# A study on Biochemical and Antioxidant Status on Blood and Urine in Preweaned Indigenous Calves treated with *Tinospora cordifolia* and *Asparagus racemosus*

**THESIS SUBMITTED FOR PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE**

**OF**

## MASTER OF VETERINARY SCIENCE

**IN**

## VETERINARY BIOCHEMISTRY

**BY**

**Prashant Singh**

**Enrollment No. V-1817/17**

**COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY**

**U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan**

**Mathura (DUVASU) - 281001 (UP)**

**(2020)**

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This is to certify that thesis entitled, **"A study on Biochemical and Antioxidant Status on Blood and Urine in Preweaned Indigenous Calves treated with *Tinospora cordifolia* and *Asparagus racemosus*"** submitted by **Dr. Prashant Singh, Enrollment No. V-1817/17** in partial fulfillment of the requirements for the award of the **Master of Veterinary Science** in **Veterinary Biochemistry** of the **Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura (UP), India,** is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated..10-6-2020

**(Pawanjit Singh)**

**Major advisor and Assistant Professor**
**Department of Veterinary Biochemistry**

## CERTIFICATE

It is certified that the thesis entitled, **"A study on Biochemical and Antioxidant Status on Blood and Urine in Preweaned Indigenous Calves treated with *Tinospora cordifolia* and *Asparagus racemosus*"**, submitted by **Dr. Prashant Singh, Enrollment No. V-1817/17** in partial fulfillment of **Master of Veterinary Science Degree in Veterinary Biochemistry** at **College of Veterinary Science & Animal Husbandry, Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura (UP),** India, embodies the original work done by the candidate himself. The candidate has carried out his work sincerely and methodically.

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which being presented by him for the award of the degree of this University.

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Name of Student : **Dr. Prashant Singh**

Enrollment No. : V-1817/17

Subject : Veterinary Biochemistry

College : College of Veterinary Science and Animal Husbandry DUVASU, Mathura.

Title of the Thesis : A study on Biochemical and Antioxidant Status on Blood and Urine in Preweaned Indigenous Calves treated with *Tinospora cordifolia* and *Asparagus racemosus*

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| 2. | Dr. Rajesh Nigam | Member | |
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| 4. | Dr. Mukul Anand | Member | |

11/09/2020

**Signature:**

**Name: Dr. A. K. Jain**
**Designation: Retd. Professor**
**Dept. of Veterinary Biochemistry,**
**DUVASU, Mathura**

11/09/2020

**HOD**
**Veterinary Biochemistry**

| | |
|---|---|
| % | Per cent |
| / | Per |
| @ | At the rate of |
| < | Less than |
| ≤ | Lesser than or equal to |
| °C | Degree centigrade |
| µg/ml | Microgram per milliliter |
| µl | Microlitre |
| µmol | Micro mole |
| ADG | Average daily gain |
| ALP | Alkaline phosphatase |
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| BHBA | Beta-hydroxy butyric acid |
| BUN | Blood urea nitrogen |
| BW | Body weight |
| CAT | Catalase |
| cm | Centimeter |
| DUVASU | Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan |
| e.g. | Exempli gratia |
| *et al* | Et alli/alia. |
| etc. | And so forth. |
| FRAP | Ferric reducing antioxidant power |
| g | Gram |
| g/dl | Grams per decilitre |
| Hb | Haemoblobin |
| HIT | Haematocrit |
| *i.e.* | id est (that is) |

| | |
|---|---|
| IU | International Unit |
| kg | Kilogram |
| l | Litre |
| LFC | Livestock farm complex |
| LPO | Lipid peroxidation |
| MDA | Malondialdehyde |
| meq/l | Miliequivalent per litre |
| mg | Milligram |
| mg | Milligram |
| mg/dl | Milligram per deciliter |
| mg/l | Milligram per litre |
| mg/ml | Milligram per milliliter |
| ml | Millimeter |
| mM | Millimole |
| NEFA | Non-esterified fatty acid |
| OD | Optical density |
| PCV | Packed cell volume |
| pH | Negative logarithm of hydrogen ion concentration |
| RBCs | Red blood cells |
| rpm | Revolution per minute |
| SE | Standard Error |
| SEM | Standard error of mean |
| SOD | Super oxide dismutase |
| TAS | Total antioxidant status |
| WBCs | White blood cells |

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## ACKNOWLEDGEMENTS

Gratitude cannot be seen or expressed, it can only be felt in heart and soul, and is beyond description. Although thanks are poor expression of the deep debt and gratitude that one feels, yet there is no better way to express it. The publishing of this manuscript is a complete team effort and I would like to express my sincere thanks to all individuals who have played a part in creation of manuscript

I feel privileged to express deepest gratitude to my guide **Dr. Pawanjit Singh,** M.V.Sc., Ph.D., Assistant Professor, Department of Veterinary Biochemistry, College of veterinary Science & Animal Husbandry, U. P. Pt. Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwa Vidyalaya Evam Go Anusandhan Sansthan, Mathura. I thank him for his esteemed and prudent guidance and supervision, valuable suggestions, constant moral support, constructive advice and criticism, untiring interest throughout the pursuit of this study,

No appropriate words could be traced in the presently available lexicon to avouch the excellent guidance given by, **Dr. Rajesh Nigam,** M.V.Sc., Ph.D., Professor & Head, Department of Veterinary Biochemistry; **Dr. Mukul Anand,** M.V.Sc., Ph.D. Assistant Professor, Department of Veterinary Physiology; **Dr. Muneendra Kumar,** M.V.Sc., Ph.D., Assistant Professor, Department of Animal Nutrition; **Dr. Vijay Pandey,** M.V.Sc., Ph.D., Assistant Professor, Department of Veterinary Biochemistry; **Dr. Ambika Sharma** M.V.Sc., Ph.D., Assistant Professor, Department of Veterinary Biochemistry and **Dr. Rajneesh Sirohi,** M.V.Sc., Ph.D., Assistant Professor, Department of Livestock Production and Management, College of Veterinary Science & Animal Husbandry, U. P. Pt. Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwa Vidyalaya Evam Go Anusandhan Sansthan, Mathura. I always felt them as a constant source of inspiration for his erudite suggestions, constructive counsel and unreserved help that served as a beckon light throughout the period of research work.

Author expresses his sincere regards to **Prof. G. K. Singh, Hon'ble Vice Chancellor**, UP Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, and Mathura for his dedication and motivation towards academic excellence.

I express my sincere thanks to **Prof. P. K. Shukla, Dean PGS,** U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam-Go Anusandhan Sansthan, Mathura (U.P.) and **Prof. Rajesh Nigam, Dean,** College of Veterinary Science and Animal Husbandry, for providing facilities and help to carry out this research work.

I am exceeding thankful to **Dr. Vinod Kumar, Dr. Brijesh Yadav, Dr. Avinash Kumar, Dr. Raju Kushwaha and Dr. Amit Jaiswal,** of DUVASU, Mathura for their unremitting help during the course of this study.

I am highly thankful to all the staff of the Department of Veterinary Biochemistry and Veterinary College Mathura namely, **Mr. Kedar, Mr. Rajbir, Mr. Yaadram, Mr. Badan** for his help and excellent co-operation.

I have no sufficient word to express my heartiest gratitude to my seniors **Dr(s) Santosh Kumar Yadav, Anil Kumar Singh, Lokesh, Praveen Gupta, Vivek Kumar Yadav, Anuj Singh Rajpoot, Sandeep Bana, Anand Singh Rana, Mohammad Saif, Rahul Dhariya** and my juniors **Dr(s) Akash Tyagi, Savare Bajrang** for their nice company during my post graduation.

I feel proud in acknowledging for my colleagues **Dr(s) Sharish Kumar, Ashwani Kumar Verma and Vivekanand Chaudhary,** for their nice company and help during my Post Graduation.

I express my gratitude to **Mr. Ravi Chauhan (M/S Students Photostat & Computer)**, Mathura, for this excellent typing in making the manuscript

My deepest gratitude goes to my parents **Mr. Dharamveer Singh** & **Mrs. Krishna Devi** without them none of the accomplishments of my life would have been possible, my uncle **Mr. Narendra Pal Singh**, my brother **Sachin** and my friends **Dheeraj Singh, Jaideep Singh, R.D Singh, Dharmendra Verma, Ashish Singh, Vishwajeet Yadav, and Vimal Pandey** have been standing for me not only during the period of this study but always throughout my carrier.

When, I felt alone and in trouble, a special blinking star near my heart provided me encouragement and determination to face the critical situation. To that inspiration, I am highly grateful as it is everything for me. It would be like a drop in the ocean of the world. Last but not least, I once again thank all the living and nonliving objects, emotions and wishes who directly or indirectly helped me to cross a milestone of study.

I thank almighty for blessing and providing me with this opportunity.

Date: 10-6-2020

Place: Mathura (U.P.)

**(Prashant Singh)**

# CONTENTS

## ABSTRACT

The aim of this study was to determine the effect of Giloy (*Tinospora cordifolia*) and Shatavari (*Asparagus racemosus*) supplementation on the growth performance, antioxidant status, immune response and other bio-chemical attributes in growing pre-weaned indigenous calves. Eighteen growing pre-weaned indigenous calves were randomly allocated into three groups having six calves in each groups and fed for 90 days. Feeding regimen was similar in all three groups. Giloy and Shatavari unsupplemented group serve as Control. The treatment groups were supplemented with 50 mg of Giloy/kg body weight (BW) ($T_1$) and 50 mg of Shatavari/kg BW ($T_2$). The experimental calves were monitored daily for calf diarrhea, joint ill, calf mortality, fortnightly for body weight gain and monthly for body condition score (BCS), length and height gain. Peripheral blood samples and urinary samples were collected at 0, 30, 60 and 90 days post-treatment and analyzed for haematological attributes, biomarker of energy and lipid metabolism, biomarker of protein metabolism, biomarker of liver and kidney function, biomarker of antioxidant status and oxidative stress, biomarkers of immune response and urine parameters. Adding Giloy or Shatavari in the diet of experimental calves did not exert any effect on the growth performance. There was no incidence of calf diarrhea and calf mortality while incidence of joint ill was significantly lower ($P<0.05$) in treatment groups. RBCs count was significantly higher ($P<0.05$) in Shatavari supplement group whereas, Hb concentration and PCV values were higher in Giloy supplemented calves. WBCs, lymphocyte and neutrophil counts showed non significant effect of treatment. Mean plasma total protein, albumin, A/G ratio and plasma urea nitrogen (PUN) levels did not showed any significant effect between groups. However, mean plasma globulin level was significant ($P<0.05$) higher in Giloy supplemented calves. The plasma cholesterol, triglyceride and lipid peroxide (LPO) levels were found to be significantly lower ($P<0.05$) in Giloy supplemented groups. However, beta hydroxyl butyric acid (BHBA) concentration was lowest ($P<0.05$) in Shatavari supplemented group. Treatment did not exert any effect on plasma glucose and non-esterified fatty acid (NEFA) concentrations. Giloy and Shatavari supplementation did not showed any effect on the biomarkers of liver functions i.e. aspartate aminotransferase (AST), alanine aminotransferase (ALT), alanine phosphatase (ALP) and biomarker of kidney function i.e. creatinine level. The superoxide dismutase (SOD) activity and total antioxidant status (TAS) were higher in Shatavari supplemented group while Catalase (CAT) activity was similar among all three groups. Adding Giloy and Shatavari to the diet of growing calves increase ($P<0.05$) plasma total immunoglobulin concentrations. Dietary treatment did not exert any effect on urinary attributes. In conclusion, dietary supplementation of Giloy and Shatavari in pre-weaned indigenous calves improves antioxidant status and immunity without any adverse effect on growth performance and liver and kidney functioning.

# Introduction

# CHAPTER-1

## INTRODUCTION

India harbor world's largest cattle population (190.90 million; DAHDF, 2012) and ranked first in milk production (140 million tones; NDDB, 2013-14) in the world. Dairy sector is a key livelihood among the farming rural families and provides food for consumption, sources of income and capital and socio-cultural needs. In spite of achieving the highest milk production, the performance of Indian cattle has been extremely poor and average milk yield is far below the yield in other countries (BAHS, 2014). Under performance of indigenous cattle is due to more population of non descript cattle, shortage of feeds and fodders and poor managemental practices.

A successful calf rearing program is essential for a profitable dairy farming operation. Good managemental practices in rearing of calf are helpful in raising high producing replacement cattle. Below three months period of calves life known as pre-weaned period, is critical to welfare and profitability of calves to transform them healthy cow. This period imposes a number of abrupt changes like parasitic infestation, reduced antioxidant status, and elevated blood cortisol which has been postulated to be responsible for immune-suppression. Impaired immune response and reduced antioxidant status during pre-weaned period has been linked to the occurrence of number of diseases which lead to calves death (Langford et al., 2003). The calf death leads to huge economic losses dominated by cost of diagnosis, treatment and additional management efforts for re-establishing normal health and performance of calf. 75% of diseases in calves occur in the first month of life while 50% of dairy cows suffer from metabolic and infectious diseases in the transition period (LeBlanc et al., 2006). Various supplements and additives have been tried to boost the immunity of calf during pre-weaned period for curtailing the incidence of various diseases.

Many studies in farm animals have underlined the importance of various supplements in improving the immune response during stress condition but carried-over effects and high cost of these supplements limits their use. Emerging evidence indicates that various Indian herbs and medicinal plants are reported to have substantial immunomodulatory effects and can be safely utilized to enhance immunity and performance of the farm animals. Herbs exert immunomodulatory and antioxidant

actions via modulation of cytokine secretion, immunoglobulin production, immunoglobulin class switching, macrophage activation, lymphocyte proliferation and phagocytosis promotion counteracting reactive oxygen species (ROS) (Mishra et al., 2017). The metabolites of active principles of herbs are having short half life and these are either secreted in milk or excreted in urine. Thus supplementation of herbs may also enhance the immunomodulatory values of calves. Various Indian herbs have been shown immunomodulatory properties among them *Tinospora cordifolia* and *Asparagus racemosus* are most common.

*Tinospora cordifolia* commonly named as Giloy or Guduchi or Amrita posses powerful health promoting, antimicrobial activity (Dorman and Deans, 2000; Kapoor et al., 2015), inhibitory activity on motility of the gastro-intestinal tract in monogastric animals (Hills and Aaronson, 1991) and dyspepsia activity (May et al., 2000). Giloy possesses anti-inflammatory, anti-stress, antioxidant, immunomodulatory, haemopoietic and rejuvenating properties. The key active components isolated from the giloy roots include alkaloids, steroids, phenyl propanoid glycosides such as Cordifolioside A, Cordifolioside B and syringin (Maurya et al., 1996), and immunostimulatory compound d-glucan (Nair et al., 2006). Immune boosting and antioxidant properties of Giloy is due to presence of cordifolioside A and syringing which stimulates phagocytic activity of macrophages and neutrophil, increases the granulocyte-macrophage colony-stimulating factor (GM-CSF). Giloy also reported to stimulates bone marrow cellularity and proliferation of stem cells as well as the haemopoetic growth factor and IL-3 production. It enhances glutathione (GSH) and vitamin C and so function as an effective antioxidant (Prince et al., 1999). It increases the total number of WBCs and alpha esterase positive cells an indicator of increase in bone marrow cells.

*Asparagus racemosus* locally named as Satavar or Shatavari or shatamull has been demonstrated to have therapeutic interest due to its role as immunomodulant, galactogauge, adaptogen, antitusive, anticarcinogen, antioxidant, antidiarrheal and as a general tonic (Oketch-Rabah, 1998; Kumar et al., 2011). Shatavari root is the most commonly used traditional medicine in animals beings and its supplementation is recommended during stress condition to boost milk quality, immunity of both mother and fetus and to tone the reproductive system and reproductive health. Previous studies revealed that the major bioactive constituents of Asparagus are a group of

steroidal saponins. This plant also rich in vitamins and minerals. Other primary chemical constituents of Asparagus are essential oils, asparagine, arginine, tyrosine, flavonoids (kaempferol, quercetin, and rutin), resin, and tanninsteroidal glycosides (asparagosides), bitter glycosides, asparagines and flavonoids. Steroid sapogenin obtained from the root of Shatavari having potent immunomodulatory and antioxidant properties (Sharma and Pandey, 2010). Immunomodulatory action of Shatavari is by up-regulation of IL-2, IL-4, and other cytokines (Gautam et al., 2004). Shatavari is also used in transplantation immunology. Shatavari helps in prevention and management of postoperative adhesions as macrophages aids in development of intraperitoneal adhesions. It was demonstrated that Shatavari, being an immunomodulator and immunostimulant significantly suppresses the chemotactic activity and production of interleukin-1 and TNF-α by macrophages.

Nevertheless, modern animal production requires the use of safe and effective additives to stimulate feed consumption and destroy harmful microorganisms of the diet, attempt to use natural materials such as medicinal plants are widely accepted as feed additives (Aboul-Fotouh et al., 2000; Thomson, 2002). Currently, the use of plant herbs has resulted in improving rumen ecology (Kamra et al., 2005; Chaudhary et al., 2008) and supporting animal performance and health status (Manzanilla et al., 2001). Variation in immune response and immunomodulatory effect of herbs was best studied in immune compromised subject i.e. in pre-weaned calves. The plan of work were specifically designed to test the hypothesis i.e. dietary supplementation of Giloy and Shatavari in calves were improve immune response, antioxidant activity and growth performance. For the present study, Tinospora and Asparagus are selected on the basis of their immunostimulating activities and antioxidant properties.

**Objectives:**

This study has been designed with the following objectives:

1. To study the effect of *Tinospora cordifolia* and *Asparagus racemosus* on antioxidant property and biochemical parameters in blood and urine of young calves supplemented with probiotic.
2. To study the effect of *Tinospora cordifolia* and *Asparagus racemosus* on growth performance of young calves supplemented with probiotic.

# Review of Literature

## CHAPTER-2

## REVIEW OF LITERATURE

India possesses a huge wealth of livestock, 190.90 million (19th Livestock Census, DAHDF, 2012) and ranked first in milk production. Cattle population itself contributes around 37.28% of the total livestock population in India (DAHDF, 2012). Although, India ranks first in milk production but the performance of Indian cattle has been extremely poor and average body weight gain and milk yield is far below the yield in other countries (BAHS, 2014). Dairy sector is a key livelihood among the farming rural families and provides food for consumption, sources of income and capital and socio-cultural needs. Poor performance of Indian cattle is due to more population of non-descript cattle, shortage of feeds and fodders and poor managemental practices. The dairy calf faces many potential stressors in the pre-weaning period, including birth, transportation, disbudding/castration, weaning, etc. Most of these stressors appear inevitable in calf raising production systems, but they are manageable if the calf timeline is considered. Although the pre-weaning stage accounts for only a 4% of the heifer's life and 10% of a dairy calf, development of the calf's stress and immune systems may affect performance and overall well-being for an animal's entire life. Impaired immunity and diarrhea has been reported as the main reason for mortality and huge economical losses in rearing neonatal calves. Rearing healthy dairy calves requires maximizing the calf's level of immunity against disease while minimizing its exposure to infectious agents.

In order to improve calf health and immunity, numerous prophylactic tools such as antibiotics, vaccination, bio-security and good animal husbandry practices are applied (Windeyer et al., 2014; Ayrle et al., 2016). Phytotherapy is an alternative health tool since it is able to modulate the innate and adaptive immunity with positive results for herd health. In addition, herbal products are advantageous over other supplements since they do not promote bacterial resistance and also not have carry over effect (Theisen and Muller, 2012). In order to reduce the antibiotic usage in farm animals, products derived from medicinal plants are used worldwide for the prevention and treatment of infectious diseases in animals and humans. A number of Indian medicinal plants have been claimed to possess immunomodulatory activity. Some of these plants are, *Tinospora cordifolia, Asparagus racemosus, Allium*

*sativum, Morus alba, Acacia catechu, Mangifera indica,* etc. A lot more are still to be explored and offer scope for further investigation. Among them, *Tinospora cordifolia* and *Asparagus racemosus* plants generally carry the high advantages of immunomodulatory, cost effective and health benefits.

Here upon, the literature pertaining to the supplementation of medicinal plants along with probiotics in growing calves has been reviewed under following sub headings:

2.1. Immune status of calf
2.2. Common plant derived immunomodulators
2.3. Benefits of using plant derived immunomodulators
2.4. Mode of action of herbs in immunomodulation
2.5. Mode of action of herbs in antioxidant status
2.6. Role of immunomodulatory herbs in animal health
2.7. Role of immunomodulatory herbs in animal
2.8. Role of *Tinospora cordifolia* in immunity and antioxidant status of animals
2.9. Role of *Asparagus racemosus* in immunity and antioxidant status of animals

**2.1. Immune status of calf**

The management of dairy calf is an important for the economy and productivity of cattle operations. The development of the immune system in calves progresses, in small steps, from conception to maturity at approximately 6 months after birth. Neonatal calves depend on passive immunity transferred from cows as the primary basis for protection against disease. Antibody from cows, transferred with colostrum, activates and regulates the innate responses present in calves to fight infection. Developing and newborn calves are subject to several immunomodulatory effects. Although all essential immune components are present in neonates at birth, many of the components are not functional until calves are at least 2 to 4 weeks of age and may continue to develop until puberty (Reber et al., 2005). Developing and newborn calves are subject to several immunomodulatory effects. The placenta produces progesterone, prostaglandin E2, and cytokines that affect the near-term fetus and the dam and suppress cell-mediated and memory responses. In contrast, these mediators promote TH2 responses and antibody production (Morein et al., 2002). Cows also produce estrogen and cortisol before parturition that have

immunosuppressive effects. Finally, as part of the parturition process calves produce high levels of cortisol that remain elevated for the first week of life (Mao et al., 1994). The cumulative effect of these hormones is to suppress immune responses and direct the immune response away from the TH1 response.

Apart from above mentioned factors, young calves also face immune suppression due to various kind of stress like transportational stress, nutritional stress (inadequate feeding), overcrowding, environmental stress (improper climate) and poor hygienic conditions. These stress factors cause a decrease in natural immunity which in turn allows access of pathogens mainly through mucosal sites. Since the calf's immune system is not well established in the first weeks of life, adequate colostral supply and good husbandry are critical to avoid intestinal and respiratory pathogens (Ayrle et al., 2016). Impaired immune response in calves is responsible for various diseases with greatest risk for enteric and respiratory disease.

**2.2. Common plant derived immunomodulators**

Immunity and antioxidant status are the body's natural defense system against various infectious diseases. Based on the function, immune system has been categorized into innate immune system (non-specific immune system) and adaptive immune system (specific or acquired immune system). The function and efficiency of the immune system are influenced by various exogenous and endogenous factors resulting in either immunosuppression or immunostimulation. The biomolecular of synthetic or biological origin capable of modulating, suppressing and stimulating any components of adaptive or innate immunity are known as immunomodulators, immunorestoratives, immunoaugmentors, or biological response modifiers (Puri et al., 1994). Immunomodulators are generally categorized into immunoadjuvants, immunostimulants and immunosuppressants in clinical practice. A number of additives, supplements and chemically compounds are being used as immunomodulators. Due to the occurrence of chemical drugs-related adverse effects, natural immunomodulators are the potential agents to replace them in therapeutic regimens. Among the natural immunomodulators, plant derived immunomodulators are one of them. Several medicinal plants exhibit not only immunomodulatory activity but also a wide range of antioxidant, anti-inflammatory, hepatoprotective, hypocholesterolemic, antifungal, cardiotonic, diuretic, etc. A brief description of plant

derived immunomodulators is presented in Table 2.1 (adopted from Kumar et al., 2012).

**Table 2.1. Common plant derived immunomodulators and their biological functions**

| Botanical name | Common name | Part used | Chemical constituents | Biological functions |
|---|---|---|---|---|
| *Asparagus racemosus* | Shatavaari | Roots | Saponins, sitosterols | Immunomodulator, antioxidant, ulcer healing agent, nervine tonic, antigout |
| *Tinospora cordifolia* | Amrita, guduuchii | Entire herb | Alkaloidal constituents such as berberine, tinosporic acid | Immunomodulator, antioxidant, hypoglycaemic agent, antipyretic |
| *Ocimum sanctum* | Tulasi | Entire plant | Essential oils such as eugenol, cavacrol, derivatives of ursolic acid, apigenin | Immunomodulator, antioxidant, carminative, stomachic, antispasmodic, Hepatoprotective |
| *Achillea millefolium* | Yarrow | Leaves | Flavonoids, alkaloids, polyacetylenes, coumarins, triterpenes | Immunomodulator, antioxidant, anti-inflammatory, antispasmodic, antipyretic, diuretic |
| *Terminalia arjuna* | Arjuna | Leaves, bark | Flavonoids, oligomeric proanthocyanidins, tannins | Immunomodulator, cardiotonic, diuretic, prescribed for Hypertension |
| *Chlorophytum borivilianum* | Safed musli | Roots | Sapogenins | Immunomodulator, antioxidant, antifungal |
| *Ganoderma lucidum* | Reishi mushroom | Whole plant | Flavonoids, triterpenes | Immunomodulator, antioxidant, |
| *Genus Ardisia* | Marlberry | Shrub, Branches and leaves | Peptides, saponins, Isocoumarins, quinones and alkyl phenols | Immunomodulator, antioxidant, antimetastatic drug |
| *Artemisia annua* | Wormwood | Entire herb | Artemisinin | Immunosuppressive |
| *Botryllus schlosseri* | Botryllus | Tunicates | Alkaloids and cytokines | Antioxidant, antiviral, antimicrobial and Antitumoral |

| | | | | |
|---|---|---|---|---|
| *Bidens pilosa* | Beggar-ticks | Flowers, leaves | Polyacetylenes | Anti-inflammatory, immunosuppressive and antibacterial |
| *Byrsonima crassa* | Byrsonima | Leaves | Flavonoids, tannins, terpenes | Antimicrobial, antioxidant |
| *Cannabis sativa* | Common hemp | Leaves | Cannabinoids | Immunomodulator |
| *Carpobrotus edulis* | Fig Marigold | Flowers, fruit | Alkaloids | Immunomodulator |
| *Centella asiatica* | Brahmi | Entire herb | Triterpenoid saponins | Immunomodulator |
| *Eclipta alba* | Bringraja | Leaves | Triterpenoid glucoside | Anticancer, antileprotic, analgesic, antioxidant, antimyotoxic |
| *aussknechtia elymatica* | Haussknechtia | Entire herb | Herb | Immunomodulator |
| *Matricaria chamomilla* | Chamomile | Flowers | Peptides | Immunomodulator |
| *Mollugo verticillata* | Carpetweed | Entire herb | Quercetin, triterpenoid glycosides | Immunomodulator |
| *Moringa oleifera* | Sahijan | Leaves | Vitamin A, B, C, carotenoids, saponins | Antioxidant |
| *Piper longum* | Pipali | Fruits | Alkaloids | Antioxidant |
| *Silybum marianum* | Milk Thistle | Flowers | Flavonoid | Antioxidant |
| *Salicornia herbacea* | Glasswort | Entire herb | Polysaccharides | Immunomodulator |
| *Thuja occidentalis* | White cedar | Leaves | Polysaccharides | Immunomodulator |

## 2.3. Benefits of using plant derived immunomodulators

Relationship between nutrition and the immune system has been the center of attention in scientific communities in last decade. In the last few years there has been a tremendous growth in the field of herbal medicine and these drugs are gaining popularity both in developing and developed countries because of their natural origin and less side effects. In literature many plants have been listed having immunomodulatory effect and some of them have been proved by using modern scientific methodologies. The WHO has listed 21,000 plants, which are used for

medicinal purposes around the world. Among these 2500 species are in India, out of which 150 species are used commercially on a fairly large scale. Many medicinal plants showing immunomodulatory activity have been used instead of drugs because of their low toxicity for the host system, adequate absorption and capability to reach the target organ without much degradation by host enzymes (Arivuchelvan et al., 2012). The side effects of synthetic drugs such as presence of antibiotic residues leading to the problem of antibiotic resistance in humans, toxic metabolites remaining in meat and byproducts are a matter of concern in long term usage of synthetic products. Such issues have promoted use of herbal preparations which are considered to be relatively safe and affordable to rural folk. Further, absence of antibiotic or toxic residues in meat and milk products has also encouraged herb based health solutions in veterinary health care sector. Thus, traditional herbal medicines in veterinary practice have great potential as an alternate therapy (Gupta et al., 2013).

### 2.4. Mode of action of herbs in immunomodulation

Experimental research performed with extracts or active principles isolated from plants have shown that they can influence the immune response in several ways (Hoffmann and Reichhart, 1997): on cellular level by modulating the proliferation rate of immune cells (naftoquinones), on humoral level by influencing the antibody production (polysaccharides) or by modulating cellular functions to increase or decrease cytokine or other mediators production (phenolic compounds). In fact, based on proper understanding of various immunomodulatory activities of herbal plants, plants derived the secondary metabolites in natural products can be the lead molecules for the future development of immunomodulators for therapeutic use. Few researches were carried out for demonstrating the specific immunomodulatory effect of different herbs or their extracts in bovines. Most of the studies focusing on collateral effects of herbal administration – antioxidant, improvement of metabolic disorders rather than general immune status. Mastitis, a potentially fatal mammary gland infection, is the most common disease in dairy cattle. A study conducted by Bhatt et al. (2014) supports the use of alternative herbal therapy against bovine sub-clinical mastitis by enhancement of cytokine expression of somatic cells and reduction in total bacterial count in bovine mammary gland. Detailed mode of action of different plant derived immunomodulators is presented in Table 2.2 (adopted from Bukhari et al., 2015).

**Table 2.2. Mode of action of plant derived immunomodulators**

| Active principle | Plant source | Mode of action |
|---|---|---|
| **Alkaloids** | | |
| Berberine | *Coptis chinensis* | Down-regulate T-helper cells cytokines (TNF-α, IL-2, IL-4) production |
| Chelerythrine | *Chelidonium majus* | Inhibit PGE2 release by regulating cyclooxygenase-2activity |
| Piperine | *Piper longum* | Reduce level of pro inflammatory cytokines IL-1β, IL-6, and TNF-α. Down regulate expression of COX-2, NOS-2, and NF-κB. Inhibit eicosanoide generation by inhibit in gphospholipase A2 and TXA2 synthase activity |
| Koumine | *Gelsemium elegans* | Inhibit T lymphocyte proliferation. |
| Sophocarpine | *Sophora alopecuroides* | Inhibit production of NO and pro inflammatory cytokines TNF-α and IL-6. Inhibit expression of iNOS and COX-2 |
| **Essential oils** | | |
| Z-ligustilide | *Angelica sinensis* | Inhibit iNOS and COX-2 induction by regulating the NF-kB and MAPK signal pathways. |
| Tetramethylpyra-zine | *Ligusticum chuanxiong* | Inhibit pro inflammatory cytokines and reactive oxygen species production. Inhibit macrophages chemotaxis, neutrophile infiltration, and nitric oxide synthase activity. |
| **Flavonoids** | | |
| Xanthohumol and Dihydroxanthohumol | *Humulus lupulus* | Inhibit NO production which is induced by LPS and INF-γ. |
| Mallotophilippens C | *Mallotus, Philippinensis* | Inhibit mRNA gene expression of iNOS, COX-2, IL-6, and IL-1β. Inactivate NF-κB. |
| Apigenin | *Mentha longifolia* | Decreased expression of ICAM and VCAM leading to decreased neutrophile, chemotaxis |
| Chrysin | *Picea crassifolia* | Inhibited production of pro-inflammatory cytokine (TNFα, IL-1β, and IL-6). By modulation of intracellular calcium reduce histamine release from mast cells. |
| Oroxylin A | *Scutellariae* | Inhibit NO production and iNOS and |

| | | |
|---|---|---|
| | *baicalensis* | COX-2 proteins expression of via inhibiting nuclearfactor-κB pathway. Enhance antioxidant response element-luciferase reporteractivity by increasing the expression of nuclearfactorerythroid 2-related factor 2 proteins. |
| Quercetin | *Dysosma veitchii* | Decreased expression of pro-inflammatory cytokines, NF-κB, and iNOS. |
| Epigallocatechin-3-gallate | *Camellia sinensis* | Inhibit reactive oxygen species generation, MAPKs phosphorylation, adhesion molecules expression signal transducers and activators of transcription 3 (STAT-3) and activating transcription factor 2 translocation through induction of heme oxygenase-1 and suppressors of cytokine signaling -3 expression. |
| **Isoflavones** | | |
| Daidzein | *Pueraria mirifica, Pueraria lobata, Glycine max* | Decreases TNF-α, IL-1β, MCP-1, NO, and iNOS expression at mRNA level. |
| Genistein | *Glycine max* | Inhibited expression of iNOS and COX-2. Decreased IL-1β and TNF-α production via activation of PPARs |
| **Phloroglucinols** | | |
| Myrtucommulone | *Myrtus communis* | Inhibit the PGE2 production by inhibiting the mPGES-1 activity without significantly inhibiting the COX enzymes activity |
| Arzanol | *Helichrysum italicum* | Reduce eicosanoids generation by inhibiting lipooxygenase and cyclooxygenase activity in arachidonic acid metabolism pathway |
| **Quinones** | | |
| Thymoquinone | *Nigella sativa* | Inhibited LPS-induced fibroblast proliferation and $H_2O_2$-induced 4-hydroxy non enalgeneration. Inhibit IL-1β, TNF-α, MMP-13, COX-2, and PGE2 while blocking phosphorylation of |

| | | |
|---|---|---|
| | | MAPKp38, ERK1/2, and NF-kBp65. |
| Shikonin | *Lithospermum erythrorhyzon* | InhibitNF-κB activity, inhibit Th1cytokines expression and induceTh2 cytokines. |
| **Stilbenes** | | |
| Resveratrol | *Fallopia japonica,* | Decrease MPO activity and mPGES-1 to basal levels. |
| Piceatannol | *Fallopia japonica* | Decrease iNOS expression. Inhibit transcription factors activation such as NF-kB, ERK, and STAT3 |
| **Terpenoid** | | |
| 14-deoxyandrographolide | *Andrographis paniculata* | Enhanced proliferation of lymphocytes. Enhanced IL-2 induction in lymphocytes |
| Oleanolic acid | *Luffacylindrica*, *Phytolaccaamericana* | Reduce level of IL-1α, IL-6, and TNF-α, as well as their effect on complement pathway though the inhibition of C3 convertase. Inhibits adenosine deaminase activity |
| Echinocystic acid | *Luffa cylindrica* | Enhance phagocytic index of macrophages in humoral and cell-mediated immune responses |
| Triptolide | *Tripterygium wilfordii* | Inhibits lymphocyte activation and pro-inflammatory cytokines gene expression (IL-2, iNOS, TNF-α, COX-2, and IFN-γ). It also inhibits activation of transcription factors such as NF-kB, NFAT, and STAT3 |
| Demethylzelasteral | *Tripterygium wilfordii* | Inhibits proliferation of vascular endothelial cells |
| Madecassoside | *Centella asiatica* | Reduce spleen cells proliferation. Inhibition of pro-inflammatory mediators such as TNF-α and IL-6. Inhibit production of PGE2, and COX-2 |
| **Other** | | |
| Apocynin | *Apocynum cannabinum* | Inhibit NADPH oxidase activity. Suppresspro- inflammatory cytokines and $CD4^+$ and $CD8^+$ T cells production |

## 2.5. Mode of action of herbs in antioxidant status

Oxidative stress, an imbalance condition when reactive oxygen species (ROS) formation exceed cellular antioxidant capacity, has become a major issue and the

subject of production concerns and related research in the domestic animal industry. ROS are constantly produced in aerobic organisms as byproducts of normal oxygen metabolism. On the other hand, exogenous stressors may exacerbate the ROS levels more dramatically. Antioxidants are responsible for scavenging of these ROS. The most effective antioxidants are those that interrupt the free radical chain reaction. Usually containing aromatic or phenolic rings, these antioxidants donate $H^{\bullet}$ to the free radicals formed during oxidation becoming a radical themselves. The use of synthetic antioxidants in animals to improve health, performance and products quality have been seriously considered (Chirase et al., 2004). However, the potential adverse effects of these compounds make their innocuousness questioned (Lin et al., 2016). A growing body of research has been devoted to natural antioxidants that are currently receiving considerable attention in animal nutrition fields. Synthetic phenolic antioxidants (butylated hydroxyanisole; BHA, butylated hydroxytoluene; BHT, and propyl gallate) effectively inhibit oxidation; chelating agents, such as ethylene diamine tetra acetic acid (EDTA), can bind metals reducing their contribution to the process. Some vitamins (ascorbic acid and α-tocopherol), many herbs and spices (rosemary, thyme, oregano, sage, basil, pepper, clove, cinnamon, and nutmeg), and plant extracts (tea and grapeseed) contain antioxidant components as well. Natural phenolic antioxidants, such as synthetics, can effectively scavenge free radicals, absorb light in the ultraviolet (UV) region (100 to 400 nm), and chelate transition metals, thus stopping progressive autoxidative damage and production of off-odors and off-tastes. Phytochemicals have been shown to exert their positive antioxidant benefits toward animals in terms of favored performance, production quality (Ansari et al., 2012; Lee et al., 2013), and enhanced endogenous antioxidant system, possibly by directly affecting specific molecular targets or indirectly as stabilized conjugates affecting metabolic pathway (Aggarwal and Shishodia, 2006). Natural medicinal herbs have been suggested good antioxidants and have been used as feed additives in animal husbandry for over 2,000 years (Wang et al., 1998). Research over the last decade has shown that several phytochemicals improve performance and products quality. Active ingredients in phytogenic materials and their mode of antioxidant actions are presented in Table 2.3 (Adopted from Lee et al., 2017).

**Table 2.3. Active ingredients in phytogenic materials and their mode of antioxidant actions**

| Active principles | Mode of action as antioxidant |
|---|---|
| Quercetin, Myricetin, Kaempferol | DPPH scavenging activity, superoxide anion radical scavenging activity, Inhibition of lipid peroxidation acitivity |
| Quercetrin, Kaempferol-3-O-α-L-rhamopyranoside, Astragalin, Quercetin, Kaempferol, Methyl gallate, Ethyl gallate, 1, 2, 3, 4, 6-penta-O-galloyl-β-D-glucopyranose | DPPH scavenging activity, Superoxide anion scavenging activity, Reducing power, Metals ion chelating effect |
| Chlorogenic acid, Echinacoside, Alkamide8/9, Cynarin, Caftaric acid, Cichoric acid | Superoxide anion scavenging activity, Reducing power, Metals ion chelating effect |
| Delphinidin 3-O-glucoside, Cyanidin 3-O-glucoside, Petunidin 3-O-glucoside, Peonidin 3-O-glucoside, Myricetin, Quercetin, Laricitrin, Kaempferol, Isorhamnetin | DPPH scavenging activity |
| Resveratrol, catechin, epicatechin, gallocatechin, gallic acid, ellagic acid | Peroxyl radical scavenging activity |
| allic acid, Caffeic acid, Epicatechin, Gallocatechin gallate | DPPH scavenging activity, Reducing capacity, Metals ion chelating effect, Inhibition of lipid peroxidation acitivity |

The major antioxidative plant phenolics can be divided into four groups: phenolic acids (gallic, protochatechuic, caffeic, and rosmarinic acids), phenolic diterpenes (carnosol and carnosic acid), flavonoids (quercetin and catechin), and volatile oils (eugenol, carvacrol, thymol, and menthol) (Shan et al., 2005). Phenolic acids generally act as antioxidants by trapping free radicals; flavonoids can scavenge free radicals and chelate metals as well. The free radical-scavenging potential of natural polyphenolic compounds appears to depend on the pattern (both number and location) of free −OH groups on the flavonoid skeleton (Lupea et al., 2008). The

B-ring substitution pattern is especially important to free radical-scavenging ability of flavonols. Flavonoids with multiple hydroxyl groups are more effective antioxidants than those with only one. The presence of the ortho-3, 4-dihydroxy structure increases the antioxidative activity (Geldof and Engeseth, 2002).

### 2.6. Role of immunomodulatory herbs in animal health

Modulation of immune response to alleviate diseases has long since been of interest. Herbal medicines have always been a form of therapy for livestock among resource poor marginal farmers (Mirzaei-Aghsaghali, 2012). Constant exposure to various stressors, such as immune pressure, rapidly increasing population, deleterious changes in the ecosystem, climate change, infection with emerging and re-emerging pathogens are critical factors in the globally increasing incidences of immunocompromising health conditions, as well as stress. Synthetic chemotherapeutic agents, which are widely available in the commercial market, may be highly efficacious, but most are immunosuppressive and exert many side effects. The use of herbs and botanical extracts for antimicrobial property and immune enhancement has been practiced from very old days in nearly every culture across the globe. Several different plant species have been documented for the treatment and prophylaxis of immune compromised diseases like mastitis. The most frequently reported plant species are *Capsicum annuum, Lepidium sativum, Allium sativum, Sesamum indicum, Citrus limon, Zingiber officinale, Citrullus colocynthis, Curcuma longa, Amomum subulatum, Sesamum indicum, Cuminum cyminum, Rosa indica, Centratherum anthelmisticum, Triticum aestivum, Nigella sativa* and *Peganum harmala*.

### 2.7. Role of immunomodulatory herbs in animal performance

Herbal feed additive affects improve nutrient utilization and absorption or the stimulation of the immune system. The possible mechanisms of action of herb in the animal for growth promotion include changes in the intestinal microbiota, increased digestibility and nutrient absorption; enhanced nitrogen absorption, improvement of the immune response morphological and histological modifications of the gastrointestinal tract and antioxidant activity. Finally herbs can contribute to the nutrient requirements of the animals and stimulate the endocrine system and intermediate nutrient metabolism. Beneficial effects of herbs or botanicals in farm

animals may arise from activation of feed intake and secretion of digestive secretions, immune stimulation, anti-bacterial, coccidiostatic, anthelmintic, antiviral or anti-inflammatory activity and antioxidant properties.

Herbal feed additives influences feed intake, digestibility of nutrients and animal performance. Due to the wide variety of active components, different herbs and spices affect digestion processes differently. Most of them stimulate the secretion of saliva. Curcuma, cayenne pepper, ginger, anis, mint, onions, fenugreek, and cumin enhance the synthesis of bile acids in the liver and their excretion in bile beneficially effects the digestion and absorption of lipids. Garlic as an alternative growth promoter in livestock production reported improved growth rate, digestibility and carcass traits (Kongmun et al., 2011). Several studies showed strong antimicrobial activity of certain plant extracts against Gram negative and Gram positive bacteria. Plants readily synthesize substances for their defense against insects, herbivores, and microorganisms. Moreover, they may produce secondary antimicrobial metabolites as a part of their normal growth and development or in response to stress. Several researches have studied the antimicrobiol effect of oriental herbs including *Allium sativum, Angelica dahurica, Anguisorba officinalls, Artemisia argyi, Coptis chinensis, Dictamnus dasycarpus, Fraxinus rhynchophylla, Geranium thunbergii, Hydrastis canadensis, Phellodenron amurense, Polygonum cuspidatum, Scutellria baicalensis* and *Sophora flavesens*. Extracts of curcuma, red pepper, black pepper, cumin, cloves, nutmeg, cinnamon, mint and ginger showed anti-inflammatory effect. The major active molecules with anti-inflammatory action are phenols, terpenoids and flavonoids. Some plant extracts have demonstrated an activity against some chicken parasites, especially coccidian (Naidoo et al., 2008; Arczewska-Wlosek and Swiatkiewicz, 2012). Various herbal preparations of *Tinospora cordifolia* and *Asparagus racemosus* either in combination or singly have been reported to act as galactogouge.

### 2.8. Role of *Tinospora cordifolia* in immunity and antioxidant status of animals

*Tinospora cordifolia* belonging to family Menispermaceae is a genetically diverse, large, deciduous climbing shrub with greenish yellow typical flowers. It thrives easily in the tropical region, often attains a great height, and climbs up the trunks of large neem trees. The surface of the stems appears to be closely studded with warty tubercles and the surface skin is longitudinally fissured. The wood is

white, soft, and porous, and the freshly cut surface quickly assumes a yellow tint when exposed to air. Long thread-like aerial roots come up from the branches (Warrier et al., 1996). A variety of active components derived from the plant like alkaloids, steroids, diterpenoid lactones, aliphatics, and glycosides have been isolated from the different parts of the plant body, including root, stem, and whole plant (Upadhyay et al., 2010). The plant is of great interest to researchers across the globe because of its reported medicinal properties like immunomodulatory, antioxidant, antiperiodic, antispasmodic, antiinflammatory, antiallergic, antistress, antileprotic, hepatoprotective and antineoplastic activities. Details regarding chemical composition of Tinospora is presented in Table 2.4 (adopted from Saeed et al., 2020).

**Table 2.4. Chemical compositions of the Tinospora herb**

| Chemical | Active principles and their distribution |
|---|---|
| Alkaloids | Tinosporin (L), tinosporic acid (L) (W),berberine (S), palmitine (S) (R),tembatarine (S) (R), mangoflorine (S) (R),choline (S) (R), tinosporin (S) (R),isocolumbin (R), tetrahydropalmatine(R) |
| Glycosides | 18 Nonderodane glycoside (S), furanoidditerpene glycoside (S), tinocordiside (S),tinocordifoliside (S), cordioside (S),cordifolioside A, B, C, D (S), syringin (S),syringinapiosylglycoside (S), palmatosidesC and P (S), cordifoliside A, B, C, D, E (S) |
| Diterpenoid lactones | Diterpenoid (S), tinosporon columbin (S),clerodane derivatives (W), tinosporon(W), tinosporisides (W), jateorine (W),columbin (W), tinosporal, tinosporide.SteroidsSitosterol (S) (O), octacosanol (S),heptacosanol (S), nonacosan-15-one (S),tetrahydrofuran (S), hydroxyecdysone (S)(O), makisterone A (S), giloinsterol (S),ecdysterone (S) |
| Sesquiterpenoid | Einocordifolin (S |
| Other compounds | Jatrorrhizine (R), tinosporidin (W),cordifol (W), cordifelone (W), giloin (W),giloinin (W), arabinogalactan (S) |
| The letters in brackets indicate the part of the plant from which thechemical constituent has been isolated. S, stem; L, leaf; R, root; W, wholeplant; O, other aerial parts. | |

The immuomodulatory property of Tinospora is well documented. Active compounds 11-hydroxymustakone, N-methyl-2-pyrrolidone, N-formylannonain, cordifolioside A, magnoflorine, tinocordiside and syringing (Sharma et al., 2012) are responsible for potential immunomodulatory and cytotoxic effects. Active principles

function by boosting the phagocytic activity of macrophages, production of ROS in neutrophil cells, enhancement in nitric oxide (NO) production by stimulation of splenocytes and macrophages indicative of anti-tumor effects (More and Pai, 2012) Tinospora extracts has been shown to result in up-regulation of IL-6 cytokine, resulting in acute reactions to injury, inflammation, activation of cytotoxic T cells, and B cell differentiation (Sudhakaran et al., 2006). Active compounds in aqueous extracts like alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds or polysaccharides in experimental rat model have been reported for their cytotoxic action. Dry stem crude extracts of Tinospora with a polyclonal B cell mitogen, G1-4A on binding to macrophages have been reported to enhance immune response in mice by inducing secretion of IL-1, together with activation of macrophages. The (1, 4)-alpha-d-glucan (alpha-d-glucan), derived from Tinospora have been shown to activate lymphocytes with downstream synthesis of the pro- and anti-inflammatory cytokines (Koppada et al., 2009) The extract also increases the number of antibody producing cells, hence implying its role in fortifying the humoral immune system. Tinospora has positive effects on lipid metabolism, as indicated by high-density lipoprotein, low-density lipoprotein, and very-low-density lipoprotein, in addition to improving hepatic functions and oxidative stress biomarkers and increasing antioxidant enzymes (superoxide dismutase and glutathione peroxidase) in different animal models (Shirolkar et al., 2016, Chavan et al., 2017).

### 2.9. Role of *Asparagus racemosus* in immunity and antioxidant status of animals

*Asparagus racemosus* belongs to family Liliaceae and commonly known as Satawar, Satamuli, Shatavari found throughout India. In Ayurveda, this amazing herb is known as the “Queen of herbs”. This herb is highly effective in problems related with female reproductive system. Reports indicate that the pharmacological activities of Asparagus root extract include antiulcer, antioxidant, and antidiarrhoeal, and immunomodulatory activities. Asparagus is a woody climber growing to 1-2 m in height. The leaves are like pine needles, small and uniform and flowers are white and have small spikes. Asparagus is known to possess a wide range of photochemical constituents. The major bioactive constituents of Asparagus are a group of steroidal saponins. This plant also contains vitamins A, B1, B2, C, E, Mg, P, Ca, Fe, and folic acid. Other primary chemical constituents of Asparagus are essential oils, asparagine,

arginine, tyrosine, flavonoids (kaempferol, quercetin, and rutin), resin, and tannin. The use of *Asparagus racemosus* dried root powder modulates the action of the immune system. That in turn, decreases the inflammatory response. It induces the immune system to fight against immune deficiencies, infections and cancer. It may be helpful in obtaining higher protective antibody against different vaccinations including more effective cell mediated immune response for protection against various bacterial, viral, and other diseases. Several workers has studied the effect of *Asparagus racemosus* root extract in augmentation of humoral and cell mediated immune response providing better protection level against infections (Singh and Sinha, 2014). Oral administration of decotion of powdered root of Asparagus been reported to produce leucocytosis and predominant neutrophilia along with enhanced phagocytic activity of the macrophages and polymorphs. The major active constituents of Asparagus are steroidal saponins (Shatavarins I-IV) that are present in the roots. Shatavarin IV has been reported to display significant activity as an inhibitor of core golgi enzymes transferase in cell free assays and recently to exhibit immuno-modulation activity against specific T-dependent antigens in immuno compromised animals (Kamat et al., 2000). Chemical constituents present in Asparagus are presented in Table 2.5 adopted from Singh et al., 2018).

**Table 2.5. Phytochemical derived from different parts of Asparagus**

| Part of plant | Chemical constituents |
|---|---|
| Root | Rutin, asparagan, Asparagamine A, 9,10- dihydro 1, 5 methoxy-Quercetin3 glucouronides, 8-methyl-2, 7- phenenthrenediol, Racemofuron, ncoumertans, Shatavarin V. Shatavarin I, II, III,IV (steroid glycosides), Immunoside, Sitosterol, Undecanyl cellanoate, Shatavari, 4,6- dihydroxy-2-0 (2- hydroxyl isobutyl) benzaldehyde, Secoisolariciresinol, diosgenin, Racemosol, 4-trihydro isoflavine 7-0-beta-D-glucopyranoside, Sterols, Alkaloid, Tannins, carbohydrates, Flavonoids, isoflavones, coumestans, prenylated. Lactones, Amino acids and rutin. |
| Shoot | Sarsasapogenin and kaempferol Thiophenes, thiazole, aldehyde, ketone, Gamma linoleinic acids, Undecanyl cetamoate |
| Leaf | Vanillin, asparagusic acid and methyl/ethyl esters |
| Flower | Diosgenin, quercetin-3-glucuronide |
| Fruit | Quercetin, rutin, hyperoside, Racemoside A, B, and C, sarsasapogenin |

# Materials and Methods

# CHAPTER-3

# MATERIALS AND METHODS

The material used and experimental techniques followed during the investigations of the effects of herbal supplementation on the performance and antioxidant status of growing indigenous calves are presented in this section.

## 3.1. Ethics Approval

Animal care procedures were approved and conducted under the established standard of the Institutional Animal Ethics Committee (IAEC), constituted as per the article number 13 of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) rules laid down by the Government of India.

## 3.2. Experimental design

A total of eighteen growing indigenous calves were selected from the cattle herd maintained at Livestock Farm Complex (LFC), DUVASU, Mathura. All calves were housed in a well-ventilated shed having the proper arrangement for feeding and watering. Deworming of all the animals was done with drug Fendikind-plus (Fenbendazole & ivermectin) before the start of the experiment. The detail of calves used in experiment is presented in Table 3.1.

To compare the effect of herbal supplementation on growth performance, immunity, antioxidant status and blood metabolites, a feeding trial of 90 days period was conducted. Experimental calves were randomly assigned into three groups (six calves in each group) on body weight and age basis. Control group were fed on basal diet consisted of yoghurt probiotic mixed with wheat bran (1:5 proportion), milk, available green fodder and concentrate mixture without any herbal supplementation. Whereas treatment group 1 ($T_1$) and group 2 ($T_2$) were additional supplemented with 50 mg Tinospora/kg BW and 50 mg Asparagus/kg BW respectively. Both the herbs collected from authentic sources and validated by department of animal nutrition DUVASU, Mathura. The nutrient requirements of growing calves were met as per NRC (2001) guidelines. The calves were provided with fresh and clean water free of choice.

**Table 3.1. Details of the growing indigenous calves used in the experiment**

| Group | Animal no. | Initial BW (kg) | Mean BW (kg) |
|---|---|---|---|
| Control | 904/D38 | 38 | 31.5 |
| | 518/D48 | 36 | |
| | 515/D38 | 31 | |
| | 517/D38 | 21 | |
| | 280/D38 | 36 | |
| | 281/D38 | 27 | |
| $T_1$ | 902/D38 | 38 | 31.5 |
| | 516/D38 | 35 | |
| | 519/D48 | 32 | |
| | 905/D48 | 25 | |
| | 197/D48 | 30 | |
| | 282/D48 | 29 | |
| $T_2$ | 900/D38 | 41 | 31.5 |
| | 903/D38 | 34 | |
| | 556/D38 | 30 | |
| | 899/D38 | 22 | |
| | 195/D38 | 36 | |
| | 194/D38 | 26 | |

### 3.3. Chemical analysis and observation recorded

The Tinospora and Asparagus were analyzed for their chemical composition i.e. dry matter (DM), ether extract (EE), crude protein (CP), crude fibre (CF), ash and nitrogen free (NFE) by following methodology of AOAC (2005). The experimental calves were monitored daily for calf diarrhea, joint ill, calf mortality, fortnightly for body weight gain and monthly for body condition, length and height gain.

#### 3.3.1. Body weight gain

Body weight of experimental calves was recorded at start of experiment followed by at fortnightly intervals. The experimental calves were weighed before feeding and watering. Fortnightly weight gain of calves calculated by increase in body

weight in one fortnight and ADG (kg/day) was calculated by dividing the fortnightly weight gain with number of days (15).

### 3.3.2. Height, length and body condition score (BCS)

Highest height and body length were measured by following standard procedures. Body condition scoring (BCS) in dairy calf is a visual and tactile evaluation of body fat reserves using a 5-point scale with 0.25-point increments. BCS was measured at 5 point scale by following methodology of Anitha et al. (2011).

**Table 3.2. Suggested BCS for growing calf**

| Events | Age (months) | BCS | | |
|---|---|---|---|---|
| | | Goal | Minimum | Maximum |
| Calf | 0 to 4 | 2.25 | 2.00 | 2.50 |
| | 4 to 10 | 2.50 | 2.25 | 2.75 |
| Pre-Breeding | 10 to 12 | 2.75 | 2.50 | 3.00 |
| Breeding | 12 to 15 | 3.00 | 2.50 | 3.25 |
| Bred | 15 to 20 | 3.25 | 3.00 | 3.50 |
| Calving | > 20 | 3.50 | 3.50 | 3.75 |

### 3.3.3. Calf diarrhea, joint ill and calf mortality

Calf scour or diarrhea was measured as per 5 point score as presented in Table 3.2.

**Table 3.3. Calf diarrhea score**

| Score | Faecal consistency, colour and odor |
|---|---|
| 1 | Normal (firm to soft) consistency, brown to light brown color, normal odor |
| 2 | Soft to loose consistency, yellow, brown, or green color, mucus, slight odor |
| 3 | Loose to watery consistency, yellow or green color, mucus, strong odor |
| 4 | Watery consistency, yellow, green, or clear color, mucus, slight blood, strong odor |
| 5 | Watery consistency, clear color, mucus, bloody |

Joint ill was determined by observing swelling of joints, number of joints involved, severity of pain, etc.

### 3.4. Blood sampling, haematological and biochemical analysis

Peripheral blood samples were collected in heparinised vacuutainer tubes (BD Franklin, USA) by venipuncture of anterior vena cava at 0, 30, 60 and 90 days post-treatment. Day 0 represents the beginning of the experiment. A fraction of blood sample was used for analysis of blood haematology i.e. red blood cells (RBCs), white blood cells (WBCs), lymphocytes, neutrophils, haemoglobin (Hb) concentration, and packed cell volume (PCV) or haematocrit (HIT) and antioxidant enzyme determination i.e. superoxide dismutase (SOD) and Catalase (CAT). Remaining amount of blood samples were centrifuged at 3000 rpm for 30 min to separate the plasma from packed erythrocytes. Plasma samples were stored at −20°C until further analysis of biomarkers of immunity, antioxidant status, protein metabolism, energy and lipid metabolism and liver and kidney function test.

#### 3.4.1. Haematological attributes

RBCs, WBCs, Hb, and PVC or HIT value were analyzed by using protocol of automatic Celltac alpha CM, Nihon, Kohden, Pvt. Ltd, Surat, India.

#### 3.4.2. Plasma attributes

##### 3.4.2.1. Plasma total protein

Total protein was estimated in blood plasma samples by using “Modified Biuret, End point assay test kit” supplied by Span Diagnosis Ltd. The principal of the assay is that the peptide bonds of proteins react with cupric ions in alkaline solution to form a colored chelate; the absorbance was measured at 578 nm. The absorbance of final color is proportional to the concentration of total protein in the sample.

**Procedure:** 10 µl of plasma aliquot was pipette in 10×75 mm tubes in duplicate, to which 1000 µl of working Biuret reagent was added. Blank (10 µl distilled water) and standard (10 µl from protein standard 6.5 g/dl) was pipette in duplicates, to which 1000 µl of working biuret reagent was added. The content were mixed well and incubated at room temperature for 1 minute. UV- spectrophotometer was blanked with reagent. The content were mixed well and incubated at room temperature for

1minute. UV- spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured at 578 nm.

**Calculation:** Total protein concentration was calculated as per formula and expressed in gm/100ml:

$$\text{Total protein (g/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 6.5$$

**3.4.2.2. Plasma albumin**

Albumin was estimated in blood plasma samples by using "Bromocresol Green, End point assay test kit" supplied by Span Diagnosis Ltd. It is based on the principal that albumin binds with anionic dye bromocresol green (BCG) to form green color complex, which is measured at 630 nm. Kit reagent were prepared and stored as per the instruction provided with the assay kit.

**Procedure:** 10 µl of plasma aliquots were pipette in 10×75 mm tubes in duplicate, to which 1000 µl of working albumin reagent was added. Blank (10 µl distilled water) and standard (10µl from standard 4g/dl) was pipette in duplicates, to which 1000 µl of working albumin reagent was added. The content were mixed well and incubated at room temperature for 1 minute. UV- spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured at 630 nm. **Calculation:** The albumin concentration was calculated as per the formula and expressed in gm/100ml:

$$\text{Albumin (g/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 4$$

**3.4.2.3. Plasma globulin**

It was determined by subtracting the albumin content from total protein content:

$$\text{Plasma globulin (g/100ml)} = \text{Total protein concentration} - \text{albumin concentration}$$

**3.4.2.4. Blood urea nitrogen (BUN)**

BUN was estimated in plasma samples by "Urease, Berthelot, End point assay test kit" supplied by Span Diagnosis Ltd. Urea hydrolyses to ammonia in presence of urease enzyme which reacts with hypochlorite and phenolic chromogen in alkaline medium to form coloured complex which is measured at wavelength 578 nm.

**Procedure:** 10 µl of plasma aliquot was pipette in 10×75 mm tubes in duplicate, to which 1500 µl of working BUN solution-1 was added. Blank (10 µl distilled water) and standard (10 µl from standard 50 mg/dl) were pipette in duplicate to which 1500 µl of working BUN solution-1 was added. The contents were mixed well and incubated at 37°C for 3 min. 1000 µl of working BUN solution-2 was added to each tube. The content were mixed well and incubated at 37°C for 5 min. UV-spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured.

**Calculation:** BUN levels were calculated as per formula and expressed in mg/100ml:

$$\text{BUN (mg/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 50$$

**3.4.2.5. Plasma creatinine**

Creatinine was determined in plasma samples by "Modified Jaffe's Reaction, Initial rate assay test kit" from Span Diagnostics Ltd. Creatinine reacts with picric acid in an alkaline medium to form an orange colored complex. The rate of formation of this complex is measured by reading the change in absorbance at 505 nm in a selected interval of time and is proportional to the concentration of creatinine.

**Procedure:** 100 µl of plasma aliquot was pipette in 10×75 mm tubes in duplicate, to which 1000 µl of working creatinine reagent was added. Blank (100 µl distilled water) and standard (100 µl from creatinine standard 2 mg/dl) was pipette in duplicates, to which 1000 µl of working creatinine reagent was added. The content was mixed well. UV- spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured at 505 nm. Measured initial absorbance of the Standard i.e. AS1 after 30 seconds and final absorbance i.e. AS2 after an interval of another 120 seconds. After standard reading was noted, reading of Test was taken i.e. AT1 and AT2 accordingly.

**Calculation:** The concentration of plasma creatinine was calculated as per formula and expressed in mg/100ml:

$$\text{Creatinine (mg/100 ml)} = \frac{(\text{AT2} - \text{AT1})}{(\text{AS2} - \text{AS1})} \times 2$$

### 3.4.2.6. Plasma glucose

Plasma glucose was estimated by "GOD-POD, End point assay test kit" supplied by Span Diagnosis Ltd. Glucose oxidase (GOD) oxidizes glucose to gluconic acid and Hydrogen Peroxide. In presence of enzyme Peroxidase, released Hydrogen Peroxide is coupled with phenol and 4-Aminoantipyrine (4-AAP) to form coloured Quinoneimine dye. Absorbance of coloured dye is measured at 505 nm and is directly proportional to glucose concentration in the sample.

**Procedure:** 20 µl of plasma aliquot was pipette in 10×75 mm tubes in duplicate, to which 1500 µl of working glucose reagent was added. Blank (20 µl distilled water) and standard (20 µl from standard 50 mg/dl) were pipette in duplicate to which 1500 µl of working glucose reagent was added. The contents were mixed well and incubated at 37 °C for 10 min. 1500 µl of purified water was added to each tube. The contents were mixed well. UV- spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured.

**Calculation:** Plasma glucose levels were calculated as per formula and expressed in mg/100ml:

$$\text{Glucose (mg/100ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

### 3.4.2.7. Plasma cholesterol

Plasma Cholesterol was estimated in plasma samples by "CHOD-PAP, End point assay test kit" supplied by Span Diagnosis Ltd. The principle of assay was cholesterol esters are hydrolyzed by cholesterol esterase to give free cholesterol and fatty acids. In subsequent reaction, cholesterol oxidase oxidizes the 3-OH group of free cholesterol to liberate cholest-4-en-3-one and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide couples with 4-aminoantipyrine and phenol to produce red quinoneimine dye. Absorbance of coloured dye was measured at 505 nm and is proportional to amount of total cholesterol concentration in the sample.

**Procedure:** 10 µl of plasma aliquots were pipette in 10×75 mm tubes in duplicate, to which 1000 µl of working cholesterol reagent was added. Blank (10 µl distilled water) and standard (10 µl from standard 200 mg/dl) was pipette in duplicate, to which 1000 µl of working cholesterol reagent was added. The content were mixed well and incubated at 37 $^{o}$C temperature for 10 minutes. UV- spectrophotometer was blanked

with reagent blank and the absorbance of standard and test sample was measured at 505 nm.

**Calculation:** The cholesterol concentration was calculated as per the formula and expressed in mg/100ml:

$$\text{Cholesterol (ml/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

### 3.4.2.8. Plasma triglyceride

Plasma Triglycerides was estimated in plasma samples by "End point assay test kit" supplied by Span Diagnosis Ltd. The principle of assay was Triglycerides are hydrolyzed by lipoprotein lipase (LPL) to give glycerol and free fatty acids. In subsequent reaction, glycerol 3-$PO_4$ oxidase oxidizes the 3- $PO_4$ group of free glycerol to liberate dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide couples with 4-aminoantipyrine and 4-chlorophenol to produce red quinoneimine dye. Absorbance of coloured dye was measured at 505 nm and is proportional to amount of total triglycerides concentration in the sample.

**Procedure:** 10 µl of plasma aliquots were pipette in 10×75 mm tubes in duplicate, to which 1000 µl of working triglycerides reagent was added. Blank (10 µl distilled water) and standard (10 µl from standard 200 mg/dl) was pipette in duplicate, to which 1000 µl of working triglycerides reagent was added. The content were mixed well and incubated at 37 $^{o}$C temperature for 10 minutes. UV- spectrophotometer was blanked with reagent blank and the absorbance of standard and test sample was measured at 505 nm.

**Calculation:** The triglyceride concentration was calculated as per the formula and expressed in m mg/100ml:

$$\text{Triglyceride (mg/100 ml)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

### 3.4.2.9. Aspartate aminotransferase (AST) activity

AST in plasma of calves was determined by UV modified, kinetic assay AST test kit (Span diagnostic ltd. Surat, India).

**Assay Principle:** AST catalyses the transamination of L-aspartate and α-ketoglutamate and oxaloacetate. In subsequent reaction, malate dehydrogenase reduces oxaloacetate to malate with simultaneous oxidation of reduced nicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD). The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm and is directly proportional to AST activity in the sample. Lactate dehydrogenase is added to enzyme system to prevent endogenous pyruvate interference, which is normally present in the plasma.

**Reagents**

| Reagent | Composition | Concentration |
|---|---|---|
| 1 (Buffer) | Tris buffer (pH 7.8) | 80 mmol/l |
| | L- Aspartate | 240 mmol/l |
| | MDH | ≥ 600 U/l |
| | LD | ≥ 600 U/l |
| 2 (Substrate) | α- Ketoglutarate | 12 mmol/l |
| | NADH | 0.18 mmol/l |

**Procedure:** Working AST reagent was prepared, as mentioned in test kit protocol, by mixing reagent 1 and reagent 2 in proportion of 1:4. 100 µl of plasma was mixed well with 1000 µl of working AST reagent and read absorbance at 340 nm in biochemical analyzer (BS-120 chemistry analyzer). Blank the analyzer with purified water. Read absorbance after 60 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds. Determine the mean absorbance change per minute (Δ A/minute).

**Calculation**

$$\text{AST activity (IU/l)} = \Delta\,\text{A/minute} \times \text{K}$$

Where; Δ A/minute is change in absorbance per minute and K is kinetic factor

**3.4.2.10. Alanine aminotransferase (ALT) activity**

ALT in plasma of calves was determined by UV modified, kinetic assay ALT (GPT) test kit (Span diagnostic ltd. Surat, India).

**Assay principle:** ALT catalyses the transamination of L-alanine and α- ketoglutarate to form pyruvate and L-glutamate. In subsequent reaction, lactate dehydrogenase

reduces pyruvate to lactate with simultaneous oxidation of reduced NADH to NAD. The rate of oxidation of NADH is measured kinetically by monitoring the decrease in absorbance at 340 nm and is directly proportional to ALT activity in the sample. Lactate dehydrogenase rapidly and completely reduces endogenous sample pyruvate during the initial incubation period, so that it does not interfere with the assay.

**Reagents**

| Reagent | Composition | Concentration |
|---|---|---|
| 1 (Buffer) | Tris buffer (pH 7.5) | 100 mmol/l |
| | L- Alanine | 500 mmol/l |
| | LD | ≥ 1200 U/l |
| 2 (Substrate) | α- Ketoglutarate | 15 mmol/l |
| | NADH | 0.18 mmol/l |

**Procedure:** Working ALT reagent was prepared, as mentioned in test kit protocol, by mixing reagent 1 and reagent 2 in proportion of 1:4. 100 µl of plasma was mixed well with 1000 µl of working ALT reagent and read absorbance at 340 nm in biochemical analyser. Blank the analyser with purified water. Read absorbance after 60 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds. Determine the mean absorbance change per minute (Δ A/minute).

**Calculation**

$$\text{ALT activity (IU/l)} = \Delta\,\text{A/minute} \times \text{K}$$

Where; Δ A/minute is change in absorbance per minute and K is kinetic factor

**3.4.2.11. Alanine phosphatase (ALP) activity**

ALP in plasma of calves was determined by p-Nitrophenyl Phosphate (PNPP)-AMP (IFCC) kinetic assay test kit (Span diagnostic Pvt. Ltd., India).

**Assay principle:** ALP catalyses the hydrolysis of colourless PNPP to yellow coloured p-nitrophenol and phosphate. Change in absorbance due to yellow colour formation is measured kinetically at 405 nm and is proportional to ALP activity in the sample.

**Reagents**

| Reagent | Composition | Concentration |
|---|---|---|
| 1 (Buffer) | AMP | 300 mM |
| | Magnesium acetate | 2 mM |
| | Zinc sulphate | 0.8 mM |
| | Chelator | Qs |
| 2 (Substrate) | pNPP | 10 mM |
| | Stabiliser | Qs |

**Procedure:** Working ALP reagent was prepared, as mentioned in test kit protocol, by mixing reagent 2 with reagent 1. 20 µl of plasma was mixed well with 1000 µl of working ALP reagent and read absorbance at 405 nm in biochemical analyser. Blank the analyser with purified water. Read absorbance after 30 seconds. Repeat reading after every 30 seconds i.e. upto 120 seconds. Determined the mean absorbance change per minute (Δ A/minute).

**Calculation**

$$\text{ALP activity (IU/l)} = \Delta\,\text{A/minute} \times \text{K}$$

Where; Δ A/minute is change in absorbance per minute and K is kinetic factor

**3.4.3. Antioxidant parameters**

In the present study, SOD, CAT and FRAP assay were used as indicator of antioxidant status, SOD and CAT were analyzed in haemolysate and FRAP assay was performed in plasma samples.

**3.4.3.1. Preparation of haemolysate**

Blood sample of experimental calves was taken in 2 ml centrifuge tube and centrifuged at 3000 rpm in refrigerated centrifuge for 15 minute. Plasma was taken off and the buffy coat was discarded and the compact RBC pellet was used for the preparation of the lysate. The pellets were washed thrice with normal saline (0.9% NaCl) solution. Haemolysate was prepared by taking 0.1 ml of washed pellets and adding 0.9 ml of chilled distilled water and stored at -20ºC till further analysis.

**3.4.3.2. Superoxide dismutase (SOD) activity**

SOD was estimated as per method described by Madesh and Balasubramanian (1998).

**Principle:** It involves generation of super oxide by pyrogallol autoxidation and the inhibition of super oxide dependent reduction of the tetrazolium dye MTT (3-(4-5,-dimethylthiazol-2yl) -2, 5-diphenyl tetrazolium bromide) to its formazan which was measured 570 nm. The reaction was terminated by the addition of dimethyl sulfoxide (DMSO), which helps to solubilize the formazane formed. The colour evolved was stable for many hours and expressed as SOD units (one unit of SOD is the amount (mg) of hemoglobin required to inhibit the MTT reduction by 50%).

**SOD Reagents**

**1. 100 µM pyragallol** (Sisco Research Laboratories Pvt. Ltd. India): 63 mg pyragallol was dissolved in the 5 ml of distilled water. One ml from this solution was added to 100 ml of distilled water.

**2. 1.25 mM MTT** (Sigma-Aldrich, Missouri, USA) 2.58 mg MTT was dissolved in 5 ml of distilled water.

**3. 50 mM Phosphate buffer saline (PBS)**: Prepared by mixing two solutions as below: Solution A: 8.9 gm of $NaHPO_4.2H_2O$ dissolved in DW and volume was made to 1 litre. Solution B: 6.81 gm of $KH_2PO_4$ dissolved in DW and volume was made to 1 litre. To solution A, solution B was added until pH reached to 7.0.

**4. Dimethyl sulfoxide (DMSO)**

**Procedure:** The reagents were added sequentially in the sample, control and the blank as shown below:

| Reagents | Sample | Control | Blank(duplicate) |
|---|---|---|---|
| PBS | 0.65 ml | 0.65 ml | 0.65ml |
| MTT | 30 µl | 30 µl | 30 µl |
| Haemolysate | 10 µl | - | - |
| Pyragallol | 75 µl | 75 µl | 75 µl |
| Incubated for 5 minute at room temperature | | | |
| DMSO | 0.75 ml | 0.75 ml | 0.75 ml |
| Haemolysate | - | 10 µl | - |

The absorbance of sample was read at 570 nm against blank.

**Calculation**

% inhibition of MTT reduction by SOD protein (Y%) = (OD of sample ×100)/OD of control

$$\text{SOD (U)} = \frac{(\text{mg of haemoglobin} \times 50 \times \text{Dilution factor})}{\text{Y \%}}$$

**3.4.3.3. Catalase (CAT) Activity**

The enzyme activity was estimated by method of Aebi (1984).

**Principle:** Catalase catalyzes the decomposition of $H_2O_2$ to give $H_2O$ and $O_2$. Catalase activity can be measured by following either the decomposition of $H_2O_2$ or the liberation of $O_2$. The method of choice for biological material is the UV spectrophotometric method. In the ultraviolet range, $H_2O_2$ shows a continual increase in absorption with decreasing wavelength. The decomposition of $H_2O_2$ can be followed directly by the decrease in extinction per unit time at 240 nm. The difference in extinction per unit time is a measure of catalase activity.

**Reagents**

1. **Phosphate buffer (50mM; pH 7.0):** Prepared by mixing solution A & B. Solution A: 8.9 gm of $NaHPO_4.2H_2O$ dissolved in DW and volume was made to 1 litre. Solution B: 6.81 gm of $KH_2PO_4$ dissolved in DW and volume was made to 1 l. To solution A, solution B was added until pH reached to 7.0.
2. **$H_2O_2$ (30mM):** Diluted 0.34 ml of 30% $H_2O_2$ with phosphate buffer to 100ml.

**Procedure:** 100 µl haemolysate was taken in a fresh clean test tube and 4.9 ml PBS was added to it. The reaction mixture (total volume of 3.0 ml) contained 2.0 ml of RBC lysate in appropriate dilution with phosphate buffer (50mM, pH 7.0) and 1 ml of $H_2O_2$ (30mM) at $25^0$C against a blank containing 1 ml phosphate buffer instead of substrate ($H_2O_2$) and 2ml haemolysate. The reaction was started by addition of $H_2O_2$. The decomposition of $H_2O_2$ was shown by decrease in absorbance at 240 nm using double beam spectrophotometer. The initial absorbance was approximately A is equal to 0.500 and the decrease in absorbance was followed for 1 minute. The difference in absorbance per unit time was the measure of catalase activity. The enzyme activity was calculated using an extinction coefficient of 0.0394 l $mM^{-1}$ $mm^{-1}$, and expressed as moles of $H_2O_2$ consumed /min/g Hb in blood.

**Calculation**

Catalase activity = (5 second O.D - 65 second O.D)×Dilution factor×3

×100/Hb gm%×0.0394×1000

Where; 0.0394 is the extinction coefficient.

**3.4.3.4. Ferric reducing antioxidant power (FRAP) assay**

Total antioxidant status (TAS) was measured by FRAP assay procedure described by of Benzie and Strain (1999).

**Principle:** The principle of this method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex ($Fe^{3+}$-TPTZ) to its ferrous colored form ($Fe^{2+}$-TPTZ) in the presence of antioxidants. FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, employing easily reduced oxidant system present in stoichimetric excess.

**FRAP Reagent**

1. (A) Acetate buffer (3.0 mM, pH 3.6): Weighed 3.1 gm sodium acetate trihydrate and added 16 ml of glacial acetic acid and made the volume to 1.0 litre with distilled water.

(B) Ferric chloride (2 mM in 40 mM HCl)

(C) Tripyridyl triazine (10 mM)

The working FRAP reagent was prepared by mixing A, B & C in the ratio of 10:1:1, at the time of use.

2. Ascorbic acid (100 µM)

**Procedure:** 100 µl of plasma was mixed with 3 ml of working FRAP reagent and absorbance was measured at 0 minute after vortexing. Thereafter, samples were placed at 37°C in water bath and absorbance was measured after 4 minutes. Ascorbic acid standards (100 µM-1000 µM) were processed in the same way.

$$\text{FRAP value } (\mu\text{mol/l}) = \frac{A-B}{X-Y} \times 100$$

Where; A is reading of sample at 0 minute, B is reading of sample at 4 minute, X is reading of standard at 0 minute, Y is reading of standard at 4 minute and is FRAP value of 100 µM standard.

### 3.4.3.5. Lipid peroxidation (LPO)

TBARS is the measure of lipid peroxidation. The extent of lipid peroxidation was evaluated in terms of malondialdehyde (MDA) production, which was determined by the method of Rehman (1984). One ml packed erythrocyte (33%) was taken to which 1ml of 10%TCA was added and thoroughly mixed, vortex and centrifuged at 2000 rpm of 10 min. To one ml of supernatant liquid, an equal amount of 0.67% TBA was added and kept in boiling water bath for 10 min. The reaction mixture was cooled under running tap water and diluted with one ml distilled water. Absorbance was recorded ad 535 nm. Calculation was done by using the major extinction coefficient of MDA-TBA complex at 535 nm, i.e. $1.56\times 10^{8}$M/cm. The amount of lipid peroxidation was expressed as nM MDA formed /ml packed RBCs.

LPO (nM MDA/ml packed RBCs = (OD/EC) × (Total volume of the reaction mixture/Amount of sample taken) $\times 10^{9} \times$DF×2 (Incubation time)

Where, EC = $1.56 \times 10^{8}$ M/cm molar extinction coefficient.

### 3.4.4. Immune Status

### 3.4.4.1. Plasma total immunoglobulin

The total immunoglobulin in the plasma samples of calves were estimated by zinc turbidity method (Mc Ewan et. al., 1970).

**Procedure:** 100 µl of plasma sample was taken in a clean dry test tube. To this 12 ml of test reagent (prepared by taking 4.1 ml of 5% $ZnSO_4$ and the volume made to 1 l with freshly prepared distilled water) was added and incubated at room temperature (15-30 $^{o}$C) for 60 minutes. The standards (4-40 mg/ml) were prepared in fetal calf plasma and processed similar to the samples. The optical density (OD) was taken at 460 nm. The OD of samples was plotted against the standard curve (Figure 3.1) and concentration of total immunoglobulin in the plasma samples were estimated and expressed as mg/ml.

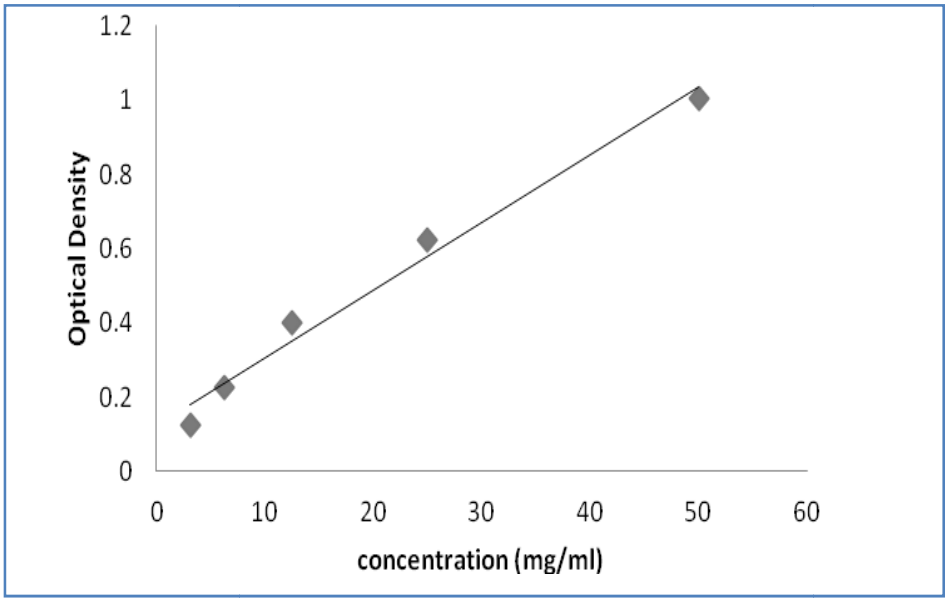



**Figure 3.1. OD and total immunoglobulin concentration for standard**

### 3.4.5. Biomarker of lipid mobilization

#### 3.4.5.1. Non-esterified fatty acids (NEFA)

The copper soap solvent extraction method modified by Shipe et al. (1980) was adopted for the estimation of plasma NEFA concentration.

**Principle:** The proteins of plasma are precipitated with hydrochloric acid and thus the fatty acids are free from proteins. The free fatty acids are subjected to react with copper reagent to convert copper soaps, which are recovered by extraction with a chloroform heptane methanol solvent. The extracted copper soaps are reacted with a colour reagent to develop a yellow colored compound, which is measured colorimetrically at 440 nm.

**Reagents**

1. **Copper reagent:** The copper reagent was a mixture of 5 ml of triethanolamine and 10 ml of 1 M aqueous cupric nitrate [Cu $(NO_3)_2.3H_2O$] diluted to 100 ml with saturated sodium chloride solution. The pH was adjusted to 8.3 with 1 N sodium hydroxide solution. The mixture was stored in the dark at room temperature, in order to ensure that the material remained stable for a period of at least 4-5 months.

2. **Colour reagent:** It was a mixture of 0.5% sodium diethyl dithiocarbamate solution in n-butanol (0.5 g per 100 ml).
3. **Solvent mixture:** Solvent mixture consisted of chloroform, heptane and methanol (all GR grade) in the ratio of 49:49:2 (v/v) respectively and the mixture was designated as CHM.
4. **Stock standard palmitic acid solution:** 5.12 gm of pure palmitic acid was dissolved in 100 ml solvent mixture giving a solution of palmitic acid concentration of 0.2 mol/l. From this solution, 1 ml was taken in a 100 ml with solvent mixture giving a final concentration of 2 mmol/l or 2000 µmol/l.
5. **Working standard palmitic acid solution:** 8 ml of stock solution was first diluted with 2 ml of solvent mixture to obtain a solution of palmitic acid concentration of 1600 µmol /l. This solution (1600 µmol/l concentration) was then diluted serially to obtain a series of working standard solutions having the concentrations of 800, 400, 200, 100, 50, µmol/l.

**Procedure:** 100 µL of 0.7 N HCl was added to 0.5 ml plasma sample in duplicate in a 16 x 125 mm screw cap test tube. The mixture was shaken on a vortex test tube mixer. Simultaneously, 0.5 ml aliquots of working standard solutions having a concentrations of 0 (blank), 50, 100, 200, 400, 800,1600 µmol/l palmitic acid were taken in another 7 screw cap test tubes (16 × 125 mm). Thereafter, 2 ml of copper reagent followed by 6 ml of the solvent mixture were added and all the test tubes were shaken for 30 minutes on a shaker at 240 rpm. Content was centrifuged for 10 minutes at 4°C at 3000 rpm in a refrigerated centrifuge. 3.5 ml of the solvent layer was transferred to an acid washed test tube containing 0.1 ml of the colour reagent. After mixing, the colour intensity (yellow colour) was measured at 440 nm within one hour using spectrophotometer (Model: Spectronics 118) against blank. The standard curve was drawn for different concentrations of palmitic acid against OD readings. The OD of samples was plotted against the standard curve and the concentrations of NEFA in the samples was estimated and expressed as µmol NEFA/l of plasma.

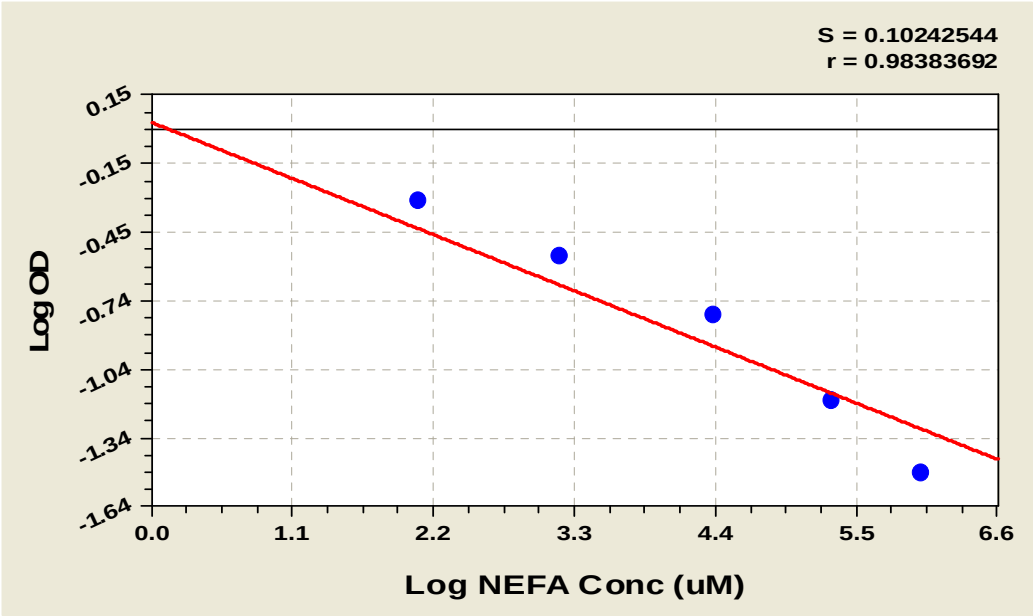



**Fig. 3.2. Standard curve for NEFA determination**

### 3.4.5.2. BHBA analysis

BHBA was analysed by distillation method Weichselbaum and Somogyi, 1941). In the determination of ketone bodies it is customary to convert β-hydroxybutyric acid into acetone for estimation. This may be brought about during distillation. When distillation is employed preformed acetone plus acetoacetic acid may be estimated separately from β-hydroxybutyric acid in the blood. Acetone in the distillates is converted into the 2:4-dinitrophenylhydrazone, which is extracted with carbon tetrachloride and estimated colorimetrically by a modification of the method of (Greenberg and Lester, 1944).

**Reagents:** Sulphuric acid (20N); potassium dichromate 2.5% (w/v); freshly filtered 2:4-dinitrophenylhydrazine 0.1% (w/v) in 4N HCl; carbon tetrachloride; NaOH (0.5N); barium hydroxide (0.3N); zinc sulphate 5% (w/v) $ZnSO_4,7H_2O$ (10 ml. of the barium hydroxide solution added drop by drop with phenolphthalein as indicator, should neutralize exactly 10 ml. of the $ZnSO_4$ solution). Distilled water is used throughout the determination.

**Preparation of standard solution of BHBA:** The calcium-zinc salt of β-hydroxybutyric acid was prepared according to Blunden (1938). Colourless needles and plates were obtained (m.p. 247.5-248.5° uncorr. with decomp.). Sodium β-hydroxybutyrate to be 98% pure (Thin and Robertson, 1952) was used also. Solutions were made from both preparations for use as standards.

**Apparatus:** An apparatus constructed from B-19 standard ground-glass joints is employed. The distillation tube (bore 8 mm.) is fused to a length of capillary tubing (bore 2 mm.) to form a delivery tube.

**Procedure**

**1. De-proteinization:** 100µl Plasma is haemolysed by mixing it with water (7 ml) in a centrifuge tube of 15 ml. The tube is stoppered and shaken. Barium hydroxide solution (0.4 ml) is added and the tube shaken to ensure complete mixing, followed by the addition of $ZnSO_4$ solution (0.4 ml) and further vigorous shaking. The mixture is centrifuged and the supernatant liquid filtered through Whatman no. 44 filter paper. The filtrate is used for the estimation of ketone bodies.

**2. Distillation** The apparatus should be assembled with silicone high-melting-point grease between the joints. It is advisable to boil it out with water after assembly and before use each day. Into a 100 ml. flask, containing three small glass beads, is placed $H_2SO_4$ (8 ml) and water (50 ml), followed by 5 ml of deproteinized filtrate.

The flask is immediately fitted to the apparatus with cold water circulating through both condensers, and then boiled gently under reflux. After refluxing for 5 min., the circulating water is drained from the reflux condenser, allowing the vapor to distil and collect in a 25 ml. stoppered-type graduated cylinder containing 1 ml. of 2:4-dinitrophenylhydrazine reagent. The rate of distillation is increased progressively and the graduated cylinder should be clamped so that the delivery tube dips beneath the surface of the reagent. When 5 ml. of distillate has collected, the graduated cylinder is lowered slightly to expose the delivery tube, which is washed externally with a fine jet of water (4 ml), and internally by allowing a further 5 ml of distillate to collect in the cylinder. Immediately, distillation is stopped by circulating cold water through the reflux condenser. Potassium dichromate solution (2 ml) is introduced into the apparatus via the tap funnel, followed by water (10 ml), which should rinse into the apparatus any dichromate remaining in the funnel. After refluxing for 40 min. a distillate is collected as described above. Boiling must be continuous throughout the procedure. The first distillate is analysed for its acetone content and represents acetone plus acetoacetic acid. The second distillate contains the acetone derived from the oxidation of BHBA.

**3. Colorimetric estimation:** The distillate which has collected in the 25 ml graduated cylinder is made up to exactly 15 ml with water. Carbon tetrachloride (6 ml) is added from an automatic burette and the cylinder is stoppered and shaken mechanically for 150 min. After it has separated, the supernatant acid solution is sucked off. Without shaking it, the cylinder is filled twice with water which is sucked off each time; finally, NaOH solution (9 ml) is added. The solutions are shaken for a further 5 min. and allowed to stand. When the carbon tetrachloride layer is perfectly clear the optical density is read in a spectrophotometer at 350 nm. BHBA in the samples was estimated and expressed as mmol/L of plasma.

### 3.5. Biochemical analysis of urine samples

**3.5.1.** Urine pH is measured by the scientific pH meter available in the department of veterinary biochemistry of DUVASU, Mathura. Urine specific gravity is measured by the scientific Dipstick (Span Array).

### 3.5.2. Uric acid and urea

Uric acid concentration in urine was measured by using kit of SPAN diagnostic Ltd. Surat

Urea concentration in urine was measured by diacatyl monoxime method of Marsh et al., (1965). Briefly, 1 ml distilled water and 1 ml of 10% TCA was added to 0.2 ml diluted urine (1;10) Mixed thoroughly and centrifuged at 3000 rpm and took 0.2 ml for supernatant with 3 ml of colour reagent. Simultaneously reagents blank and standard (5, 15, 30 and 50 m mol/l) were put replacing the urine supernatant with water and standard, respectively. Kept in boiling water both for 20 minutes and cooled at room temperature and measured optical density at 520 nm by spectrophotometer within 15 minutes. Concentration of urea was calculated by formula.

Urea (mmol/L) = (OD of unknown/OD of standard) × concentration of standard

### 3.6. Statistical analysis

The generated data were subjected to analysis of variance using the General Linear Model (GLM) procedure of the Statistical Software Package (SPSS for windows, V21.0; Inc., Chicago, IL, USA). The days and treatment effect was tested using the following model:

$$Yij = \mu + Ti + Dj + eij$$

Where; *Yij* is dependent variable, $\mu$ is overall mean of the population, *Ti* is mean effect of the treatment, *Dj* is mean effect of day of sampling (j = 0, 30, 60 and 90 days of dietary treatment), and *eij* is unexplained residual element assumed to be independent and normally distributed. Individual animals were used as the experimental unit for all data. The pair-wise comparison of means was carried out using "Tukey's honest significant difference (HSD) test". Significance was determined at $P<0.05$ and the values are presented in the tables.

Photographs 1: Feeding of experimental calves


Photographs 2: Blood collection of experimental calves


Photographs 3: Laboratory work during experimental period

Photographs 4: Laboratory work during experimental period

# Results

# CHAPTER- 4

# RESULTS

The present study has been carried out for the determination of the effect of Tinospora and Asparagus on growth performance, immunity, antioxidant property and other biochemical parameters in blood and urine of young calves. The results obtained during the course of this study have been presented and discussed in respective sections.

## 4.1. Chemical composition of Tinospora and Asparagus

The chemical composition of Tinospora and Asparagus used for the feeding of calves in the present study is presented in Table 4.1. Tinospora contains higher proportion of CP, EE and CF while less content of NFE compared to the Asparagus. Content of DM and ash are similar among Tinospora and Asparagus.

**Table 4.1. Chemical composition (% on DM basis) of Tinospora and Asparagus**

| Nutrient | Tinospora cordifolia (Giloy) | Asparagus racemosus (Shatavari) |
|---|---|---|
| DM | 68.34 | 64.18 |
| CP | 8.04 | 3.50 |
| EE | 1.43 | 0.52 |
| CF | 54.19 | 6.84 |
| Ash | 10.26 | 9.93 |
| NFE | 26.08 | 79.21 |

## 4.2. Growth performance

### 4.2.1. Body weight

BW change of growing calves of three different groups is depicted in Table 4.2. Mean value showed non-significant effect of supplementation of Tinospora and Asparagus on BW change. The BW at the beginning of the study in three different groups was 31.50, 31.50 and 31.50 kg, respectively. However, BW at the end of the 90 days study was 54.33, 53.66 and 52.66 kg, respectively. The mean BW change during study period was 41.60, 41.40 and 40.76 kg, respectively in calves of groups control, $T_1$ and $T_2$ groups, respectively. BW change showed linear increase with the days of study.

**Table 4.2. Effect of herbal supplementation on BW change (kg) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 31.50 | 31.50 | 31.50 | 2.45 | 0.251 |
| 15 | 33.83 | 34.00 | 33.66 | 2.59 | 0.514 |
| 30 | 36.75 | 36.50 | 36.33 | 2.69 | 0.325 |
| 45 | 40.50 | 40.33 | 39.66 | 2.83 | 0.153 |
| 60 | 44.83 | 44.50 | 43.66 | 2.96 | 0.547 |
| 75 | 49.50 | 49.33 | 47.83 | 3.09 | 0.326 |
| 90 | 54.33 | 53.66 | 52.66 | 3.38 | 0.128 |
| Mean | 41.61 | 41.40 | 40.76 | 2.11 | 0.571 |

Although the Average daily gain (ADG) was numerically higher in Tinospora followed by Asparagus supplemented groups but mean value showed non significant effect (Table 4.3). ADG at first fortnight of the study in three respective groups were 144, 155 and 167 g/day and at the last fortnight ($6^{th}$ fortnight) of the experiment were 322, 322 and 289 g/day, respectively. The mean ADG during 90 days study period averaged 235, 254 and 246 g/day, respectively.

**Table 4.3. Effect of herbal supplementation on ADG (g/day) in calves**

| Fortnight | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 1 | 144 | 155 | 167 | 16 | 0.268 |
| 2 | 178 | 195 | 167 | 27 | 0.688 |
| 3 | 222 | 250 | 255 | 25 | 0.103 |
| 4 | 267 | 289 | 278 | 23 | 0.852 |
| 5 | 278 | 311 | 322 | 25 | 0.229 |
| 6 | 322 | 322 | 289 | 14 | 0.682 |
| Mean | 235 | 254 | 246 | 23 | 0.582 |

### 4.2.2. Height

Height gain in calves in three different groups measured at monthly intervals and presented in Table 4.4. Similar to the BW gain, herbal supplementation did not exert any effect on height gain in calves. Height gain at the beginning of study in

three respective groups were 91.28, 91.65 and 93.81 cm respectively while at the end of experiment were 96.45, 96.50 and 98.61 cm respectively. Although the height gain is higher in the calves of $T_2$ group but mean value showed non significant effect. The mean height during 90 days study period was 93.84, 94.02 and 96.29 cm, respectively.

**Table 4.4. Effect of herbal supplementation on height gain (cm) in experimental calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 91.28 | 91.65 | 93.81 | 2.79 | 0.128 |
| 30 | 92.96 | 93.08 | 95.50 | 2.90 | 0.549 |
| 60 | 94.66 | 94.85 | 97.23 | 2.82 | 0.285 |
| 90 | 96.45 | 96.50 | 98.61 | 2.79 | 0.167 |
| Mean | 93.84 | 94.02 | 96.29 | 2.45 | 0.154 |

### 4.2.3. Length

Length gain in calves in three different groups measured at monthly intervals and presented in Table 4.5. Similar to the Height gain, herbal supplementation did not exert any effect on length gain. Length gain at the beginning of study in three respective groups were 42.3, 41.71 and 42.15 cm respectively while and at the end of experiment were 47.93, 47.85 and 48.11 cm, respectively. The mean length gain during 90 days study averaged 45.12, 44.67 and 45.10 cm, respectively.

**Table 4.5. Effect of herbal supplementation on length (cm) of experimental calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 42.30 | 41.71 | 42.15 | 2.93 | 0.239 |
| 30 | 44.28 | 43.51 | 44.03 | 2.96 | 0.825 |
| 60 | 45.95 | 45.60 | 46.08 | 2.94 | 0.358 |
| 90 | 47.93 | 47.85 | 48.11 | 2.95 | 0.568 |
| Mean | 45.12 | 44.67 | 45.10 | 2.08 | 0.240 |

### 4.2.4. Body condition score (BCS)

In the present study, BCS measured at 5 point scale ranges in between 2.60-3.0 (Table 4.6). Even though the treatment exerts significant effect (P<0.05) on the BCS at 90$^{th}$ days of the study but overall mean value showed non significant effect. Mean BCS during 90 days study period averaged 2.73, 2.83 and 2.83 in Control, $T_1$ and $T_2$ groups, respectively. BCS at day 90 of the study in three respective groups were 2.70, 3.00 and 2.90.

**Table 4.6. Effect of herbal supplementation on BCS (5 point score)**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 2.60 | 2.80 | 2.70 | 0.06 | 0.483 |
| 30 | 2.70 | 2.70 | 2.90 | 0.07 | 0.293 |
| 60 | 2.90 | 2.80 | 2.80 | 0.03 | 0. 719 |
| 90 | $2.70^{a}$ | $3.00^{b}$ | $2.90^{ab}$ | 0.09 | 0.048 |
| Mean | 2.73 | 2.83 | 2.83 | 0.03 | 0.182 |

### 4.2.5. Calf diarrhea, joint ill and calf mortality

The faecal consistency measured at 5 point scale ranges in between 2-2.8 (Table 4.7). Although the score of faeces was better in Tinospora and Asparagus supplemented calf but mean value showed non significant effect of the treatment. The mean score during 90 days study period was 2.50, 2.38 and 2.43 in Control, $T_1$ and $T_2$ groups, respectively. These score showed that faeces in three different groups was soft to loose in consistency, yellow in colour with slight odor. There was no incidence of calf scour or diarrhea in all three groups might be due to the routine feeding of probiotic.

**Table 4.7. Effect of herbal supplementation on calf diarrhea (5 point score)**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 2.80 | 2.80 | 2.70 | 0.06 | 0.966 |
| 30 | 2.60 | 2.30 | 2.40 | 0.15 | 0.882 |
| 60 | 2.20 | 2.40 | 2.60 | 0.20 | 0. 815 |
| 90 | 2.40 | 2.00 | 2.00 | 0.23 | 0.768 |
| Mean | 2.50 | 2.38 | 2.43 | 0.06 | 0.930 |

In the present study, no. of calf with joint ill is depicted in Table 4.8. At the beginning of the study there was no calf with joint ill in all three groups. Mean value showed significant effect (P<0.05) of the treatment and the incidence of joint ill was reported lower in Tinospora and Asparagus supplemented calves.

**Table 4.8. Effect of herbal supplementation on joint ill in calves (no. of calf affected)**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 0 | 0 | 0 | 0.00 | 1.000 |
| 30 | 1 | 1 | 0 | 0.33 | 0.015 |
| 60 | 0 | 0 | 1 | 0.33 | <0.001 |
| 90 | 1 | 0 | 1 | 0.33 | 0.024 |
| Mean | 0.50 | 0.25 | 0.25 | 0.083 | 0.028 |

In the present study, no death was reported in all three groups during 90 days experimental period.

## 4.3. Effect of Tinospora and Asparagus supplementation on hematological and biochemical attributes

### 4.3.1. Hematological parameters

Hematological attributes studied in present study were RBCs, WBCs, Hb, PCV or HIT, lymphocytes, and neutrophils. Mean values for RBCs showed significant effect (P<0.05) of herbal supplementation and count was higher in Asparagus supplemented calves (Table 4.9; Figure 4.1.). RBCs count at the start of experiment was 7.87, 8.81 and 9.25 × $10^6$/µl and at the end of experiment were 7.62, 9.26 and 8.43 × $10^6$/µl, respectively in three different groups. Mean RBCs count during 90 days study period was 8.45, 9.56 and 9.78 × $10^6$/µl, respectively in three respective groups.

**Table 4.9. Effect of herbal supplementation on RBCs ($10^6$/µl) count of experimental calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 7.87 | 8.81 | 9.25 | 1.03 | 0.793 |
| 30 | 7.86 | 8.82 | 8.27 | 1.02 | 0.046 |
| 60 | 7.88 | 8.68 | 7.83 | 0.95 | 0.072 |
| 90 | 7.62 | 9.26 | 8.43 | 0.79 | 0.058 |
| Mean | $8.45^{a}$ | $9.56^{ab}$ | $9.78^{b}$ | 0.86 | 0.040 |

Mean with different superscript in row differs significantly (P<0.05)

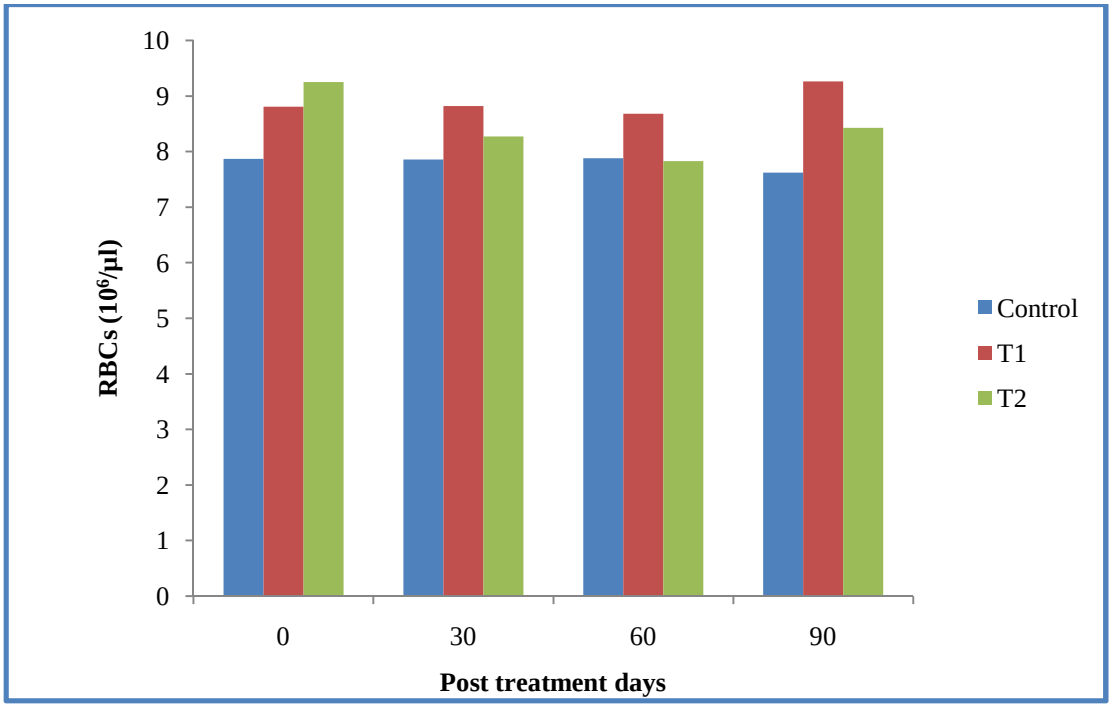



**Figure 4.1. Effect of herbal supplementation on RBCs count of calves**

Mean WBCs count of C, $T_1$ and $T_2$ groups were 12.48, 12.20, 13.53 × $10^3$/µl, respectively showed non-significant effect of treatment (Table 4.10). WBCs count in three respective groups at the start and end of the study were 13.65, 14.50, 13.53 × $10^3$/µl and 14.35, 12.80, 13.77 × $10^3$/µl. Mean WBCs count in three respective groups was 12.48, 12.20 and 13.53 × $10^3$/µl.

**Table 4.10. Effect of herbal supplementation on WBCs ($10^3$/µl) count of experimental calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 13.65 | 14.50 | 13.53 | 1.94 | 0.246 |
| 30 | 11.94 | 11.32 | 11.32 | 2.09 | 0.265 |
| 60 | 9.98 | 10.17 | 10.52 | 2.03 | 0.305 |
| 90 | 14.35 | 12.80 | 13.77 | 2.02 | 0.549 |
| Mean | 12.48 | 12.20 | 13.53 | 1.30 | 0.310 |

Mean values for Hb showed significant effect (P<0.05) of herbal supplementation (Table 4.11; Figure 4.2.). During 90 days study period, mean Hb concentration was found highest in group supplemented with Tinospora ($T_1$). Mean Hb concentration among three respective groups were 9.28, 9.80 and 9.50 g/100 ml blood. Hb concentrations at the beginning of experiment were 9.12, 9.33 and 9.08 g/100 ml blood and at the end of experiment were 9.71, 10.31 and 9.71 g/100 ml of blood, respectively.

**Table 4.11. Effect of herbal supplementation on Hb (g/100 ml) concentration of calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 9.12 | 9.33 | 9.08 | 0.41 | 0.069 |
| 30 | 9.18 | 9.56 | 9.46 | 0.58 | 0.083 |
| 60 | 9.11 | 9.98 | 9.73 | 0.53 | 0.978 |
| 90 | 9.71 | 10.31 | 9.71 | 0.57 | 0.449 |
| Mean | $9.28^{a}$ | $9.80^{b}$ | $9.50^{ab}$ | 0.23 | 0.049 |

Mean with different superscript in row differs significantly (P<0.05)

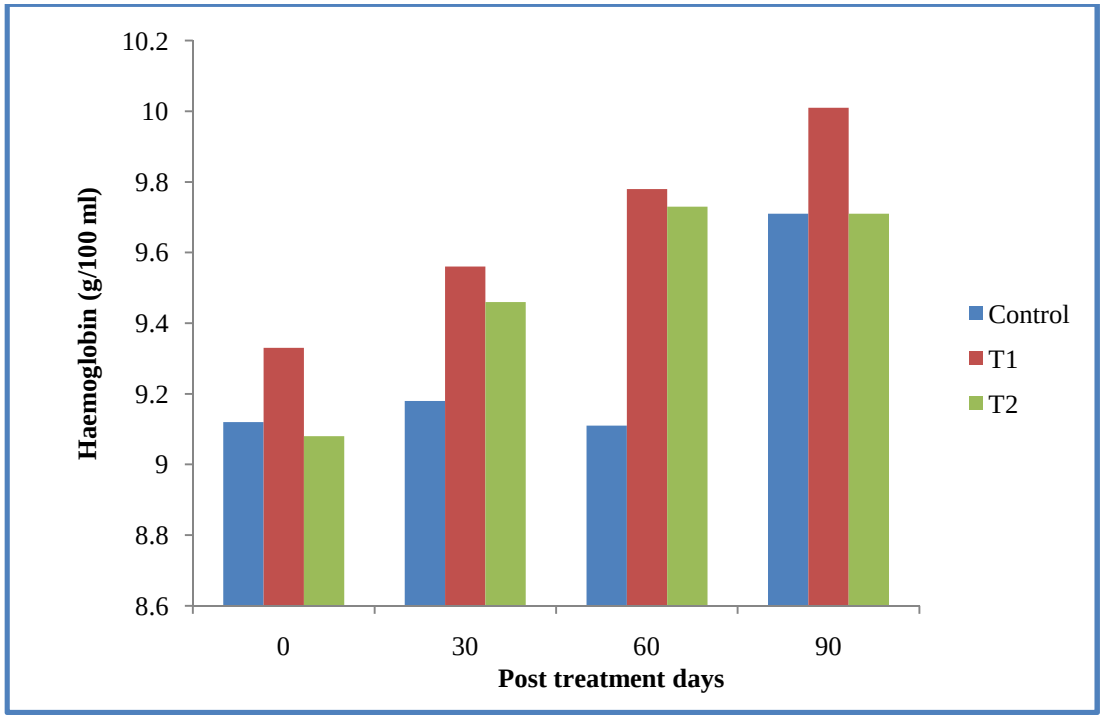



**Figure 4.2. Effect of herbal supplementation on Hb concentration of calves**

Similar to the Hb concentration, mean values for PCV or HIT also showed significant effect (P<0.05) of treatment (Table 4.12; Figure 4.3.). The overall mean PCV values were found to be 28.97, 29.55 and 31.72 % packed cells in control, $T_1$ and $T_2$ groups, respectively. PCV values in control, $T_1$ and $T_2$ groups averaged 30.53, 31.37 and 31.47 % packed cells, respectively at the start of study whereas; 31.85, 27.68 and 35.18 % packed cells, respectively at the 90$^{th}$ day of study.

**Table 4.12. Effect of herbal supplementation on PCV (%) value in experimental calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 30.53 | 31.37 | 31.47 | 3.04 | 0.436 |
| 30 | 27.60$^{a}$ | 31.37$^{b}$ | 30.53$^{ab}$ | 3.37 | 0.454 |
| 60 | 25.88 | 27.80 | 29.70 | 1.65 | 0.329 |
| 90 | 31.85 | 27.68 | 35.18 | 3.62 | 0.561 |
| Mean | 28.97$^{b}$ | 29.55$^{b}$ | 31.72$^{ab}$ | 2.14 | 0.319 |

Mean with different superscript in row differs significantly (P<0.05)

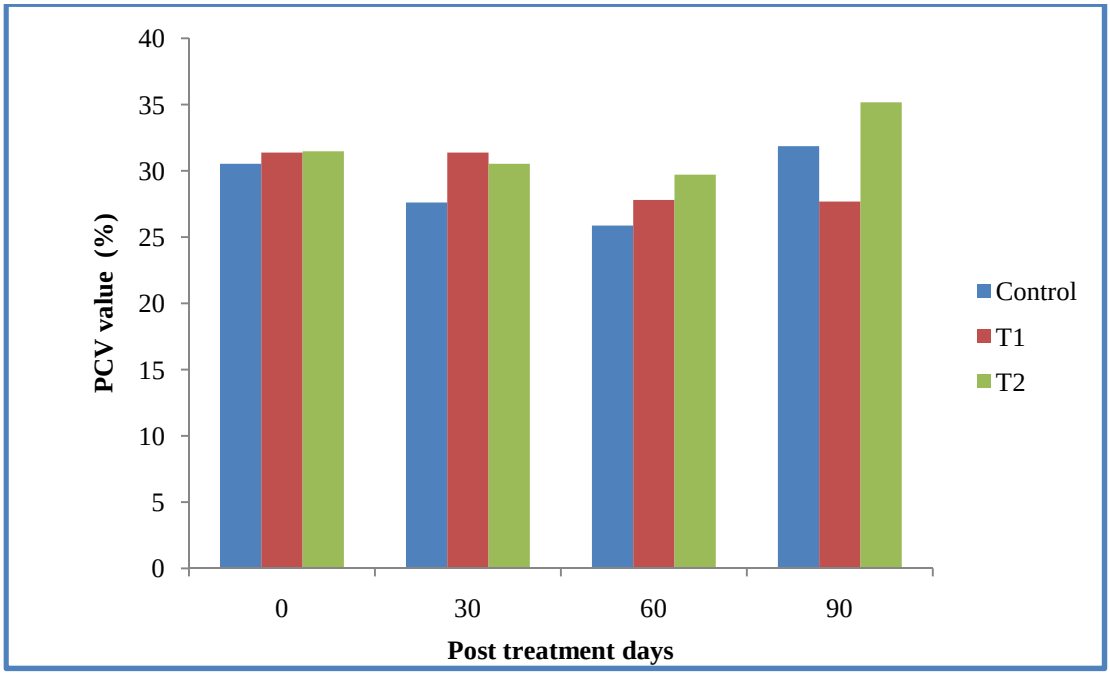



**Figure 4.3. Effect of herbal supplementation on PCV value of calves**

Mean values for lymphocytes does not show any significant effect of herbal supplementation (Table 4.13). Blood lymphocytes concentration in three respective groups averaged 65.16, 64.01 and 65.68 % of WBCs. Lymphocytes concentration at the start of experiment was 68.51, 69.53 and 68.93 % and at the end of experiment were 65.13, 61.70 and 64.88 %, respectively.

**Table 4.13. Effect of herbal supplementation on lymphocytes (%) count of calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 68.51 | 69.53 | 68.93 | 3.63 | 0.421 |
| 30 | 58.10 | 63.48 | 65.53 | 7.43 | 0.446 |
| 60 | 68.90 | 61.31 | 63.38 | 5.16 | 0.385 |
| 90 | 65.13 | 61.70 | 64.88 | 7.30 | 0.878 |
| Mean | 65.16 | 64.01 | 65.68 | 3.94 | 0.491 |

Similar to lymphocytes concentration, mean values for neutrophils does not show any significant effect of herbal supplementation (Table 4.14). Blood neutrophil concentration in Control, $T_1$ and $T_2$ groups averaged 29.00, 27.25 and 26.71 % of WBCs. Neutrophil concentration at the start of experiment was 23.33, 23.00 and 23.83 % and at the end of experiment were 31.50, 30.16 and 30.83 %, respectively.

**Table 4.14. Effect of herbal supplementation on neutrophils (%) count of calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 23.33 | 23.00 | 23.83 | 1.95 | 0.562 |
| 30 | 29.00 | 25.33 | 23.50 | 1.60 | 0.315 |
| 60 | 32.16 | 35.00 | 29.66 | 2.46 | 0.874 |
| 90 | 31.50 | 30.16 | 30.83 | 2.92 | 0.312 |
| mean | 29.00 | 27.25 | 26.71 | 1.22 | 0.065 |

### 4.3.2. Biochemical parameters

#### 4.3.2.1. Biomarkers of protein metabolism

In the present study, plasma concentrations of total protein, albumin, globulin and PUN were used as biomarkers of protein metabolism. Mean plasma total protein values does not showed significant effect between groups (Table 4.15). Mean plasma total protein levels in three respective groups averaged 7.10, 7.42 and 7.31 g/l, respectively. Plasma total protein levels at the start of study were 6.82, 7.45 and 7.18 g/l and at the 90$^{th}$ day of study were 7.50, 7.72, 7.47 g/l, in the calves of three respective groups.

**Table 4.15. Effect of herbal supplementation on plasma total protein concentration (g/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 6.82 | 7.45 | 7.18 | 0.44 | 0.077 |
| 30 | 6.85 | 7.28 | 7.32 | 0.25 | 0.052 |
| 60 | 7.21 | 7.20 | 7.27 | 0.26 | 0.056 |
| 90 | 7.50 | 7.72 | 7.47 | 0.22 | 0.082 |
| Mean | 7.10 | 7.42 | 7.31 | 0.19 | 0.196 |

Similar to the plasma total protein concentration, plasma albumin concentrations does not show any significantly effect of Tinospora and Asparagus supplementation in all groups (Table 4.16). Overall plasma albumin concentration among three different groups averaged 3.55, 3.65 and 3.50 g/l, respectively. Mean

plasma albumin levels in three respective groups at start of experiment were 3.58, 3.68 and 3.57 g/l, respectively whereas; mean plasma albumin concentration at the end of the study was averaged 3.60, 3.74 and 3.61 g/l, respectively.

**Table 4.16. Effect of herbal supplementation on plasma albumin level (g/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 3.55 | 3.65 | 3.50 | 0.22 | 0.508 |
| 30 | 3.56 | 3.71 | 3.60 | 0.12 | 0.140 |
| 60 | 3.58 | 3.60 | 3.55 | 0.14 | 0.148 |
| 90 | 3.60 | 3.74 | 3.61 | 0.18 | 0.140 |
| Mean | 3.58 | 3.68 | 3.57 | 0.08 | 0.335 |

Plasma globulin concentration in present study was determined by subtracting plasma albumin levels from plasma total protein levels. The periodic changes in plasma globulin concentration (g/l) during 90 days experimental period have been depicted in Table 4.17 and Figure 4.4. Mean plasma globulin concentration was significant (P<0.05) higher in Tinospora supplemented calves and averaged 3.38, 3.73 and 3.55 g/l, respectively. Mean plasma globulin concentration starts increasing at $90^{th}$ days of study in $T_1$ and $T_2$ groups.

**Table 4.17. Effect of herbal supplementation on plasma globulin level (g/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 3.51 | 3.61 | 3.47 | 0.40 | 0.198 |
| 30 | 3.54 | 3.65 | 3.50 | 0.39 | 0.209 |
| 60 | 3.32 | 3.77 | 3.54 | 0.36 | 0.216 |
| 90 | $3.31^{a}$ | $3.90^{b}$ | $3.67^{b}$ | 0.29 | 0.036 |
| Mean | $3.38^{a}$ | $3.73^{b}$ | $3.55^{ab}$ | 0.08 | 0.047 |

Mean with different superscript in row differs significantly (P<0.05)

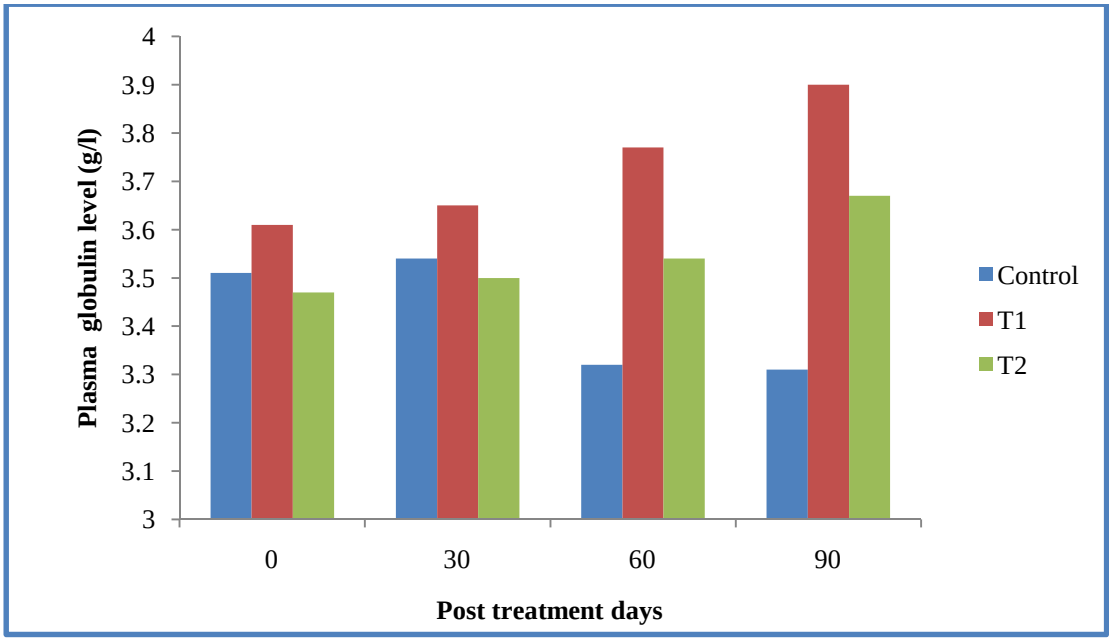



**Figure 4.4. Effect of herbal supplementation on plasma globulin level in calves**

Mean plasma A/G ratio does not showed significant effect and averaged 1.01, 0.98 and 0.95, respectively (Table 4.18). Plasma A/G ratio at the start of experiment was 1.05, 0.96 and 0.97 and at the end of experiment was 0.93, 0.94 and 0.92, respectively.

**Table 4.18. Effect of herbal supplementation on plasma A/G ratio in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 1.05 | 0.96 | 0.97 | 0.14 | 0.129 |
| 30 | 1.10 | 1.03 | 0.94 | 0.11 | 0.105 |
| 60 | 0.96 | 1.01 | 0.97 | 0.10 | 0.210 |
| 90 | 0.93 | 0.94 | 0.92 | 0.10 | 0.403 |
| Mean | 1.01 | 0.98 | 0.95 | 0.06 | 0.244 |

The mean values of BUN concentration did not differed significantly among C, $T_1$ and $T_2$ groups (Table 4.19), mean values being 16.78, 16.52 and 16.11 mg/dl in blood plasma, respectively. Mean BUN concentration in three different groups during 90 days study period was ranges in between 16.11-17.12 mg/dl.

**Table 4.19. Effect of herbal supplementation on BUN level (mg/dl) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 16.54 | 16.72 | 16.15 | 0.41 | 0.683 |
| 30 | 16.98 | 16.45 | 16.12 | 0.38 | 0.371 |
| 60 | 17.12 | 16.42 | 15.98 | 0.39 | 0.817 |
| 90 | 16.47 | 16.48 | 16.19 | 0.47 | 0.564 |
| Mean | 16.78 | 16.52 | 16.11 | 0.20 | 0.098 |

### 4.3.2.2. Biomarkers of energy and lipid metabolism

In the present study, plasma concentration of glucose, cholesterol, triglycerides, NEFA, BHBA and LPO were used as biomarkers of lipid metabolism. Plasma glucose concentration across 90 days study in three different groups is presented in Table 4.20. Statistical analysis revealed non significant effect of herbal supplementation on plasma glucose concentration. Mean plasma concentration of glucose in Control, $T_1$ and $T_2$ groups were 48.72, 51.88 and 49.70 mg/100 ml, respectively.

**Table 4.20. Effect of herbal supplementation on plasma glucose level (mg/100 ml) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 47.71 | 50.51 | 50.45 | 0.94 | 0.798 |
| 30 | 46.34 | 52.35 | 50.06 | 0.39 | 0.528 |
| 60 | 49.32 | 51.97 | 48.86 | 1.11 | 0.469 |
| 90 | 51.52 | 52.35 | 49.54 | 0.69 | 0.758 |
| Mean | 48.72 | 51.88 | 49.70 | 1.06 | 0.076 |

The effect of herbal supplementation of plasma cholesterol is presented in Table 4.21 and Figure 4.5. Although, the plasma cholesterol concentration was found to be significantly lower ($P<0.05$) in $T_1$ group but values ranges between physiological limits. Mean plasma cholesterol concentration averaged 102.00, 96.00 and 96.34 mg/100 ml, respectively.

**Table 4.21. Effect of herbal supplementation on plasma cholesterol level (mg/100 ml) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 98.30 | 100.68 | 99.04 | 5.20 | 0.615 |
| 30 | 102.00 | 94.15 | 94.70 | 5.35 | 0.837 |
| 60 | 103.20 | 95.00 | 93.61 | 6.34 | 0.147 |
| 90 | $104.00^{b}$ | $94.15^{a}$ | $98.00^{ab}$ | 6.49 | 0.012 |
| Mean | $102.00^{b}$ | $96.00^{a}$ | $96.34^{ab}$ | 3.33 | 0.001 |

Mean with different superscript in row differs significantly (P<0.05)

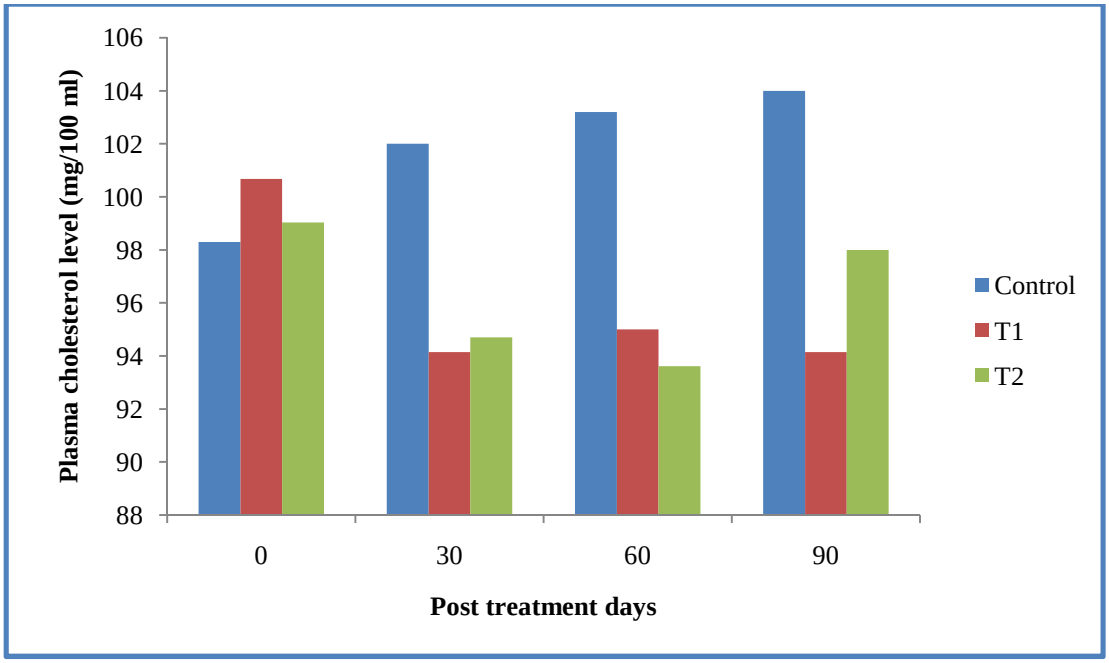



**Figure 4.5. Effect of herbal on plasma cholesterol level in calves**

In the findings of the present study, plasma triglyceride concentration also showed significant effect of herbal supplementation (Table 4.22; Figure 4.6). The mean plasma triglyceride level was lowest in Tinospora supplemented calves ($T_1$). Mean values for plasma triglycerides concentration in three different groups were 30.26, 26.86 and 27.28 mg/100 ml, respectively. Plasma triglycerides concentration in three different groups at start and end of experiment was 28.66, 29.18, 28.97 and 31.82, 25.51, 26.07 mg/100 ml of plasma, respectively.

**Table 4.22. Effect of herbal supplementation on plasma triglyceride level (mg/dl) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 28.66 | 29.18 | 28.97 | 2.57 | 0.169 |
| 30 | 29.80 | 26.84 | 27.57 | 1.79 | 0.232 |
| 60 | 30.74 | 25.88 | 26.49 | 1.88 | 0.186 |
| 90 | $31.82^{a}$ | $25.51^{b}$ | $26.07^{b}$ | 1.99 | 0.021 |
| Mean | $30.26^{b}$ | $26.86^{a}$ | $27.28^{ab}$ | 1.28 | 0.001 |

Mean with different superscript in row differs significantly (P<0.05)

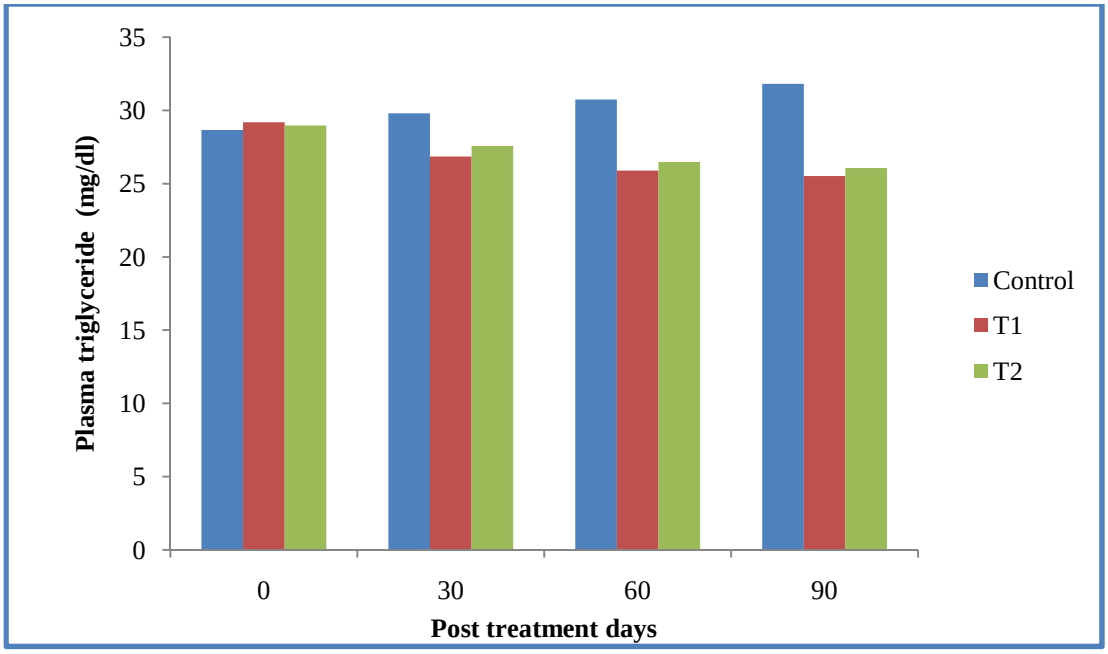



**Figure 4.6. Effect of herbal supplementation on plasma triglyceride level in calves**

NEFA and BHBA used as biomarker of lipid mobilization. NEFA showed non significant effect of treatment (Table 4.23). Plasma NEFA concentration found within physiological limit and averaged 0.102, 0.110 and 0.110 nmol/l, respectively in Control, $T_1$ and $T_2$ groups. Plasma NEFA concentration in Control, $T_1$ and $T_2$ groups at start and end of experiment were 0.101, 0.101, 0.102 and 0.104, 0.112, 0.104 nmol/l, respectively.

**Table 4.23. Effect of herbal supplementation on plasma NEFA concentration (nmol/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 0.101 | 0.101 | 0.102 | 0.005 | 0.190 |
| 30 | 0.103 | 0.104 | 0.112 | 0.004 | 0.769 |
| 60 | 0.091 | 0.111 | 0.107 | 0.006 | 0.501 |
| 90 | 0.104 | 0.112 | 0.104 | 0.008 | 0.406 |
| Mean | 0.102 | 0.110 | 0.110 | 0.006 | 0.552 |

Plasma BHBA concentration showed significant effect ($P<0.05$) of treatment and averaged 0.26, 0.24 and 0.22 mmol/l. BHBA concentration was lowest in calves fed on diet supplemented with Asparagus (Table 4.24; Figure 4.7).

**Table 4.24. Effect of herbal supplementation on plasma BHBA concentration (mmol/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 0.24 | 0.23 | 0.22 | 0.021 | 0.308 |
| 30 | 0.26 | 0.22 | 0.24 | 0.015 | 0.295 |
| 60 | 0.23 | 0.25 | 0.22 | 0.026 | 0.060 |
| 90 | 0.27 | 0.25 | 0.24 | 0.025 | 0.965 |
| Mean | $0.26^{b}$ | $0.24^{ab}$ | $0.22^{a}$ | 0.014 | 0.040 |

Mean with different superscript in row differs significantly ($P<0.05$)

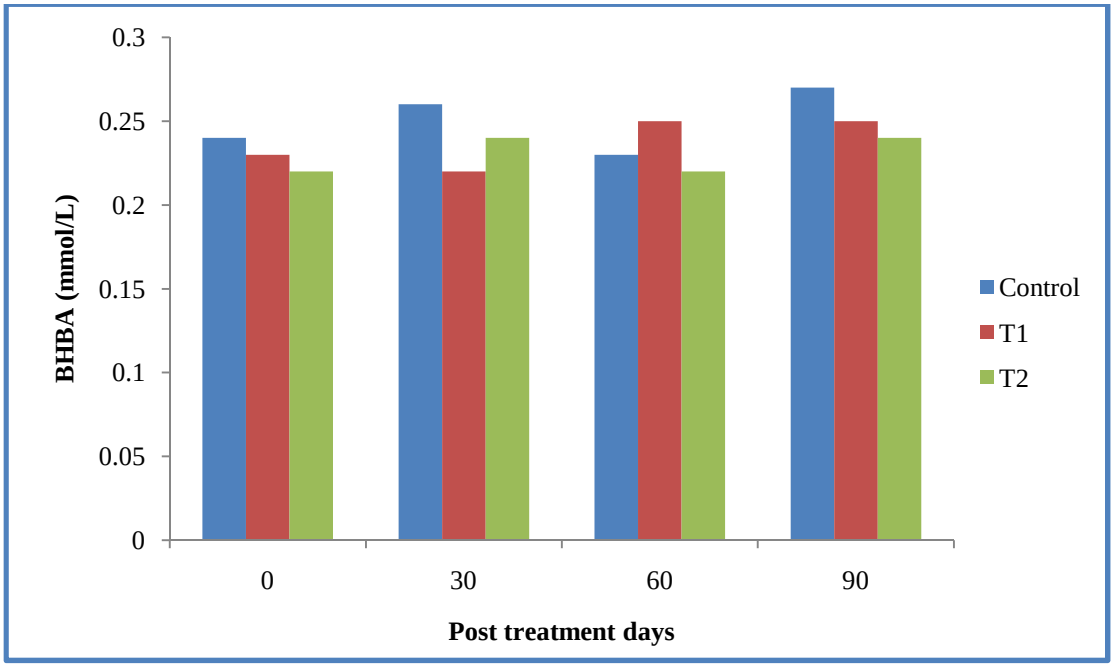



**Figure 4.7. Effect of herbal supplementation on plasma BHBA concentration in calves**

Plasma LPO concentration showed significant effect (P<0.05) of treatment (Table 4.25; Figure 4.8). Level of LPO was lower in calves fed on diet supplemented with Tinospora ($T_1$) followed by Asparagus ($T_2$) and unsupplemented groups. Plasma LPO levels begins decreasing 90$^{th}$ days of study. Mean plasma LPO concentration averaged 6.93, 6.46 and 6.75 µmol/L. LPO levels were 6.28, 5.97 and 6.66 µmol/L at the day of initiation of experiment and were 7.43, 6.61 and 7.28 µmol/L at the end of the study.

**Table 4.25. Effect of herbal supplementation on plasma LPO concentration (µmol/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 6.28 | 5.97 | 6.66 | 0.53 | 0.125 |
| 30 | 6.84 | 6.66 | 6.40 | 0.28 | 0.057 |
| 60 | 7.18 | 6.59 | 6.67 | 0.32 | 0.061 |
| 90 | $7.43^{b}$ | $6.61^{a}$ | $7.28^{b}$ | 0.37 | 0.011 |
| Mean | $6.93^{b}$ | $6.46^{a}$ | $6.75^{ab}$ | 0.18 | 0.008 |

Mean with different superscript in row differs significantly (P<0.05)

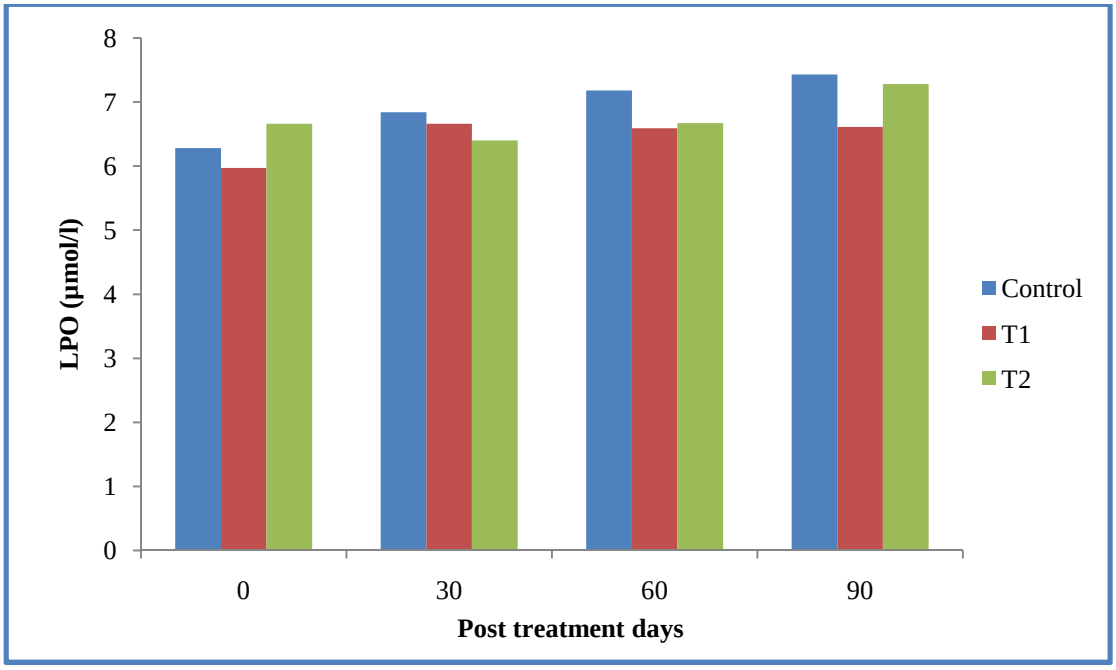



**Figure 4.8. Effect of herbal supplementation on plasma LPO concentration in calves**

### 4.3.2.3. Liver and kidney function test

Studied biomarkers of liver function test in present study are ALT, AST and ALP whereas; plasma creatinine was used as biomarker of kidney function test. No significant differences in the ALT, AST and ALP were observed among Control, $T_1$ and $T_2$ groups (Table 4.26 – 4.28). Mean plasma concentration of ALT, AST and ALP observed within physiological range. Mean plasma concentration of ALT and AST in Control, $T_1$ and $T_2$ groups were 6.01, 5.73 and 6.12 IU/l and 26.14, 25.40 and 26.89 IU/l, respectively.

**Table 4.26. Effect of herbal on plasma ALT level (IU/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 6.50 | 6.06 | 6.51 | 0.26 | 0.160 |
| 30 | 5.99 | 6.32 | 6.88 | 0.21 | 0.550 |
| 60 | 5.85 | 5.24 | 5.38 | 0.24 | 0.154 |
| 90 | 5.70 | 5.30 | 5.69 | 0.19 | 0.937 |
| Mean | 6.01 | 5.73 | 6.12 | 0.23 | 0.247 |

**Table 4.27. Effect of herbal supplementation on plasma AST level (IU/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 25.64 | 25.05 | 27.11 | 0.81 | 0.057 |
| 30 | 26.75 | 25.54 | 26.28 | 1.10 | 0.202 |
| 60 | 26.03 | 25.53 | 27.05 | 1.02 | 0.289 |
| 90 | 26.12 | 25.07 | 27.09 | 0.76 | 0.235 |
| Mean | 26.14 | 25.40 | 26.89 | 0.58 | 0.006 |

Similar to the ALT and AST, plasma concentration of ALP was also showed non-significant effect among three respective groups (Table 4.28). ALP concentration among three different groups at start and end of experiment was 28.82, 27.23, 29.40 and 31.76, 26.67, 26.07 IU/l, respectively. However, plasma ALP concentration during 90 days study period averaged 30.22, 26.85 and 27.32 IU/l, respectively in calves of Control, $T_1$ and $T_2$ groups.

**Table 4.28. Effect of herbal supplementation on plasma ALP level (IU/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 28.82 | 27.23 | 29.40 | 1.76 | 0.508 |
| 30 | 29.15 | 26.45 | 27.29 | 1.45 | 0.146 |
| 60 | 31.11 | 26.05 | 26.51 | 3.85 | 0.132 |
| 90 | 31.76 | 26.67 | 26.07 | 1.28 | 0.335 |
| Mean | 30.22 | 26.85 | 27.32 | 1.08 | 0.288 |

The periodic changes in plasma creatinine concentration during 90 days experimental period is depicted in Table 4.29. The concentration of plasma creatinine ranged between 0.43-1.57 mg/100 ml across study period. Although, mean plasma creatinine concentration was found to be lower in Asparagus supplemented groups ($T_2$) but showed non-significant difference among three groups. The mean values of creatinine were found to be 1.21, 1.14 and 0.85 mg/100 ml in three respective groups.

**Table 4.29. Effect of herbal supplementation on plasma creatinine (mg/100 ml) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 1.23 | 1.20 | 1.11 | 0.14 | 0.235 |
| 30 | 1.51 | 1.05 | 0.45 | 0.28 | 0.286 |
| 60 | 1.22 | 1.09 | 0.85 | 0.08 | 0.398 |
| 90 | 0.83 | 1.24 | 1.04 | 0.10 | 0.115 |
| Mean | 1.21 | 1.14 | 0.85 | 0.24 | 0.165 |

### 4.3.2.4. Biomarker of antioxidant status and immune response

In the present study, SOD, CAT and FRAP assay (TAS) were used as biomarkers of antioxidant status whereas; plasma total immunoglobulin level was used as biomarker of immune response. Plasma concentration of antioxidant enzymes i.e. SOD and CAT during 90 days study are presented in Table 4.30 and Table 4.31. Significant differences ($P<0.05$) in the blood SOD activity were observed between Control, $T_1$ and $T_2$ groups (Figure 4.9). Corresponding mean values for SOD activity among three different groups averaged 0.41, 0.41, 0.45 µmol MTT formazan/mg Hb. No significant difference was observed in CAT activity in all the three groups and mean value of CAT averaged 157.83, 160.73, 159.00 nmol/ml haemolysate.

**Table 4.30. Effect of herbal supplementation on SOD activity (µmol MTT formazan/mg Hb) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 0.31 | 0.31 | 0.33 | 0.13 | 0.445 |
| 30 | 0.37 | 0.38 | 0.36 | 0.13 | 0.240 |
| 60 | 0.47 | 0.48 | 0.53 | 0.05 | 0.238 |
| 90 | 0.48 | 0.48 | 0.56 | 0.09 | 0.354 |
| Mean | $0.41^a$ | $0.41^a$ | $0.45^b$ | 0.06 | 0.0480 |

Mean with different superscript in row differs significantly ($P<0.05$)

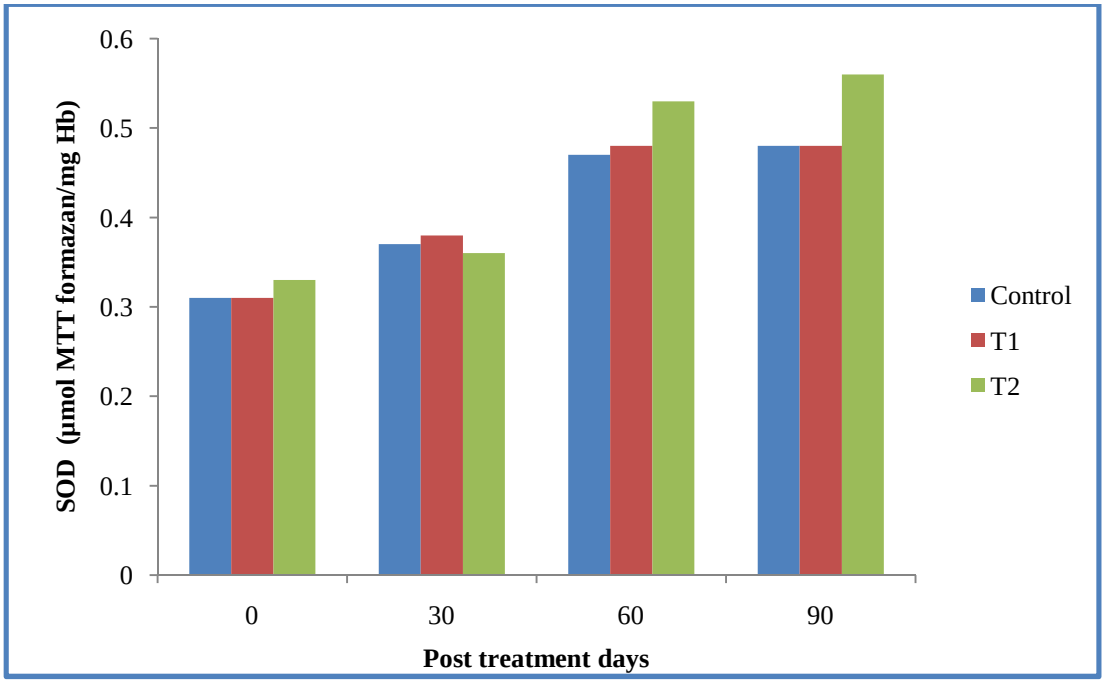



**Figure 4.9. Effect of herbal supplementation on SOD activity in indigenous calves**

**Table 4.31. Effect of herbal supplementation on CAT activity (nmol/ml haemolysate) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 159.27 | 162.24 | 158.76 | 6.17 | 0.699 |
| 30 | 157.54 | 164.34 | 155.62 | 5.73 | 0.098 |
| 60 | 155.65 | 160.42 | 160.23 | 6.41 | 0.196 |
| 90 | 158.84 | 155.93 | 161.36 | 8.53 | 0.517 |
| Mean | 157.83 | 160.73 | 159.00 | 4.31 | 0.435 |

The FRAP assay used as biomarker of total antioxidant status (TAS) of animals. Mean values of FRAP assay showed significant difference ($P<0.05$) between control and treatment groups but values were found within normal physiological level (Table 4.32; Figure 4.10). Overall mean value for TAS was significantly higher in Asparagus supplemented group ($T_1$) than Tinospora ($T_2$) and unsupplemented (Control) groups. The overall mean value averaged 1250.53, 1304.44 and 1273.26 µmol/l, respectively in the Control, $T_1$ and $T_2$ groups.

**Table 4.32. Effect of herbal supplementation on FRAP activity (µmol/l) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 1165.17 | 1179.27 | 1155.31 | 35.51 | 0.541 |
| 30 | 1273.45 | 1259.86 | 1309.34 | 33.14 | 0.372 |
| 60 | 1252.55 | 1269.57 | 1355.84 | 48.40 | 0.374 |
| 90 | $1310.99^{a}$ | $1384.33^{ab}$ | $1397.25^{b}$ | 50.98 | 0.042 |
| Mean | $1250.53^{a}$ | $1273.26^{a}$ | $1304.44^{b}$ | 17.39 | 0.047 |

Mean with different superscript in row differs significantly (P<0.05)

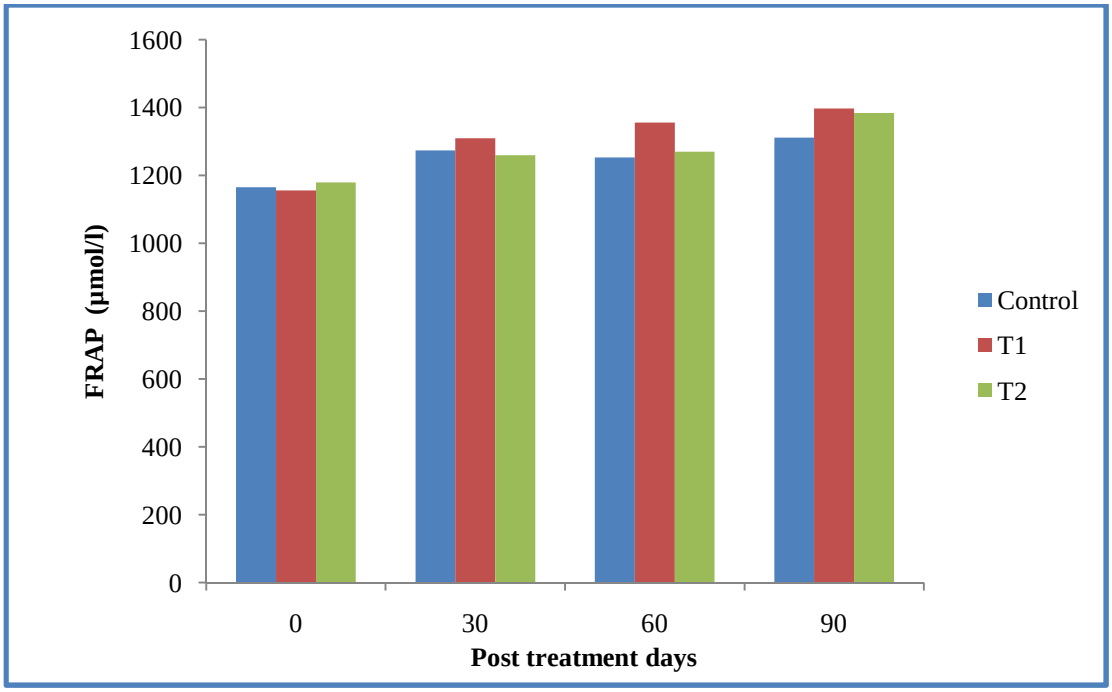



**Figure 4.10. Effect of herbal supplementation on FRAP value in calves**

Mean values of plasma total immunoglobulin showed significant effect (P<0.05) of herbal supplementation (Table 4.33; Figure 4.11). The mean values during 90 days study period averaged 20.28, 22.33 and 21.84 mg/ml, respectively in Control, $T_1$ and $T_2$ groups. Plasma total immunoglobulin concentration was higher in both the treatment groups and values in Control, $T_1$ and $T_2$ groups at initiation and termination of experiment were 20.46, 19.84, 19.89 and 21.78, 24.20, 23.74 mg/ml, respectively.

**Table 4.33. Effect of herbal supplementation on plasma total immunoglobulin level (mg/ml) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 20.46 | 19.84 | 19.89 | 2.86 | 0.919 |
| 30 | 19.08 | 22.53 | 21.15 | 2.21 | 0.064 |
| 60 | 19.76 | 22.71 | 22.58 | 2.65 | 0.065 |
| 90 | $21.78^{a}$ | $24.20^{b}$ | $23.74^{b}$ | 1.80 | 0.148 |
| Mean | $20.28^{a}$ | $22.33^{b}$ | $21.84^{b}$ | 1.31 | 0.069 |

Mean with different superscript in row differs significantly (P<0.05)

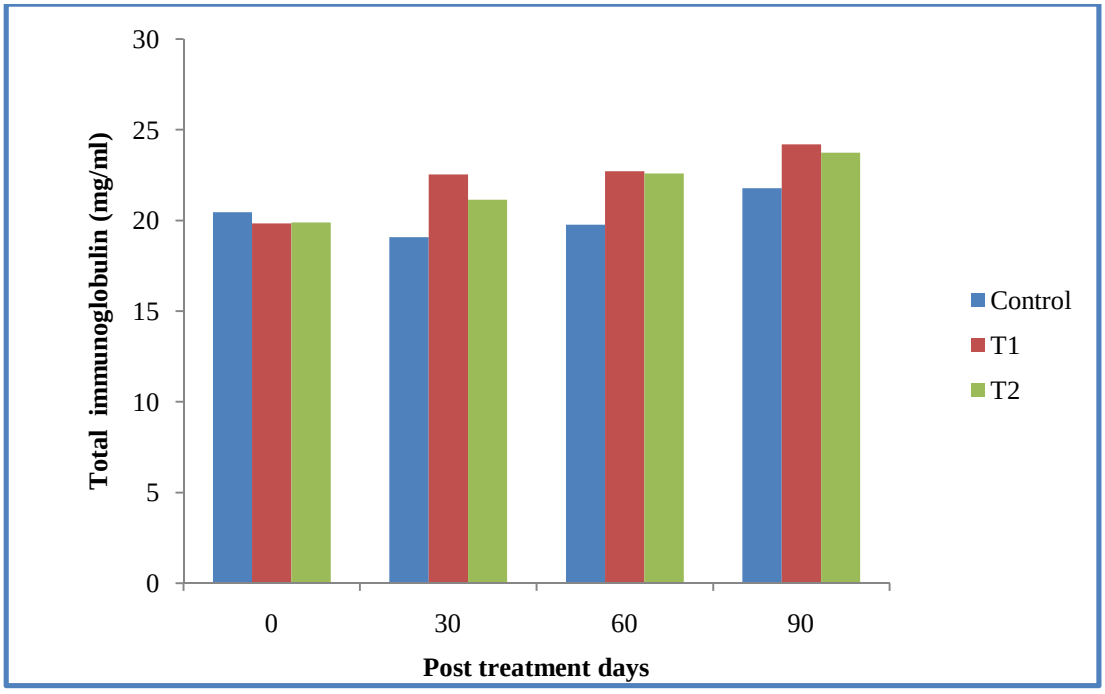



**Figure 4.11. Effect of herbal supplementation on plasma total immunoglobulin level in calves**

### 4.4. Urinary parameters

In present study urinary attributes like pH, specific gravity, urea and uric acid does not show any significant effect of the supplementation of Tinospora and Asparagus in experimental calves. Urinary pH at beginning and at the end of the study was almost similar (Table 4.34). Urinary pH at day 0 and at day 90 was 7.19, 7.32 and 7.33 and 7.58, 7.64 and 7.64 in three respective groups. The mean pH was 7.42, 7.57 and 7.61 in Control, $T_1$ and $T_2$ groups, respectively.

**Table 4.34. Effect of herbal supplementation on urinary pH in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 7.19 | 7.32 | 7.33 | 0.45 | 0.231 |
| 30 | 7.42 | 7.76 | 7.64 | 0.31 | 0.541 |
| 60 | 7.48 | 7.54 | 7.80 | 0.28 | 0.124 |
| 90 | 7.58 | 7.64 | 7.64 | 0.22 | 0.310 |
| Mean | 7.42 | 7.57 | 7.61 | 0.19 | 0.089 |

Similar like urinary pH, specific gravity of urine does not showed any significant effect of Tinospora and Asparagus supplementation (Table 4.35). Mean specific gravity in three different groups was 1.024, 1.021 and 1.026, respectively.

**Table 4.35. Effect of herbal supplementation on the specific gravity of urine in indigenous calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 1.012 | 1.015 | 1.002 | 0.011 | 0.0843 |
| 30 | 1.025 | 1.012 | 1.022 | 0.015 | 0.0521 |
| 60 | 1.016 | 1.019 | 1.005 | 0.013 | 0.0917 |
| 90 | 1.019 | 1.020 | 1.021 | 0.015 | 0.0854 |
| Mean | 1.029 | 1.021 | 1.026 | 0.017 | 0.0597 |

In present study, urinary urea level does not showed any significant effect of treatment (Table 4.36). Urinary urea level during 90 days study period ranges in between 4.81-5.31 mmol/l. The mean urea level in urine was 5.01, 5.08 and 5.16 mmol/l in Control, $T_1$ and $T_2$ groups respectively.

**Table 4.36. Effect of herbal supplementation on urinary urea (mmol/l) level in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 4.81 | 5.11 | 4.94 | 0.26 | 0.263 |
| 30 | 5.10 | 5.14 | 5.15 | 0.17 | 0.292 |
| 60 | 5.11 | 5.02 | 5.22 | 0.30 | 0.567 |
| 90 | 5.01 | 5.04 | 5.31 | 0.28 | 0.572 |
| Mean | 5.01 | 5.08 | 5.16 | 0.16 | 0.183 |

Similar like urea the uric acid concentration in urine also does not showed any significant effect of the treatment (Table 4.37). Urinary uric acid concentration at day 0 was 121.83, 122.50 and 119.16 mg/dl and at the end of the study were 129.16, 126.66 and 132.83 mg/dl, respectively. The mean value of urinary uric acid levels was 120.92, 123.08 and 125.96 mg/dl in Control, $T_1$ and $T_2$ groups, respectively.

**Table 4.37. Effect of herbal supplementation on urinary uric acid (mg/dl) in calves**

| Days | Group | | | Pooled SEM | P value |
|---|---|---|---|---|---|
| | Control | $T_1$ | $T_2$ | | |
| 0 | 121.83 | 122.50 | 119.16 | 6.18 | 0.696 |
| 30 | 114.00 | 121.16 | 126.16 | 10.18 | 0.572 |
| 60 | 118.66 | 122.00 | 125.66 | 6.05 | 0.285 |
| 90 | 129.16 | 126.66 | 132.83 | 7.12 | 0.568 |
| Mean | 120.92 | 123.08 | 125.96 | 4.96 | 0.567 |

# Discussion

**CHAPTER- 5**

# DISCUSSION

In this section, the findings of the effect of Tinospora and Asparagus supplementation on the growth performance, hematological parameters and biochemical attributes in growing calves are discussed with the findings of other researchers worked on herbal supplements in dairy animals.

## 5.1. Chemical composition of Tinospora and Asparagus

The Tinospora contains higher proportion of CP, EE and CF however, less amount of NFE than Asparagus. Content of DM and ash are similar in Tinospora and Asparagus. The findings in the study are similar with the results obtained by Akande et al. (2012). More fibre content (55.32%) in Tinospora than Asparagus (2.54%) was also observed by Ali et al. (2019). Hussain et al. (2009) reported that Tinospora contains 13.32% CP, 23.30% fibre and 1.99% fat. Tinospora leaves of the plant are rich in protein (11.2%) and are fairly rich in calcium and phosphorus. Tinospora contains high fibre (15.9%), sufficient protein (4.5%-11.2%), carbohydrate (61.66%), and low fat (3.1%). Its nutritive value is 292.54 calories per 100 g. It has high potassium (0.845%), high chromium (0.006%), sufficient iron (0.28%) and calcium (0.131%) (Nile and Khobragade, 2009).

## 5.2. Effect of herbal supplementation on growth performance

### 5.2.1. Growth performance

Herbal feed additives could either effect feeding pattern, or effect the growth of favorable microorganisms in the rumen, or stimulate the secretion of different digestive enzymes, which in turn may improve the efficiency of nutrients utilization and growth performance of dairy animals (Bakshi and Wadhwa, 2000). In the present study, Tinospora and Asparagus supplementation in growing calves does not exert any effect on the growth performance. The mean ADG in three respective groups were 198.16, 207.50 and 203.83 g/day. Most of the study regarding the effect of the supplementation of the immunomodulatory herbs is restricted in dairy animals during transition period rather than growth performance. Similar to the findings of the present study, no effect of supplementation of *Tinospora cordifolia* (Giloy) @ 4% of

concentrate was reported by Raj et al. (2020) in crossbred calves. In opposite to the findings of the present study, significant ($P<0.05$) higher body weight gain was reported in Murrah buffalo calves supplemented with combination of *Emblica officinalis* and *Tinospora cordifolia* (Patel et al., 2017). Gautam et al. (2013) also reported that the herbs used in the self-made rumenotoric drugs (*Tinospora cordifolia* is one of the constituent) not only improve the appetite and digestion process but also stimulate growth parameters. Increase in body weight in *Tinospora cordifolia* supplemented group may be due to its ability to reduce the loss of lipids secondary to its hypoglycemic effect (Stanley et al., 2000). Higher growth rate in Sahiwal heifers supplemented with Asparagus @150 mg/kg BW/day were reported by Jamara et al. (2014). Similar to the findings of the present study, Somkuwar et al. (2005) also observed non significant effect of herbs on body weight gain of buffalo calves. But according to Seidzadeh et al. (2016) average daily weight gain was affected in new born calves by feeding herbal additives.

The supplementation of Tinospora and Asparagus also did not showed any effect on height and length gain in experimental calves. No information available regarding the effect of the supplementation of immunomodulatory herbs on height and length gain in calves.

### 5.2.2. Body condition score (BCS)

In the present study, BCS measured at 5 point scale ranges in between 2.60-3.0 and showed non significant effect either of Tinospora and Asparagus supplementation. Mean BCS during 90 days study period averaged 2.73, 2.83 and 2.83 in three respective groups. No information available regarding the effect of immunomodulatory herbs supplementation on BCS of dairy calves. In peripartum buffaloes, treatment group have lost less BCS as compared to control group may be due to an efficient microbial digestion enhanced by the feeding of polyherbal mixture (Patel et al., 2013). In dairy cows, BCS was increased in herbs supplemented cows compared with control cows at day 30 and 50 of experiment (Cigari et al., 2015). Tedesco et al. (2001) also reported that transition cows supplemented with herbs had lower body condition loss compared to un-supplemented cows.

### 5.2.3. Calf diarrhea, joint ill and calf mortality

In the present study, faecal consistency was measured as indicator of calf diarrhea. Supplementation of Tinospora and Asparagus does not exert any effect on faecal consistency. There was no incidence of calf diarrhea during 90 days experimental period in all three groups might be due to the regular feeding of probiotic. Mean value showed significant effect ($P<0.05$) of the treatment. The incidence of joint ill was reported lower in Tinospora and Asparagus supplemented calves. In the present study, no death was reported treatment as well as in control group. The incidence of neonatal diarrhea depends on immune status specifically on the concentration of antibodies of young once. The claimed use in non-specific diarrhea is supported by evidence from pharmacological studies in guinea pig showing that supplementation of *Tinospora cordifolia* reduce incidence of diarrhea (Kamble et al., 2008; Lather et al., 2011). Antispasmodic activity was demonstrated against spasm induced by acetylcholine, histamine, and barium chloride (Lather et al., 2011**)**, supporting the claimed use (Tambekar et al., 2009**)** as gastrointestinal protective in nonspecific diarrhea. Smooth muscle relaxing action could be further explored for uterine muscle for confirmation of supposed usefulness in dysmenorrhea. Higher incidence of calf mortality in control group than Tinospora supplemented Karan Fries calves were reported by Mallick and Prakash (2011,b).

## 5.3. Effect of Herbal supplementation (Tinospora and Asparagus) on hematological and biochemical attributes

### 5.3.1. Hematological parameters

In the present study, supplementation of Tinospora and Asparagus did not exert any effect on the most of the hematological attributes. Mean RBCs count, Hb concentration and PCV value showed significant effect ($P<0.05$) of treatment. RBCs count was higher in Asparagus supplement calves whereas, Hb concentration and PCV values were higher in Tinospora supplemented calves. The blood content of RBCs, WBCs, Hb, PCV or HIT value, lymphocytes and neutrophil counts were within normal physiological range (Singh et al., 2019). Kamble et al. (2008) also observed similar findings as reported in the present study. However, increased Hb, PCV, TLC, lymphocyte and neutrophils counts in Holstein and Tharparkar heifers after Tinospora supplementation was noted by Prakash et al. (1978). In contrary to the

present findings, Rekhate et al. (2010) found no effect on WBCs counts and Hb concentration in broiler birds supplemented with Asparagus. Sarker et al. (2010) observed that the alteration in the Hb content were within normal range though there was mild improvement in the hematological parameters in the Asparagus treated rats. Raj et al. (2020) reported increase in neutrophils counts while feeding with Tinospora in cross bred calves. While a decrease in the values of WBCs counts were observed by Lather et al. (2011) in cross bred calves treated with herbal extract. A higher total leukocyte, lymphocyte, neutrophil count along with increased neutrophil lymphocyte ratio was recorded in Tinospora supplemented cows in comparison to untreated cows (Mallick and Prakash, 2011,a).

### 5.3.2. Biochemical parameters

In this study, biomarkers of protein, lipid and energy metabolism, liver, kidney function test along with antioxidant status and immune response have been used to evaluate the effect of Tinospora and Asparagus supplementation in growing calves. Generally, in the present study plasma parameters values measured were within the normal range for healthy dairy animals as recorded by El-Aidy (2003), Eweedah et al. (2007), Singh (2016) and Singh et al. (2019).

#### 5.3.2.1. Biomarkers of protein metabolism

The plasma concentrations of total protein, albumin, globulin, A/G ratio and BUN were used as biomarkers for protein metabolism in the present study. Plasma total protein and albumin concentration showed non significant effect of the treatment. However, plasma globulin concentration showed significant effect ($P<0.05$) of treatment. Higher plasma globulin level in $T_1$ and $T_2$ groups might be due to the higher immunomodulatory properties of Tinospora and Asparagus. Similar findings were reported by Bishavi et al. (2002) after Tinospora supplementation in rats. Increase in globulin level and no effect on albumin after Asparagus supplementation in broilers has been observed by Rekhate et al. (2010). Similarly, increase in plasma globulin fraction was noted by Vihan et al. (1988) in Sheep and Goats after herbal supplementation. Serum level of total proteins, albumins and globulins does not showed any significant difference on Tinospora supplementation in lactating cross bred cows (Sharma et al., 2018). Contrary to the present study, a

significant increase in plasma total protein and albumin concentration was observed by Rekhate et al. (2010) in Asparagus supplementation broilers.

The blood or serum urea concentration is a marker of protein intake and digestion, degradation of protein sources, and energy availability in the rumen (Roseler et al., 1993). In present study, BUN concentration showed no effect of Tinospora and Asparagus supplementation in calves. No information is available in support of findings of the present study. In opposite to the findings of present study, Dudi and Dutt (2015) in Murrah buffalo calves, Nascimento et al. (2015) in Nellore cattle, Kelly et al. (2011) in Limousin × Friesian heifers and Sharma et al. (2014) in Sahiwal calves observed lower BUN concentration.

**5.3.2.2. Biomarkers of energy and lipid metabolism**

The plasma concentration of glucose, cholesterol, triglycerides, NEFA, BHBA and LPO were used as biomarkers of lipid metabolism. Supplementation of Tinospora and Asparagus did not exert any effect on plasma concentration of glucose, NEFA and BHBA. Inclusion of Asparagus root powder in rat diet, resulted in a dose-dependant reduction in plasma and hepatic lipid profiles, increased fecal excretion of cholesterol, neutral sterol and bile acid along with increases in hepatic HMG-CoA reductase activity and bile acid content (Nishant et al., 2009). However, plasma cholesterol, triglyceride and LPO concentrations were lower in Tinospora supplemented calves. However, decreased plasma glucose concentration was reported by Stanley et al. (2000) in rats. Similar study showed by feeding of Tinospora stem powder leads significant decrease in blood glucose in lactating dairy cows revealed by Sharma et al. (2018). Whereas, increased plasma glucose level in ewes and rams was observed by Rincon et al. (2011) and in broiler was noted by Rekhate et al. (2010). Similarly, higher blood glucose in Tinospora supplemented Murrah Buffaloes were observed by Mir et al. (2014). Variation in blood glucose concentration in different finding might be due to difference in the species and age of the animals used in the different studies.

In present study there is decrease in the plasma cholesterol and triglycerides levels in treatment groups which are agreed with the findings of Stanley et al. (2000). Contrary to the present study, Sharma et al. (2018) found that plasma cholesterol concentration on Tinospora feeding does not reveal any significant differences among

treatment groups. Lower plasma triglycerides levels in Asparagus supplemented broilers were also noted by the Rekhate et al. (2010). Visavadiya and Narasimhacharya (2009,a) also reveal similar results in rats supplemented with Asparagus.

In present study there is no change in plasma NEFA and BHBA values but LPO values shows gradient decrease from Control, $T_2$ then $T_1$ groups having mean values of 6.93, 6.75 and 6.46 µmol LPO/l, respectively. BHBA and NEFA is the product of tissue fatty acid catabolism, and systemic concentrations increase in proportion to the degree of fat mobilization. These two biomarkers used to access the energy balance of animals. No more information available regarding the circulatory concentration of BHBA and NEFA by herbal feeding in calves but contrary to the findings of the present studies there is decline in NEFA values in herbal supplemented cows (Grummer et al., 2004).

**5.3.2.3. Liver and kidney function test**

ALT, AST and ALP were used as biomarker of liver function test whereas; plasma creatinine was used as biomarker of kidney function test. Dietary treatment did not exert any effect on liver and kidney function tests in experimental calves. Data on AST, ALT and ALP concentrations were within normal physiological limits and corroborate to those reported by Sharma et al. (2014) and Singh et al. (2016) in healthy indigenous calves. Similar result was observed by Sharma et al. (2010) in rats and Vakili et al. (2013) in calves after Tinospora feeding. No effect on liver and kidney function test in human study was reported by Maruganandan et al. (2001) in after Tinospora supplementation. The present study showed that the herbal supplementation had no significant effects on creatinine, but values decreases in both the treatment groups and similar result seen in studies of Khanam et al. (2011) and Wesam et al. (2018) in rats after Tinospora supplementation.

**5.4.2.4. Biomarker of antioxidant status and immune response**

When ROS and other radical generation exceed the detoxification or antioxidant capacity of a system, it is regarded as oxidative stress (Chirase et al., 2004). Various stressors are like environmental stress, nutritional stress, managemental stress, transportational stress, production stress, etc are responsible for oxidative stress. Body antioxidant system is responsible for neutralization of ROS

that's why antioxidant system in responsible for proper functioning of immune system. In the present study, SOD, CAT and TAS were used as biomarkers of antioxidant status whereas; plasma total immunoglobulin level was used as biomarker of immune response. Significantly higher ($P<0.05$) activity of SOD and TAS were reported in Asparagus supplemented calves however, treatment did not showed any effect on the CAT activity in different groups. The roots of Asparagus possess an antioxidant property and the antioxidant compound was identified as racemofuran along with two known compounds-asparagamine A and racemosol (Wiboonpun et al., 2004). Methanolic root extracts of Asparagus were found to markedly increase superoxide dismutase, while decreasing lipid peroxidation in rats (Sairam et al., 2003; Bhatnagar et al., 2005). The antioxidant activity of individual herbal ingredients, i.e. *W. somnifera* (Bhattacharya et al., 1987), *E. officinalis* (Kumaran and Karunakaran, 2006), *A. racemosus* (Visavadiya and Narasimhacharya, 2009,b), *T. Cordifolia* (Prince and Menon, 1999), *O. sanctum* (Geetha et al., 2004) and *N. sativa* (Burits et al. 2001; Neveen and Iman 2010) in laboratory animals supports findings of better antioxidant status after supplementation with herbs. No significant differences in the plasma SOD, and CAT was observed by Reddy et al. (1993) and Sharma et al. (2010) in rats after Tinospora feeding and Maruganandan et al. (2001) in human after Asparagus feeding. Increased value of FRAP assay as reported in Asparagus supplemented calves was also reported by Visavadiya and Narasimhacharya, (2009 a,b) in rats. However, Prince et al. (1999) reported higher total antioxidant activity in Tinospora supplemented human. However, a similar plasma total antioxidant activity among unsupplemented and Tinospora supplemented cows was observed by Mallick and Prakash (2011). Similarly, no significant difference in antioxidant attributes i.e. activity of SOD, CAT and total antioxidant status occurred in the rats administered with Asparagus root powder (Nishant et al., 2009).

Plasma total immunoglobulin concentration was higher in both the treatment groups and mean values in three respective groups were 20.28, 22.33 and 21.84 mg/ml. A similar result regarding to the plasma total immunoglobulin as in present study was recorded in the calves fed on diet supplemented with Tinospora by the Gupta et al. (2016). Similar increase in plasma total immunoglobulin reveals in the study of Kehrli et al. (1989) and Saad et al. (1989) in cattle supplemented with herbal mixture. Among different treatment groups, mean plasma IgG concentration at the

day of calving did not differ significantly but at 15th day before and after calving, plasma IgG concentration in cows of poly herbal mixture supplemented group was significantly ($P \leq 0.05$) higher than control group (Sharma et al., 2014). Many researchers have reported possible immunostimulation activity with steroidal saponins; however possible correlation of immunostimulation and saponin content in *Asparagus racemosus* remains to be established (Thatte and Dahanukar, 1988; Rege et al., 1999). Higher plasma total immunoglobulin concentration in treatment groups might be due to better antioxidant activity in these groups. Higher immunoglobulin concentration in treatment groups were supported by the higher globulin content in these groups.

### 5.4. Urine parameters

In present study, effect of Tinospora and Asparagus supplementation in calves were studied on various urine attributes like pH, specific gravity, urea and uric acid content. Treatment did not exert any effect on the urinary attributes. Limited information available regarding the effect of supplementation of Tinospora and Asparagus in dairy animals. However, similar result were seen in study of Purandare et al. (2007) after Tinospora feeding in human and Russel et al. (1983) in rats after Asparagus feeding.

# Summary and Conclusions

## CHAPTER-6

## SUMMARY AND CONCLUSIONS

This study was conducted to evaluate the effect of Tinospora and Asparagus supplementation on the Growth performance, antioxidant status and immune response and other blood and urinary attributes in growing pre-weaned indigenous calves. A total of 18 growing indigenous calves were selected from the herd maintained at LFC, DUVASU, Mathura, India for a period of 90 days. Calves were randomly allocated into three groups (n=6) on body weight and age basis. Experimental calves either received a basal diet devoid of Tinospora and Asparagus (control) or supplemented with either Tinospora ($T_1$ group) or Asparagus ($T_2$ group). Nutrients requirement of experimental calves were met by feeding probiotic mixed with wheat bran (1:5 proportion), milk, available green fodder and concentrate mixture as per the guidelines of NRC (2001). The experimental calves were monitored daily for calf diarrhea, joint ill, calf mortality, fortnightly for body weight gain and monthly for BCS, length and height gain.

Peripheral blood samples were collected in heparinised vacuutainer tubes (BD Franklin, USA) by venipuncture of jugular vein at 0, 30, 60 and 90 days post-treatment. A fraction of blood sample was used for analysis of blood haematology i.e. RBCs, WBCs, lymphocytes, neutrophils, Hb concentration, and PCV or HIT and antioxidant enzyme determination i.e. SOD and CAT. Remaining amount of blood samples were centrifuged at 3000 rpm for 30 min to separate the plasma from packed erythrocytes. Plasma samples were stored at −20°C until further analysis of biomarkers of energy and lipid metabolism (glucose, cholesterol, triglycerides, BHBA and NEFA), protein metabolism (plasma total protein, albumin, globulin and BUN), liver and kidney function test (ALT, AST, ALP and creatinine) and biomarker of anti-oxidant status and oxidative stress (FRAP and LPO). Spot urine samples were collected and analyzed for total protein, uric acid, urea, pH and specific gravity.

Tinospora contains higher amount of CP, EE and CF while less content of NFE in comparison to the Asparagus. Growth performance was found non-significant in calves receiving diet supplemented with Tinospora and Asparagus. ADG in Control, $T_1$ and $T_2$ groups were averaged 198.16, 207.50 and 203.83 g/day,

respectively. Similarly, treatment did not have any effect on body height and length gain and BCS of experimental calves. There was no incidence of calf diarrhea in all three groups and the mean faecal consistency score during 90 days study period was 2.50, 2.38 and 2.43 in Control, $T_1$ and $T_2$ groups, respectively. However, incidence of joint ill was reported lower in treatment groups. No calf mortality was observed in all three groups during 90 days experimental period.

In the present study, RBCs count was significantly higher ($P<0.05$) in Asparagus supplement group whereas, Hb concentration and PCV values were higher in Tinospora supplemented calves. WBCs, lymphocyte and neutrophil counts showed non significant effect of treatment. Mean RBCs count during 90 days study period was 8.45, 9.56 and 9.78 × $10^6$/µl, respectively in three respective groups. Mean blood Hb concentration across 90 days study was averaged 9.28, 9.70 and 9.50 g/100 ml blood, respectively in control, $T_1$ and $T_2$ groups. The mean PCV values averaged 28.97, 29.55 and 31.72 % packed cells in control, $T_1$ and $T_2$ groups, respectively.

The plasma total protein, albumin, globulin, A/G ratio and BUN were used as biomarkers of protein metabolism. Mean plasma total protein, albumin, A/G ratio and PUN levels did not showed any significant effect between groups (Table 4.15). However, mean plasma globulin level was significant ($P<0.05$) higher in Tinospora supplemented calves and averaged 3.38, 3.73 and 3.55 g/l, respectively.

In the present study, plasma concentration of glucose, cholesterol, triglycerides, NEFA, BHBA and LPO were used as biomarkers of lipid metabolism. Feeding of diet supplemented with Tinospora or Asparagus did not exert any effect on plasma glucose and NEFA levels. The plasma cholesterol, triglyceride and LPO levels were found to be significantly lower ($P<0.05$) in Tinospora supplemented groups. However, BHBA concentration was lowest ($P<0.05$) in calves fed on diet supplemented with Asparagus and mean value averaged 0.26, 0.24 and 0.22 mmol/l in three respective groups.

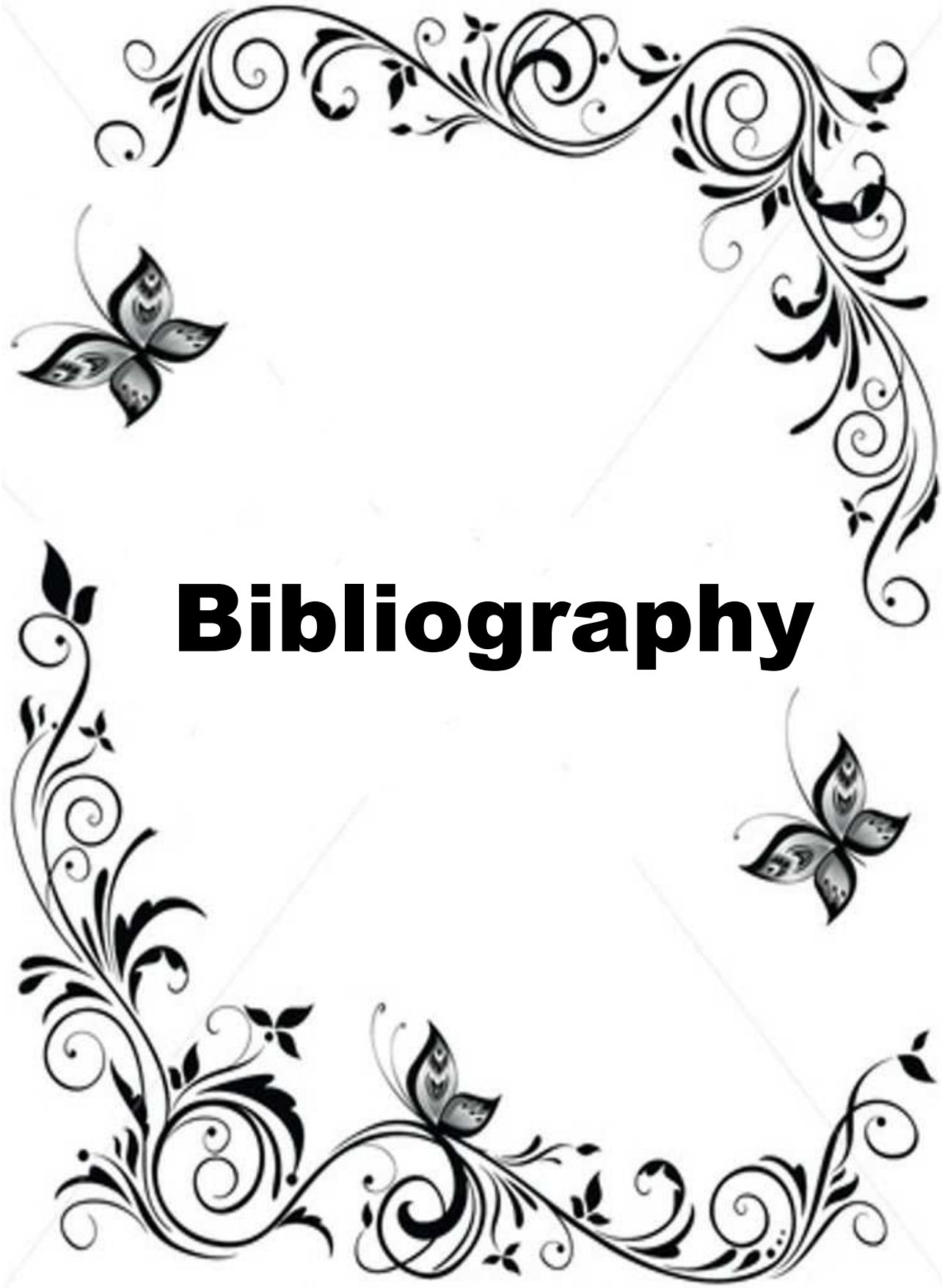
In the present study, ALT, AST and ALP were used as biomarker of liver function test and plasma creatinine level was used as biomarker of kidney function test. Treatment did not exert any effect on ALT, AST, ALP and creatinine level in three different groups.

In present study, SOD, CAT and FRAP (TAS) were used as biomarkers of antioxidant status and plasma total immunoglobulin level was used as biomarker of immune response. The SOD activity and TAS were higher in Asparagus supplemented group while CAT activity was similar among all groups. Corresponding mean values for SOD activity and FRAP assay in three respective groups were 0.41, 0.41, 0.45 µmol MTT formazan/mg Hb and 1250.53, 1304.44 and 1273.26 µmol/l. Dietary Herbal supplementation of the herbs exerted an increased effect on plasma total immunoglobulin concentrations. Plasma total immunoglobulin concentration is higher ($P<0.05$) in both the treatment groups Mean values of plasma total immunoglobulin averaged 20.28, 22.33 and 21.84 mg/ml, respectively in control, $T_1$ and $T_2$ groups.

In present study, urine parameters studied were urine pH, specific gravity, urea, uric acid and all of them does not showed significant effect ($P>0.05$) of the treatment. Urinary pH ranges, specific gravity, urea and uric acid ranges in between 7.19-7.80, 1.002-1.026, 4.81-5.31 mmol/l and 114.00-132.83 mg/dl, respectively.

**Conclusions**

In conclusion, dietary supplementation of Tinospora and Asparagus did not exert any adverse effect on the performance, liver and kidney function test and urinary attributes in pre-weaned indigenous calves. In the present study, RBCs count was higher in Asparagus supplement group whereas, Hb concentration and PCV values were higher in Tinospora supplemented calves. Dietary supplementation of Tinospora and Asparagus improved antioxidant status and immune response of experimental calves.



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# CV OF STUDENT

1. **Name** : **Dr. Prashant Singh**
2. **Date of Birth** : 01/04/1980
3. **Place of Birth** : Jewar, Uttar Pradesh
4. **Mother's Name** : Smt. Krishna Devi
5. **Father's Name** : Shri Dharamveer Singh
6. **Permanent Address** : 645, Omaxe city, Palwal (HR) 121102
7. **Mobile No.** : 9996691552
8. **E-mail** : prashant.vet2006@gmail.com
9. **Academic Qualifications:**

| Degree | University/ Board | Year of Passing | Percentage /OGPA of marks | Subjects |
|---|---|---|---|---|
| **Graduation (B.V.Sc. & A.H.)** | CSAUA& T Kanpur | 2006 | 7.12 out of 10 | As per VCI |
| **Intermediate** | UP Board Allahabad | 1997 | 60.06 % | Physics, Chemistry, Biology, Hindi, English |
| **High School** | UP Board Allahabad | 1995 | 57.66 % | Hindi, English, Science, Social Science, Math, Science |

10. **Number of Seminar/Conference/Workshop/Training attended** : 2
11. **Medals/Honours/Fellowships Received** : Nil
12. **List of Publications (related to thesis work only)** : Nil

**Date:** 10-6-2020
**Place:** mathura

P Singh
**(Signature of Students)**

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P Singh

Signature of Student