± 0.9^{Aa}	13.72 ± 1.89^{aB}	14.82 ± 1.15^{aB}
Treatment	8.59 ± 0.45^{Aa}	8.90 ± 0.43^{bA}	7.99 ± 0.2^{bA}
Lymphocytes (%)			
Control	59.3 ± 1.12^{Aa}	57.1 ± 2.36^{Aa}	53.9 ± 4.01^{Aa}
Treatment	55.9 ± 1.66^{Aa}	58.4 ± 1.69^{Aa}	57.7 ± 3.22^{Aa}
Neutrophils (%)			
Control	40.3 ± 1.13^{Aa}	42.8 ± 2.28^{Aa}	45.6 ± 3.94^{Aa}
Treatment	43.8 ± 1.65^{Aa}	41.3 ± 1.76^{Aa}	42.0 ± 3.05^{Aa}

Values are expressed as Mean±S.E.M.

Values bearing dis-similar superscript in capital letters differ significantly within a row (ANOVA; Significance difference: $p \leq 0.05$).

Values bearing dis-similar superscript in small letters differ significantly within a column (t test; Significance difference: $p \leq 0.05$).

As opposed to these results, Kumar et al. (2018) reported that supplementing a polyherbal formulation at different dose rates in different groups of lactating sub-mastitic cattle, there was significant increase in haemoglobin, TEC and PCV. Hendawy et al. (2019) reported no significant change in Hb, PCV, MCV, MCHC, MCH but significant increase in TEC was observed after adding different medicinal herbs to basic diet of lactating ewes at a level of 5 grams/ewe/day for 3 months after parturition. These sharp variations might be due to difference in species, physiological state of animal as well as different dose rates used for supplementation.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present investigation was designed for "Evaluation of efficacy of polyherbal formulation for augmentation of milk production in buffaloes". The objective of the present study was to evaluate efficacy of polyherbal formulation on buffaloes in respect to production parameters and assess its safety profile through biochemical, antioxidant and haematological parameters.

Twenty healthy lactating buffaloes were selected on the basis of their daily milk yield record and also animals in same parity and same stage of lactation were preferred. Buffaloes were divided into two groups of ten animals each and groups were tried to be made homogenous. Group I served as control group in which no supplement was given and animals were fed normal diet based on feeding schedule followed at GADVASU dairy farm only, whereas animals of group II were treated with the prepared polyherbal formulation (@20 grams/100kg B.W.) with their scheduled diet for 45 consecutive days orally. Animals were observed for another 30 days post treatment.

Polyherbal formulation used in current study contained 13 ingredients namely *Asparagus racemosus, Cuminum cyminum, Carum carvi, Anethum sowa, Swertia chirata, Picrorhiza kurroa, Zingiber officinale, Piper longum, Piper nigrum, Embelia ribes, Allium sativum, Pistacia integerrima* and Red Ochre.

The summary of different findings in the present experiment is presented below:

1. There was no significant difference ($p \leq 0.05$) in body weight of treatment group as compared.
2. Polyherbal formulation supplemented to animals in the treatment group did not cause any change in physiological parameters of animals i.e. Temperature, Heart rate and Respiration rate.
3. Polyherbal formulation did not show any significant effect ($p \leq 0.05$) on milk yield of animals.
4. There was no significant difference ($p \leq 0.05$) seen in average of milk fat%, lactose%, solid not fat % and specific gravity of animals between control and

treatment group. However, there was significant increment in milk protein % and total solids % within treatment group over time.

5. Somatic cell count showed significant decrease ($p \leq 0.05$) in treatment group as compared to control group and among individual animals over time. Meanwhile, mean SCC for control group numerically raised over time.
6. Mean values of electrical conductivity in control group showed a significant rise ($p \leq 0.05$) over time whereas electrical conductivity of animals in treatment group showed significant decrease as compared to that of control group animals and within group as well.
7. There was no noteworthy change in milk pH of any group.
8. In sensory evaluation, colour, appearance, odour, flavor and overall acceptability of the milk were tested and given score. The scores coming within 6 to 8 were of good category. The milk from treatment group showed a slight decrease in odour and acceptability score but the change was not significant ($p \leq 0.05$) and therefore, milk was fit for human consumption.
9. Various biochemical parameters were assayed to see effect of herbal treatment on general health and safety of animals. Supplementation of polyherbal formulation in buffaloes did not cause any change in levels of hepatic functioning parameters like ALT, AST, GGT, ALKP or in renal functioning parameters like Creatinine and BUN or LDH and CK which clearly indicated that novel supplement did not have any negative impact on animal's health.
10. The results indicated that in treatment group, there was significant increase ($p \leq 0.05$) in calcium levels on day 45 and 60 as compared to buffaloes in control group.
11. Phosphorus level in plasma of treatment animals was significantly higher than those of control animals on day 45 ($p \leq 0.05$). On day 60, though the difference was not significant, phosphorus was higher in treatment buffaloes than those in control group.
12. Although there was no significant difference ($p \leq 0.05$) between glucose levels of both the groups but within the treatment group, there was significant increment in amount of glucose in plasma on day 30 and 60. This indicated to glucogenic properties of novel polyherbal formulation used in this study.

13. Non-significant decline was observed in mean total protein values of control group animals whereas non-significant incline observed in treatment group buffaloes. Moreover, albumin of treatment group animals showed a positive significant difference on day 30 and day 60 ($p \leq 0.05$) as compared to those of control.
14. LDL and total cholesterol levels showed a significant decrement ($p \leq 0.05$) on day 30 and 60 in treatment group as compared to control group. HDL values showed a non-significant decline among individual animals within treatment group. This indicated towards hypocholesteremic action of polyherbal supplementation used in present study.
15. There was significant increase in LPO levels ($p \leq 0.05$) within control group over time whereas no such change was observed in treatment group which indicated towards oxidative stress in animals during production period in non-supplemented animals.
16. SOD levels increased within both groups over time however, no significant difference ($p \leq 0.05$) was seen between the groups. This indicated towards efforts of animal's body to deal with oxidative radicals formed. The average values were higher in treatment group than control group.
17. Average GSH values in treatment group were higher than control group animals although the difference was not significant ($p \leq 0.05$).
18. Catalase values within treatment group animals showed a significant increase ($p \leq 0.05$) over time which indicated towards antioxidant properties possessed by polyherbal formulation supplemented.
19. Hemoglobin, TEC, PCV, MCV, MCH and MCHC values were all within normal physiological range for both groups which indicated that polyherbal formulation did not have any negative impact on hematology of animal.
20. There was significant decrease ($p \leq 0.05$) in total leucocyte count of animals in treatment group on day 60 as compared to those in control group and significant increment in TLC within control group animals over time.

CONCLUSION

Supplementation of polyherbal formulation showed maintenance of body weight in animals indicating that formulations helps animal maintain health even during stressful phase of production. It did not have significant effect on milk yield or milk fat % of animals but significantly improved milk protein % and total solids % of supplemented animals. Due to its potential antimicrobial effect indicated by decreased somatic cell count and electrical conductivity, the formulation might prove to be beneficial in maintaining udder health of lactating animals. Milk from treatment group did not have any noteworthy objectionable colour, smell or flavour and was fit for human consumption. The results of blood parameters clarified that there was no negative impact of supplementation on animals' health and it in fact, had positive effect on some aspects of health in lactating buffaloes supplemented with polyherbal formulation such as glucose and calcium levels. Formulation also helped animals cope with production stress in better way indicated by change in antioxidant enzymes. Overall it is concluded that the polyherbal formulation has a positive influence on ruminant health and digestion. Further studies on the mechanism of action of polyherbal formulation are needed by observing its activity on various hormones and glands.

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