AMELIORATIVE POTENTIAL OF POLYHERBAL FORMULATION ON THROMBOCYTOPENIA IN DOGS WITH EHRLICHIOSIS

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OF

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MEDICINE

BY

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Enrolment No. V- 2131/19

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

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(2021)

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This is to certified that the thesis entitled **"AMELIORATIVE POTENTIAL OF POLYHERBAL FORMULATION ON THROMBOCYTOPENIA IN DOGS WITH EHRLICHIOSIS"** submitted by **Dr. Gulmire Suraj Kumar,** Enrollment No. V-2131/19 in partial fulfillment of the requirements for the award of the **Master of Veterinary Science in Veterinary Medicine** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (U.P.)** 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.

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<	:	Less than
>	:	More than
%	:	Percent
/	:	Per
@	:	At the rate
µ	:	micron
µg	:	microgram
µl	:	micro liter
^{0}C	:	Degree centigrade
^{0}F	:	Degree Fahrenheit
aPTT	:	Activated Partial Thromboplastin Time
Alb	:	Albumin
ALP	:	Alkaline phosphatase
ALT	:	Alanine aminotransferase
AST	:	Aspartate aminotransferase
APA	:	Antiplatelet antibodies
b.wt.	:	Body weight
BCT	:	Buffy coat technique
BUN	:	Blood urea nitrogen
CKD	:	Chronic kidney disease
CME	:	Canine monocytic ehrlichiosis
Cr	:	Creatinine
Cu.mm	:	Cubic millimeter
DIC	:	Disseminated intravascular coagulation
dL	:	Deci liter

DLC	:	Differential leucocyte count
DNA	:	Deoxyribonucleic acid
DW	:	Distilled water
E.canis	:	*Ehrlichia canis*
e.g.	:	Exempli gratia
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbent assay
et al.	:	Et alli/alia
etc.	:	And so forth
Fig.	:	Figure
gm/dl	:	Gram per 100 milliliters
GSD	:	German Shepherd
Hb	:	Hemoglobin
HCT	:	Hematocrit
I/M	:	Intramuscular
I/V	:	Intravenous
IFN	:	Interferon
Inj.	:	Injection
IU	:	International unit
IU/ml	:	International unit per milliliter
Kg	:	Kilogram
L	:	Liter
mcg	:	microgram
MCV	:	Mean corpuscular volume
mEq	:	mille equivalent
mg	:	milligram
ml	:	milliliter

mm	:	millimeter
MPV	:	Mean platelet Volume
N	:	number
ND	:	Non-descript
nPCR	:	Nested PCR
OD	:	Optical density
PCR	:	Polymerase chain reaction
PCV	:	Packed cell volume
PCT	:	Plateletcrit
PDW	:	Platelet distribution width
pH	:	Negative logarithm of hydrogen ion
PLT	:	Platelets
PMIF	:	Platelet migration inhibition factor
PT	:	Prothrombin Time
RBC	:	Red blood cells
rpm	:	Revolution per minute
S.N	:	Serial number
SE	:	Standard Error
Sec	:	Second
Sp.	:	species
Tab	:	Table
TEC	:	Total erythrocyte count
Th 1	:	Type 1 T-Helper cell
Th 2	:	Type 2 T-Helper cell
TLC	:	Total leucocyte count
TNF	:	Tumor necrosis factor
TP	:	Total protein

TVCC	:	Teaching veterinary clinical complex
WBC	:	White blood cells
X100	:	Magnification 100 times
X40	:	Magnification 40 times

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ABSTRACT

In present investigation total 62 dogs were screened for ehrlichiosis based on observation of 2-3 classical clinical symptoms of ehrlichiosis in dogs. All screened dogs underwent for blood smear, buffy coat smear examination and PCR to confirm the disease status. On blood smear examination 3 dogs were positive for ehrlichiosis; buffy coat smear examination shows 6 dogs were positive; primary PCR revealed 18 positive dogs, however nested PCR confirmed 42 dog's positive for ehrlichiosis of all screened dogs. Important clinical symptoms exhibited by the CME positive dogs in decreasing frequency were fever and mucosal pallor followed by lymph adenomegaly, melena, depression, tick infestation and weight loss. Clinical symptoms with moderate frequency were epistaxis, ascites, ocular abnormalities, vomiting, ecchymotic and petechial hemorrhages, hind limb/scrotal/facial edema and icterus. Symptoms with least frequency were hematuria, hematemesis and CNS signs. Vital parameter like heart rate, rectal temperature, respiration rate were elevated with concurrent decrease in total leucocyte count, total erythrocyte count, hemoglobin, platelets, packed cell volume, however the mean values of neutrophils was considerably increased with decrease in lymphocytes. Mean values of monocytes, eosinophil, MCV, MCH, MCHC were non-significant from the control. The mean values of ALT, AST, ALP and BUN were elevated while total protein, albumin were reduced in ehrlichiosis affected dogs. Mean values of globulin and serum creatinine were non-significant from the control. Percentage positivity of ehrlichiosis was recorded on the basis of age, breed, sex and season. Age group with highest positivity was category 3 (> 1 year) with a positivity of 61.90 %, breed with highest positivity was German shepherd 38.09 %, sex with highest positivity was male 54.76 % and season with maximum incidence of cases was summer 78.57%. In present study it was found that there was significant increase activated partial thromboplastin time in all treatment groups of dogs in comparison with control. There was non-significant increase in canine platelet migration factor on post-treatment in all treatment groups. Therefore, in terms of improvement among all the treatment groups of dogs, best recovery was seen in group which was treated with conventional treatment combined with Polyherbal platelet booster containing extract of *Carica papaya, Tinospora cordifolia, Withinia somnifera* and *Asparagus racemosus*.

Introduction

CHAPTER- 1

INTRODUCTION

Canine monocytic ehrlichiosisis (CME) is a potentially fatal disease in dogs caused by lipopolysaccharide deficient, obligatory intracellular, gram-negative bacteria of the genus *Ehrlichia* and family Anaplasmataceae, which have affinity for the haemopoietic cells (Rikhisa et al, 1985). *E. canis* is one of the most important organisms affecting wild and domesticated dogs of the family Canidae worldwide (Ebani et al., 2011). It is also called tropical canine pancytopenia with a worldwide prevalence (Skotarczak, 2003). Ehrlichiosis was defined for the first time in Algeria in 1935 by Donatien and Lestoquard (Donatien and Lestoquard, 1935).

Ehrlichia canis is naturally transmitted transstadial and interstadial but not transovarial by the tick *Rhipicephalus sanguineous* (Grooves et al., 1975). After an incubation period of 8-20 days, the course of *E. canis* infection can be sequentially divided into acute (2-4 weeks), subclinical (several months to years) and chronic phases but the distinction among these phases is not straight forward in the naturally-occurring disease (Mylonakis and Theodorou, 2017). The acute phase is characterized by high fever, depression, lethargy, anorexia, lymph adenomegaly, splenomegaly, hemorrhagic tendencies (Leiva et al., 2005). The latter are usually exhibited by dermal petechiae and ecchymoses and epistaxis. Untreated dogs and those treated inappropriately enter the subclinical phase and dogs show no clinical signs but may remain persistent carriers of *E. canis* (Waner et al., 1997). During the chronic phase, symptoms similar to those seen in the acute phase may occur but with a greater severity. Pale mucous membranes, weakness, bleeding and significant weight loss are common findings in this phase (Harrus et al., 1997). Dogs in chronic phase of disease with severe symptoms may be less responsive to therapy (Neer et al., 2002; Harrus et al., 2012). Therefore, early and accurate diagnosis of suspected cases in dogs is essential to alter the course of this disease (Neer et al., 2002). Immunocompetent dogs may eliminate the infection during the acute or subclinical phases, but some will eventually develop the chronic phase, characterized by bone marrow aplasia,

peripheral blood pancytopenia and high mortality due to septicemia and/or severe bleeding (Mylonakis and Theodorou, 2017).

Ulcerative stomatitis, necrotic glossitis, hind limb and/or scrotal oedema, bacterial pyoderma, icterus and central nervous system signs such as seizures, ataxia, vestibular dysfunction, ocular lesions and cervical pain have been more frequently reported in chronic CME, in addition to this bleeding diathesis is also more common and severe in the chronic phase of CME (Mylonakis et al., 2019).

Diagnosis of the disease can be challenging due to its different phases and multiple clinical manifestations. CME should be suspected when a compatible history (living in or traveling to an endemic region, previous tick exposure), typical clinical signs and characteristic hematological and biochemical abnormalities are present. Traditional diagnostic techniques including hematology, cytology, serology and isolation are valuable diagnostic tools. However, a definitive diagnosis of *E. canis* infection requires molecular techniques (Harrus and Waner, 2011).

Indirect fluorescent antibody (IFA) testing is considered the “gold standard” for the detection and titration of anti-*E. canis* antibodies, although enzyme-linked immunosorbent assays (ELISA) is also a commonly employed technique for confirmation of clinical cases (Mylonakis and Theodorou, 2017).

The thrombocytopenia being one of the most common and consistent hematological abnormality of dogs in Canine Monocytic Ehrlichiosis is attributed to different mechanisms in the different stages of the disease in naturally or experimentally *E. canis* infected dogs (Dubie et al., 2014). Various studies have stated that platelet indices especially MPV and PDW can provide clinical information on the mechanisms of thrombocytopenia (Bowles et al., 2005). Mechanisms thought to be involved in the pathogenesis of thrombocytopenia in the acute phase of the disease include increased platelet consumption due to inflammatory changes in blood vessel endothelium, increased splenic sequestration of platelets and immunologic destruction or injury resulting in a significantly decreased platelet life span. In addition, a platelet migration inhibition factor was isolated and characterized. This factor is proposed to play a role in enhancing platelet sequestration and stasis leading to reduced peripheral-blood platelet counts (Harrus et al., 1999).

Tests such as Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT), Thrombin Time (TT) and assay of clotting factors are considered as "Specific tests" for evaluation of dogs with bleeding tendencies (Pasa et al., 2017). The aims of Canine Monocytic Ehrlichiosis antimicrobial treatment include achievement of clinical remission, resolution of clinicopathologic abnormalities and eradication of the organism (Mylonakis et al., 2010).

Ethnoveterinary medicine (EVM) is also known as traditional animal health care practices is defined as local or indigenous knowledge and methods for caring, healing and managing livestock (Bhatt et al., 2013). The EVM drugs are used in the management of bacterial, viral, protozoan, fungal, parasitic and non-infectious causes of diseases (Moreki et al., 2012). Ethno- veterinary practices are widely accepted globally as complementary to the existing modern practices. These medicines have been developed through the experience of many generations assimilating the knowledge, in course of time, from fragments of Siddha, Ayurvedic, Yunani as well as Tribal systems of medicine (Bhuvaneswari et al., 2015). The knowledge base of EVM differs not only from region to region but also among and within communities. Therefore, it is less systematic, less formalized and not universally recognized as a valid method of disease control in animals (Matekaire and Bwakura, 2004). The practice of ethnomedicine is a complex multidisciplinary system constituting the use of plants in a spiritual way in the natural environment and has been the source of healing for people for millennia. It has been reported that as many as 70% (90% in some communities) of the world population continue to rely mainly on their own localized ethnomedicine for personal as well as veterinary healthcare (McCorkle, 1995).

Carica papaya belongs to the family Caricaceae which is widely used for consumption as fresh fruit, dried and crystallized fruit. It is successfully employed in folk medicine for the treatment of dengue infections with hemorrhagic manifestations, using suspensions of powdered leaves in palm oil. A lot of work has been carried out on plant parts like fruits, seeds and roots, indicating the presence of biologically active compounds (Sathasivam et al., 2009). The leaves of papaya have been shown to contain many active components such as papain, chymopapain, cystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucocynolates (Seigler et al., 2002). Papaya leaf contains anti-oxidant, vitamins, minerals, cardiac glycosides and

alkaloids which play an important role in curing of thrombocytopenia and also help to increase the hemoglobin, hematocrit, red blood cells, thrombocytes and total protein contents (Kathiresan et al., 2009).

Tinospora cordifolia commonly named as amrita "Guduchi" in sanskrit belonging to family Menispermaceae which is climbing shrub (Rana et al., 2012). In Hindi, the plant is commonly known as Giloya, which is a Hindu mythological term that refers to the heavenly elixir that have saved celestial beings from old age. It has number of chemical constituents has number of different groups such as Alkaloids, Terpenoids, Lignans, Steroids and many other active arabinogalactan (Kakkar et al., 2013). It probably acts by improving the production of platelets from bone marrow due to its antioxidant and immunomodulatory properties (Eberly et al., 2008).

Withania somnifera or Ashwagandha, Indian ginseng is an important herb in the Ayurvedic and indigenous medical systems for over 3000 years. The roots of the plant are categorized as rasayanas, which are reputed to promote health and longevity by augmenting defense against disease, arresting the ageing process, revitalizing the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors (Gupta and Rana, 2007). It exhibits antimicrobial, anti-inflammatory, immunomodulation, anti-oxidative, hepatoprotective and life prolonging properties. Ashwagandha have profound effects on the hematopoietic system, acting as an immunoregulator and a chemoprotective agent (Uddin et al., 2012).

Asparagus racemosus / Satavar, shatavari, or shatamull is an important medicinal herb, belongs to family Asparagaceae (Choudhary and Sharma, 2014). In the literal sense, the word 'Shatavari' means 'one who possesses a hundred husbands or acceptable to many'. It has been used in the traditional systems of medicine like Ayurveda, Siddha and Unani, as a general health tonic. The word Asparagus is derived from Greek and it denotes 'stalk' or 'shoot' (Goyal et al., 2003). The root extract of *Asparagus racemosus* possess anti-anemic and anti- thrombocytopenic properties and these could be related to the phytoconstituents such as alkaloids, saponins, phytosterols, triterpenoids, polyphenols, carbohydrate, flavonoids, glycosides, phenolic compounds and tannins present in the extract (Chaudhary et al., 2015).

Objectives:

Keeping in view the importance of ehrlichiosis and disease induced thrombocytopenia and its management, the study is planned with following major objectives:

1. To investigate the diagnostic relevancy of canine Specific Platelet Migration Inhibition Factor in dogs with ehrlichiosis.
2. To investigate the coagulation profile (Prothrombin Time and Activated Partial Thromboplastin Time) and platelet indices in dogs with ehrlichiosis.
3. To investigate the effect of self-prepared polyherbal formulation on thrombocytopenia in dogs with ehrlichiosis.

Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

This chapter reviews the literature associated with the main areas of interest in present investigation for the purpose of getting an impression about the important aspects of canine ehrlichiosis in dogs. The review of literature pertaining to various aspects of canine ehrlichiosis in dogs is described under following headings.

2.1. Clinical propaedeutic of canine ehrlichiosis.
2.2. Haemato-biochemical alterations in canine ehrlichiosis.
2.3. Serum Platelet migration inhibitor factor and its clinical utilization to detect thrombocytopenia.
2.4. Coagulation Profile (Prothrombin Time and Activated Partial Thromboplastin Time) and platelet indices.
2.5. Diagnostics and therapeutics of canine ehrlichiosis including herbs for increasing platelet count.

2.1. Clinical Propaedeutics of Canine Ehrlichiosis.

Ehrlichia canis infection is a tick-borne disease of dogs. Other wild canids (foxes, wolves, jackals) can also become infected with *E. canis* (Torina et al., 2013; Ebani et al., 2011; Fishman et al., 2004). All breeds are prone to CME. However, German Shepherd dogs and Siberian Huskies are prone to develop more severe clinical signs of ehrlichiosis. Therefore, these breeds have a worse prognosis (Harrus et al., 1997).

Ehrlichia canis is a gram-negative obligatory intracellular bacterium with a tropism for monocytes and macrophages (Aguiree et al., 2004). It was firstly described in 1935 in Algeria with acute cases of fever and anemia in several dogs (Donatien and Lestoquard, 1935). Later, during the Vietnam War, many military working dogs passed to Vietnam by the US army revealed a severe disease called Tropical Canine Pancytopenia (Huxsoll et al., 1970).

The genus Ehrlichia has been estranged into three genogroups because of strong genetic affinities between its species. Genogroup I integrates three species i.e., *E. canis, E. chaffeensis and E. ewingii*. Genogroup II contains *E. phagocytophila, E. equi* and the human granulocytic ehrlichiosis (HGE) agent. Genogroup III covers two species: *E. sennetsu and E. risticii*. The name of each genogroup is consistent with the name of the first species described. The said classification was enduring on the gene encoding 16S rRNA (Baumgarten et al., 1999; Stich et al., 2002; Dumler and Bakken, 1995).

E. canis is transmitted by the brown dog tick *Rhipicephalus sanguineus* (Lewis et al., 1977; Groves et al., 1975). It has also been shown to be experimentally transmitted by the tick *Dermacentor variabilis*. Transmission in *Rhipicephalus sanguineus* tick happens trans-stadial but not transovarial. Larvae and nymphs have been shown to become ailing while feeding on acutely ill dogs (Waner and Harrus, 2000). This tick is worldwide disseminated but particularly frequent in tropical and subtropical regions (Suksawat et al., 2001; Suto et al., 2001; Hua et al., 2000). The distribution of this vector is likely related to the prevalence of the disease triggered by E. canis (Neer et al., 2002).

Following an incubation period of 1-3 weeks, Canine monocytic ehrlichiosis (CME) is characterized by 3 stages: acute, subclinical and chronic (McClure et al., 2010). The acute phase can last 2 to 4 weeks, clinical signs may vary or go imprudently even without treatment. However, some dogs that show clinical enhancement may remain persistent subclinical carriers for months and years (Harrus et al., 1998b). The subclinical phase follows the acute stage. Dogs in subclinical phase prevailing no clinical signs. However, when screened for hematological abnormalities, these dogs may have scarce platelet concentrations (Waner et al., 1997). Some, but not all infected dogs may advance to a chronic phase. In the chronic phase clinical signs are more severe. Dogs in the chronic phase exhibit bone marrow hypoplasia and severe pancytopenia (Mylonakis et al., 2004).

Various researcher specified that symptoms of ehrlichiosis are following: ticks are commonly found on dogs during the acute stage. The clinical signs in the acute phase may be mild and non-specific, however some cases may present as severe and life threatening. Signs may include depression, lethargy, anorexia, pyrexia, lymph adenomegaly, splenomegaly and mild weight loss. Bleeding tendencies mostly

petechiae and ecchymoses of the skin and mucous membranes and occasional epistaxis may exist in affected dogs with ehrlichiosis. Ocular signs are not unusual and consist of anterior uveitis ± corneal opacity (oedema and/or deposition of cellular precipitates), hyphema, tortuous retinal vessels and focal chorio-retinal lesions containing of central pigmented spots with surrounding areas of hyperreflectivity. Sub retinal hemorrhages ensuing in retinal detachment and blindness may occur. Other clinical signs may include like serous to purulent oculo-nasal discharge, vomiting, lameness, ataxia and dyspnea. The common clinical signs of the chronic disease are fever, depression, weakness, anorexia, chronic weight loss, pale mucous membranes and peripheral oedema specifically of the hind limbs and the scrotum. Secondary bacterial and protozoal infections, interstitial pneumonia, renal failure and arthritis may occur throughout chronic severe disease. Some reproductive disorders have also been associated with chronic CME including prolonged bleedings during estrus, inability to conceive, abortions and neonatal deaths. Polymyositis has also been related with CME. Neurological signs may arise during the acute and chronic disease. Neurological signs include meningoencephalitis e.g., arched back, severe neck or back pain, paraparesis or tetra paresis, ataxia, cranial nerve deficits and convulsions. Neurological signs may be ascribed to hemorrhages, extensive plasma cell infiltration and perivascular cuffing of the meninges (Waner and Harrus, 2000).

Although some older studies have included lameness due to polyarthritis as a sign of CME, no evidence-based studies have supported this sign. Therefore, in cases of lameness and/or polyarthritis in dogs infected with *E. canis*. It is advisable to rule out co-infections with other pathogens such as *A. phagocytophilum* or *B. burgdorferi* (Cowell et al., 1988; Bellah et al., 1986).

According to the study on CME in Madras, pyrexia was present in 71.87%, anorexia in 71.87%, depression in 34.37%, vomiting in 29.68%, skin lesions in 20.31%, peripheral lymphadenopathy in 9.37% and splenomegaly in 6.25%. Bleeding was displayed as epistaxis in 4.68% of the dogs, melena in 14.06%, hematemesis in 3.13% and hematuria in 3.13%. Decreased levels of hemoglobin, PCV and RBCs with normal values for mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentrations were noted which indicated normocytic normochromic anemia (Thriunavukkarasu et al., 1994). *E canis* causes a potentially fatal disease in dogs that wants rapid and accurate diagnosis in order to recruit

appropriate therapy leading to a favorable prognosis (McBride et al., 2003). Dogs which become infected with *E. canis* keep on infected for their entire lives even if they received antibiotic treatment with doxycycline reported by Wen et al., 1997

2.2. Haemato-Biochemical alterations In Canine Ehrlichiosis.

Hematology revealed that the mean values of hemoglobin, total erythrocyte counts (Sharma et al., 2010), platelet count (Srikala et al., 2012; Oliveira, 2000) and packed cell volume was declined in dogs with ehrlichiosis. Decreased Hb and TEC levels could be due to epistaxis, petechial hemorrhages, myelosuppression or due to severe anemia (Bhardwaj, 2013; Dixit et al., 2012). Among differential leucocyte count, mean values of lymphocytes diminished (Oliveira et al., 2000), neutrophils increased (Agnihotri et al., 2012), eosinophils reduced and basophils diminished (Castro et al., 2004; Srikala et al., 2012) significantly in dogs with ehrlichiosis. The significant decline in levels of monocytes in cases positive for ehrlichiosis (Oliveira et al., 2000). Among various red blood cells indices, the mean values of mean corpuscular hemoglobin concentration were stated to augmented significantly in dogs (Castro et al., 2004; Mulla et al., 2007) along with a decline in levels of MCH in dogs with ehrlichiosis. Serum biochemistry reported significantly upsurge in serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase and creatinine levels as well as decline in total protein levels in dogs. The rise in creatinine levels may be due to immune complex-mediated glomerulonephritis (Agnihotri et al., 2012). A mild-to-moderate thrombocytopenia and/or anemia are the most reliable hematological findings in subclinical CME (Harrus et al., 1998a).

Hyperproteinemia, hyperglobulinemia, hypoalbuminemia and mildly raised alkaline phosphatase and alanine aminotransferase activities are common biochemical deviations associated with CME (Castro et al., 2004). Hypoalbuminemia seen in CME may be the consequence of peripheral loss of albumin due to edematous inflammatory fluids as a result of improved vascular permeability, blood loss or diminished protein production due to synchronized mild liver or may be due to minimal-change glomerulopathy disease (Reardon and Pierce, 1981).

Hematology and coagulation trial was considered by Ingole, (2003) in five cases of *Ehrlichia canis*, they detected that there was decline in Hb, PCV, TEC and

buccal mucosal bleeding time (BMBT), activated clotting time (ACT), prothrombin time (PT) and thrombin time (TT) were within the reference ranges. C-reactive protein, haptoglobin, serum amyloid A, a1-acid glycoprotein and ferritin gradient to rise (positive acute phase proteins), while albumin (negative acute phase protein) and paraoxonase-1 (oxidative stress indicator) have a tendency to decline in dogs with acute and chronic but not in the subclinical phase of the disease (Rudoler et al., 2015).

2.3. Serum Platelet migration inhibitor factor and its clinical utilization to detect thrombocytopenia

Thrombocytopenia is measured to be the most common and reliable hematological abnormality of dogs naturally or experimentally affected with *E. canis* (Waner et al., 1995). The thrombocytopenia in CME is accredited to different mechanisms in the different stages of the disease. Mechanisms thought to be involved in the pathogenesis of thrombocytopenia in the acute phase of the disease contain increased platelet consumption due to inflammatory changes in blood vessel endothelium, augmented splenic sequestration of platelets and immunologic demolition or injury ensuing in a significantly reduced platelet life span (Kakoma et al., 1978). Studies using radioisotopes have revealed that platelet survival time declined from a mean of 9 days to 4 days, 2 to 4 days after infection with *E. canis* (Smith et al., 1975). A platelet migration inhibition factor was also isolated and characterized. This factor is credible to play a role in enhancing platelet sequestration and stasis foremost to reduced peripheral-blood platelet counts (Abeygunawardena et al., 1990).

Demonstration of serum platelet-bindable antiplatelet antibodies (APA) in dogs after experimental infection with *E. canis* supports the hypothesis that immune destruction may also subsidize to the pathogenesis of thrombocytopenia in acute ehrlichiosis (Harrus et al., 1996a; Waner et al., 1995). The earliest detection of APA was made on day 7 post infection (p.i.) in one of six dogs, on day 13 in three and on day 17 in the two remaining dogs (Harrus et al., 1996b). The initial appearance of APA prior to arrival of *E. canis* antibodies indorsed that B cells carrying natural autoantibody receptors may be tempted to undergo propagation and maturation by interface with ehrlichial antigens which are antigenically similar to self-antigens. The alternative theory planned that APA develop secondarily to platelet mechanisms undergoing devastation and massive release of platelet structural proteins brought

about by nonimmunologic platelet destruction (Waner et al., 1995). Complement consumption was revealed to occur during the thrombocytopenic phase of acute ehrlichiosis and partial decomplementation of diseased dog sera vapid the severity of the thrombocytopenia further corroborating the dispute for an immunopathologic component in the pathogenesis of thrombocytopenia in CME (Lovering et al., 1980). Concurrently with the progress of the thrombocytopenia during the acute phase, a significant increase in the mean platelet volume is usually seen and replicates active thrombopoiesis (Waner et al., 1995). During severe chronic phase of disease diminished platelet production due to bone marrow hypoplasia is measured to be the reason for the thrombocytopenia (Woody and Hoskins, 1991). In this stage, dogs commonly exhibit pancytopenia as a result of this hypoplastic bone marrow which further complicating their clinical status. Platelet adhesiveness was shown to decrease in dogs acutely infected with *E. canis* (Lovering et al., 1980).

The platelet dysfunction arises in the acute stage of CME and together with thrombocytopenia may be a factor conducive to the bleeding tendency detected in the disease. The presence of extreme concentrations of serum APA concurrent with platelet dysfunction (in days 17 to 24 after experimental infection) proposed that APA played a role in causing platelet dysfunction in the acute stage of canine ehrlichiosis. Interaction of APA with platelet membrane glycoproteins was anticipated to cause the platelet dysfunction (Harrus et al., 1996b).

Severe thrombocytopenia is a major manifestation of the pancytopenia in canine ehrlichiosis triggered by *Ehrlichia canis*. Platelet counts began to deterioration when high migration inhibition activity was evident. Declined platelet survival time is apparently due to extreme platelet destruction (Smith et al., 1975).

A platelet migration inhibitory factor produced by lymphocytes of E. canis infected dogs contribute to the thrombocytopathia by inhibiting platelet pseudopod formation. Hyperglobulinemia may also interfere with platelet migration and adhesiveness. Antiplatelet antibodies have been established in dogs with experimentally induced ehrlichiosis (Greene, 1995). Inhibition of platelet aggregation and antiplatelet antibodies were detected concurrently suggesting a causal relationship. However, in the same study, platelet aggregation was inhibited in some dogs at a time in which antiplatelet antibodies were absent, indicating that other factors are also important (Harrus et al., 1996b). A platelet migration inhibition factor

proposed to play a role in enhancing platelet sequestration and stasis, leading to reduced peripheral-blood platelet counts (Abeygunawardena et al., 1990).

Both cellular and humoral responses (Ristic et al., 1972) are persuaded during the disease and it has been projected that the immune responses may be unfavorable to the host (Buhle et al., 1974). Humoral factors were produced against normal canine platelets during ehrlichiosis and were noticeable by the migration inhibition test. The activity concurred with the severe phase of thrombocytopenia but continued at high levels during the chronic phase of the disease. The humoral antibody does not appear to be associated with protection and may contribute to the pathogenesis of the disease (Weisiger et al., 1975). A platelet migration inhibition test was established (Duquesnoy et al., 1975) to measure antiplatelet activity.

Platelet migration is a definite physiological phenomenon distinct from simple diffusion and Brownian movement (Lowenhaupt et al., 1973) and offers a good index of platelet function. Inhibition of platelet migration in vivo could have important pathological effects. Sequestration or consumption of platelets by the vasculature and their premature devastation by the reticuloendothelial system are among the various mechanisms for the development of thrombocytopenia (Wilkins et al., 1973). Migration inhibition could affect the entry of platelets into the circulatory system from the bone marrow and also enable adherence of platelets to the endothelium in a manner analogous to the thrombocytopenia associated with Rocky Mountain spotted fever (Park et al., 2011).

Scanning electron microscopy shows the inhibitory effect persuades prominent surface changes and may render exaggerated platelets vulnerable to direct demolition by the reticuloendothelial system. The appearance of normal and inhibited platelets examined by scanning electron microscopy. Uninhibited platelets had numerous pseudopods whereas inhibited platelets generally were rounded and smooth. Occasionally, evidence of membrane impairment with apparent shrinkage and loss of intracellular material was seen with inhibited platelets. Platelet migration inhibition headed visible levels of specific antibody (Kakoma et al., 1978).

2.4. Coagulation profile (Prothrombin Time and Activated partial Thromboplastin Time) and platelet index:

Thrombocytopenia is the most commonly acquired hemostatic disorder in dogs and can become potentially life-threatening (Bommer et al., 2008; Grindem et al., 1991). Severe thrombocytopenia in dogs is mostly caused by immune-mediated thrombocytopenia. However, low platelet counts are also commonly associated with inflammatory, infectious or neoplastic diseases (Botsch et al., 2009).

RBC indices provide important information on anemia in dogs. Therefore, platelet indices can provide clinical information on thrombocytopenia. Platelet volume indices such as Mean Platelet Volume (MPV) which represents average platelet size and Platelet Distribution Width (PDW) which signifies platelet size heterogeneity are now routinely accessible (Souza et al., 2012)**.**

The connection between MPV, PDW and platelet counts in a population of clinically healthy and systemically ill thrombocytopenic dogs and confirmed that both MPV and PDW were functions of platelet count and changes in platelet volume indices should be understood in relation to platelet number (Bommer et al., 2008).

The quantification of platelets in peripheral blood was a well-recognized means in veterinary diagnostics and recently new indices associated to platelet counts have been provided by hematologic analyzer namely plateletcrit (PCT) and mean platelet volume (MPV) where platelet volume characterizes a marker of platelet function and activation (Boomer et al., 2008; Kaito et al., 2005). Elevated MPV values have been accompanying with an adequate/ increased number of megakaryocytes on bone marrow cytology in human beings and dogs (Sullivan et al., 1995).

The mean platelet volume (MPV) in 68 dogs with thrombocytopenia attributable to various causes. Platelet size was high or low in some dogs and the most clinically useful observation was that low MPV (micro-thrombocytosis) was a specific indicator of immune mediated thrombocytopenia (IMTP) in these thrombocytopenic dogs (Northern and Tvedten, 1992).

Platelet counts results of bone marrow analysis and MPV might be helpful in dogs for the differentiation between primary IMTP and thrombocytopenia resulting from other diseases (Dircks et al., 2009). PCT is an indicator of platelet mass in the

body whereas MPV exemplifies a marker of platelet function and activation. PDW is the measure of the relative size of platelets and has been used to discriminate between immune mediated thrombocytopenia and thrombocytopenia in aplastic anemia in human beings and less understood in veterinary literature (Kaito et al., 2005).

An increased platelet size increases PDW levels just as an increased erythrocyte size increases RDW levels (Schwartz et al., 2014). PDW can rise due to small variations in platelet morphology before MPV values are changed, similar to what occurs with the anisocytosis index. RDW showing the greater sensitivity of PDW (Matos et al., 2008).

Various studies have specified that platelet indices especially MPV and PDW can provide clinical information on the mechanisms of thrombocytopenia (Bowles et al., 2005, Kaito et al., 2005).

The study conducted on 46 dogs (36 were naturally infected with vector-borne diseases and 10 were healthy) from different breed, age and of both sexes. Venous blood samples were attained to detect PLT counts and antibodies of ehrlichiosis. The diagnosis of vector-borne disease was reached by using a commercial ELISA assay kit. PLT count was accomplished with an automated blood cell counter analyzer (Pasa et al., 2017). In addition, PT and APTT concentrations were measured using a micro coagulometer. Statistically significant reduced PLT count was found in dogs mono infected with ehrlichiosis compared to healthy dogs ($P < 0.001$). Changes in mean PT value in the studied animals did not illustrate statistically significant alterations among the groups ($P > 0.05$). APTT values in the ehrlichiosis mono-infection group were significantly higher than that of the healthy control ($P < 0.01$). Coagulation tests are very important in evaluating dogs with bleeding tendencies. Evaluation of complete blood count, platelet numbers, serum biochemistry, Buccal Mucosal Bleeding Time (BMBT) and Activated Clotting Time (ACT) are measured as "Screening Tests" and Tests such as Prothrombin Time (PT) and activated Partial Thromboplastin Time (aPTT), Thrombin Time (TT) and assay of clotting factors are considered as "Specific tests" for evaluation of dogs with bleeding tendencies (Sumathi et al., 2012).

Interpretation of the PT and aPTT is of prime important to clinicians. If PT was abnormal and aPTT was normal then an acquired "deficiency" due to warfarin

(coumadin) therapy is suspected. If the PT was normal and aPTT was extended which could be due to deficiency of factors VIII (Hemophilia A and von Willebrand Disease), IX and XI. Prolongation of both aPTT and PT recommends a deficiency or inhibition of the common pathway coagulation factors (Factor X, V, and II) or a qualitative or quantitative fibrinogen defect. aPTT is also used to display levels of anticoagulant therapy (Vlasin et al., 2004; Lopes et al., 2005).

For knowing the prognostic factors for survival in canine monocytic ehrlichiosis (CME) records of 40 cases were retrospectively studied. The dogs were allocated as survivors ($n = 21$) and non-survivors ($n = 19$) and their signalment, anamnesis, clinical and clinicopathological signs and treatment protocols were compared. Pale mucous membranes, bleeding tendencies and weakness were more predominant in the non-survivors compared to the survivors. Dogs in the non-survivor group had meaningfully lower white blood cell (WBC), hematocrit (HCT) and platelet (PLT) counts. Pronounced pancytopenia ($WBC < 4 \times 10^3\ \mu L^{-1}$; $HCT < 25\%$; $PLT < 50 \times 10^3\ \mu L^{-1}$) was found as a risk factor for mortality. In this study severe leukopenia ($WBC < 0.93 \times 10^3\ \mu L^{-1}$), severe anemia ($PCV < 11.5\%$), prolonged activated partial thromboplastin time ($APTT > 18.25$ s) and hypokalemia ($K < 3.65$ mmol/L) were each initiate to estimate mortality with a probability of 100%. In contrast, WBC counts above $5.18 \times 10^3\ \mu L^{-1}$, platelet counts above $89.5 \times 10^3\ \mu L^{-1}$, $PCV > 33.5\%$, $APTT < 14.5$ s and serum potassium concentration above 4.75 mmol/L, each provided 100% prediction for survival. These prognostic indicators can be easily obtained at presentation are inexpensive and may be useful supports when treatment and prognosis are being considered (Shipov et al., 2008).

2.5. Diagnostics and therapeutics of canine ehrlichiosis including herbs for increasing platelet count

Diagnosis of the Canine Monocytic Ehrlichiosis can be exciting due to its different phases and multiple clinical manifestations. CME should be alleged when a compatible history (living in or travel to an endemic region, previous tick exposure), typical clinical signs and representative hematological and biochemical abnormalities are present (Nakaghi et al., 2008). Traditional diagnostic techniques (hematology, cytology, serology and isolation) are valuable diagnostic tools for CME. However, a definitive diagnosis of *E. canis* infection requires molecular techniques (Harrus and Waner, 2011).

During the acute stage, moderate to severe thrombocytopenia is a distinctive hematological finding. During the subclinical phase, a mild thrombocytopenia may be present in the absence of clinical findings. In experimentally infected dogs reduced platelet counts by up to 42% have been detected with counts as low as 1,40,000/µL (Harrus et al., 1998b; Waner et al., 1997). Leukocyte and erythrocyte counts may also be decreased, although these changes may be relatively mild and difficult to notice in a clinical setting (Waner et al., 1997). In the chronic phase, thrombocytopenia is usually severe and convoyed by marked anemia and leukopenia. Marked pancytopenia due to bone marrow hypoplasia is a hallmark of the chronic severe form (Harrus et al., 1997). Platelet counts and their magnitude have been proposed and are being used as a screening test for CME in endemic regions. While only 1/71 (1.4%) non-thrombocytopenic dogs (platelet counts >200,000/ µL) were positive for E. canis DNA (16S rRNA) by polymerase chain reaction (PCR), 13/62 (21%) dogs with platelet counts of 1,00,000–2,00,000/ µL and 53/84 (63.1%) dogs with platelet counts ≤ 1,00,000 µL were PCR positive for *Ehrlichia canis* (Bulla et al., 2004).

CME should not be reigned out solely on the basis of a normal platelet count (Mylonakis et al., 2011b; Codner and Farris-Smith, 1986). A non-regenerative anemia, leukopenia, neutropenia (mild-to-moderate leukocytosis, neutrophilia with or without a mild left shift may rarely be seen) and lymphopenia or mild lymphocytosis are additional deviations. Granular lymphocytosis with T-cell expansion in the blood and other tissues may occur in the subclinical and chronic phase of the disease emulating lymphocytic leukemia. Thus, in endemic areas CME should be a top differential for persistent lymphocytosis in the dog (Harrus et al., 1998b).

Demonstration of typical cytoplasmic E. canis morulae in monocytes, macrophages and lymphocytes in Romanowsky type stained smears from buffy coat and less frequently lymph node, BM, spleen, liver and cerebrospinal fluid smears by light microscopy strongly supports a diagnosis of CME. Morulae are membrane bound vacuoles which are usually densely packed with bacteria as seen by electron microscopy (Harrus and Waner, 2011; Mylonakis et al., 2010; Faria et al., 2010; Meinkoth et al., 1998; Hildebrandt et al., 1973). Blood smears of infected dogs may contain reactive monocytes, erythrophagocytosis, thrombophagocytosis, phagocytosis of nuclear material and mega platelets. However, red blood cell morphology is unremarkable in most cases (Harrus et al., 1997). Large granular lymphocytosis may

be present in progressive stages. There is approximately 50% of 200 lymphocytes estimated in Wrights-stained blood smears had prominent azurophilic granules and the cell nuclei were often cleaved or depressed with mature nuclear chromatin consistent with large granular lymphocytes (Heeb et al., 2003).

Blood smear evaluation is also necessary to appraise co-infections with other tick-borne pathogens (e.g., *Babesia canis* and *Hepatozoon canis*) which influence disease manifestation, severity and treatment outcome (Gal et al., 2007). In a study with dogs naturally infected by *E. canis* (presumptive acute CME), the diagnostic sensitivity of buffy coat (review of 1000 oil immersion fields, 100x objective lens), lymph node (500 oil immersion fields, 100x objective lens) or their combination was 66%, 61% and 74%, respectively (Mylonakis et al., 2003). Diagnostic sensitivity of spleen cytology in dogs naturally infected by *E. canis* was 49% (Faria et al., 2010).

Hyperproteinemia, Hypoalbuminemia, hyperglobulinemia and hypergamma-globulinemia are common serum biochemical alterations in CME. Mild increases in alanine aminotransferase (ALT) and alkaline phosphatase (ALP) are common in dogs during the acute phase. (Mylonakis et al., 2011b; Frank and Breitschwerdt, 1999; Harrus et al., 1997). Acute phase protein concentrations may rise during clinical CME. Plasma C-reactive protein (CRP) concentrations have been shown to increase between 4 and 16 days and peaked between 15 and 42 days after inoculation with *E. canis* in five dogs (Shimada et al., 2002). Creatinine concentration is elevated in some dogs while glomerular proteinuria may be present attributable to glomerulonephritis with or without immune-complexes deposition in the chronic and acute CME, respectively (Frank and Breitschwerdt, 1999).

Several serological methods have been established for the diagnosis of CME and are measured appreciated screening and/or diagnostic tools. The indirect immune-fluorescence antibody (IFA) test for anti- *E. canis* IgG antibodies is considered the serological gold standard indicating exposure to *E. canis.* IgM is not considered a consistent indicator of *E. canis* exposure due to the inconsistent development of IgM antibodies in the course of the disease (McBride et al., 2003). In contrast, IgG titer P1:40 are considered positive for *E. canis* exposure. Anti-Ehrlichia IgG antibodies persevere for several months to years after treatment and elimination of the rickettsia (Bartsch and Greene, 1996).

Enzyme-linked immunosorbent assays (ELISA) have been established and found useful in the diagnosis of the disease. Dot-ELISA kits for the detection of *E. canis* IgG antibodies are commercially available and are frequently used as point-of-care tests (Harrus et al., 2002).

Western immunoblot is a more definite serological technique which can assist in depicting the infecting agent by giving 'fingerprints' of its immunogenic protein profile. Usually, a homogeneous pattern is retrieved among different strains of *E. canis*. However, antigenic variety among different strains in various regions of the world has been suggested. Western immunoblotting has been used to illustrate and distinguish between infection with different organisms causing ehrlichiosis, anaplasmosis or neo-rickettsiosis and has the potential to explain dilemmas relating serologic cross-reactivities (Hegarty et al., 1997).

PCR and sequencing are sensitive methods for noticing and illustrating *E. canis* DNA. Detection of *E. canis* DNA can be accomplished as early as 4–10 days post-inoculation (Iqbal et al., 1994). Several assays are instituted on different target genes (e.g., *16S rRNA, p28, p30, dsb, VirB9*). However, *16S* rRNA and *p30*-based PCR assays are most commonly used. The *p30*-based nested PCR assay has been shown to be more subtle than the *16S* rRNA-based nested PCR assay. The circumstance that *E. canis* contains multiple copies of the *p30* gene, but only one copy of the *16S* rRNA gene rises the probabilities for recognition when *p30* is targeted (Stich et al., 2002).

Quantitative real-time PCR (qPCR) is more subtle than conventional PCR and consents for quantification of bacterial load. It has been used for the quantification of ehrlichial load in naturally and experimentally infected dogs (Baneth et al., 2009). It is less prone to contaminations than conventional PCR and when contamination is present, it can be easily noticed by using melting curve analyses. Therefore, real-time PCR is quickly becoming the favored method for diagnosis of *E. canis*. The capabilities of real-time PCR instruments permit for the construction of multiplex assays for the simultaneous detection of several pathogens in one sample. Several multiplex qPCR assays have been established for the simultaneous detection of *E. canis* and other organisms. A multiplex qPCR has been established for simultaneous detection of *E. chaffeensis, E. canis, E. ewingii, A. phagocytophilum,* and *A. platys* (Sirigireddy and Ganta, 2005). A new molecular approach using real-

time PCR is High resolution melt (HRM) analysis. HRM real-time PCR is a molecular approach originally developed for single nucleotide polymorphism (SNP) genotyping (Wittwer et al., 2003).

CME is successfully treated with antibiotics that belong to the tetracycline family. The treatment of choice is doxycycline at 5 mg/kg twice daily or 10 mg/kg once daily for 4 weeks. This regime assurances a complete response in the majority of cases (McClure et al., 2010; Harrus et al., 2004). Doxycycline, unlike other tetracyclines does not seem to cause enamel discoloration in puppies. Moreover, one of the most common side effects of tetracyclines is vomiting which can be eliminated by excruciating the antibiotic dose into two half-doses every 12 h (q12h) or by administering the antibiotic after feeding. When the dog has liver problems, treatment regime should be stopped (Little, 2010; Neer et al., 2002; Wen et al., 1997). Chloramphenicol has been used in dogs below 1 year of age. Imidocarb dipropionate has also been selected as a likely treatment for ehrlichiosis in dogs. It should only be used in cases of co-infection with *Babesia canis or Hepatozoon canis.* (Sainz et al., 2000; Matthewman et al., 1994; Price and Dolan, 1980). Other antibiotics such as rifampicin or levofloxacin have been effective in In-vitro studies (Schaefer et al., 2008). Rifampicin subsidized to refining laboratory findings, but it was not effective in abolishing the infection (Theodorou et al., 2013).

Treatment of ehrlichiosis should not be started with glucocorticoids combined with antibiotics. Steroids should only be considered when no acceptable response is noticed or when immune-mediated problems arise. Ehrlichia intercede an immune response specified by hemolytic anemia, thrombocytopenia, uveitis, glomerulonephritis, vasculitis, etc. In those cases, treatment with glucocorticoids (usually prednisone or prednisolone) may be indicated; doses of prednisone should range from 0.5 to 2 mg/ kg/day and the treatment duration should vary according to the type and seriousness of the accompanying immune-mediated condition (Kohn et al., 2008; Massa et al., 2002; Varela et al., 1997; Maretzki et al., 1994; Perille and Matus, 1991). The use of immunosuppressive glucocorticoids for treatment of secondary immune-mediated diseases must be measured very carefully. The administration of immunosuppressive doses of glucocorticoids or other drugs to subclinically infected dogs may result in the re-emergence of bacteremia although the dog may not show clinical signs (Egenvall et al., 2000).

Supportive treatment in dogs with CME-induced aplastic pancytopenia is critically important if the limited chances for survival are to be pursued. It contains the administration of balanced crystalloid solutions and/or the periodic blood-typed and cross-matched packed red blood cells or whole blood transfusions, oral platelets boosters, steroid therapy for few days and prophylactic (asymptomatic dogs with moderate-to-severe neutropenia (neutrophil count <1,000/ μl) or therapeutic (symptomatic neutropenic dogs) use of bactericidal antibiotics (Mylonakis et al., 2010).

Carica papaya/Papaya pear is belongs to the family Caricaceae. Papaya is known as "A powerhouse of nutrients" and was reliably called the "The fruit of Angels" by Christopher Columbus in the 20th century. It is a polygamous, dicotyledonous and diploid species and found in the most tropical and subtropical countries of the world (Yunita et al., 2012). It is being cultivated widely for consumption as fresh fruit, dried and crystalized fruit as well as for use in drinks, jams and candies. Green fruit, the leaves and flowers may also be used as a cooked vegetable. Several proteins and alkaloids present in papaya leaves and fruit contains important pharmaceutical (ElMoussaoui et al., 2001). Several studies were supported out to appraise the biological activities of various parts of papaya plant such as fruits, shoots, leaves, rinds, seeds, roots and latex (Kovendan et al., 2012). Papaya leaves comprise active components such as papain, chymopapain, cystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates that surges the total antioxidant power in blood and diminish lipid peroxidation level (Seigler et al., 2002). Papaya leaf extracts hold biological membrane stabilization properties and also avoid the stress-induced demolition of the plasma membrane. Flavonoids and other phenolic compounds present in papaya leaf extracts were accountable for the observed membrane stabilizing property and thereby prevent the internal bleeding in the blood vessels (Ranasinghe et al., 2012). Flavonoids present in Carica papaya prevents NS2B-NS3 protease and thereby avoid the DEN-2 Virus assembly. Papaya leaf juice significantly increases the platelet count (Senthilvel et al., 2013).

Papaya leaf encompasses anti-oxidant, vitamins, minerals, cardiac glycosides and alkaloids which shows an important role in curing of thrombocytopenia and also help to increase the hemoglobin, hematocrit, red blood cells, thrombocytes and total protein contents (Halim et al., 2011; Imaga et al., 2010; Ayoola and Adeyeye, 2010;

Kathiresan et al., 2009). Papaya leaf encompasses carbohydrates, dietary fiber, fat, protein and metals. Vitamins present in papaya leaf are thiamin (B1), riboflavin (B2), niacin (B3), vitamin B6, ascorbic acid (C) and especially B17 (laetrile) which is supposed to be used for treatment of cancer. Minerals and metals present in leaf are calcium, iron, magnesium, potassium and sodium. Vitamin A holds bile production normal and Vitamin B9 assistances in blood DNA synthesis, cell growth and development. Vitamin B12 aids in maintaining the normal count of thrombocytes and assistances to fight against thrombocytopenia (Kathiresan et al., 2009; Betty et al., 2009). Vitamin C may act as anti-oxidant to scavenge the oxygen radicals (superoxide, hydroxyl, peroxyl), sulphur radicals and nitrogen-oxygen radicals (Sebastian et al., 2003). Calcium ions helps in the proliferation of lymphocyte cells which show key role in platelet aggregation when combine with Vitamin D and avoids thrombocytopenia (Emilio et al., 2009; Cabrera-Cortina et al., 2008). Magnesium ions improves erythrocyte hydration. Sodium ions aids in upholding electrolyte balance and avoids hyponatremia during dengue infection (Jutrat et al., 2005). The alkaloids present comprise carpaine, pseudocarpaine and dehydrocarpaine I and II. These constituents can act on the bone marrow avoid its destruction and augment its ability to produce platelets. Moreover, it can also avoid platelet destruction in the blood and thereby increase the life of the platelet in circulation (Patil et al., 2013).

Tinospora cordifolia is a widely used shrub in ayurvedic systems of medicine. It is a large, glabrous, deciduous climbing shrub belonging to the family menispermaceae. In Hindi, the plant is commonly known as Giloe which means to the heavenly elixir that has kept celestial beings from old age and kept them eternally young. Guduchi, its Sanskrit name, means one which protects the entire body. The term amrita is ascribed to its ability to impart youthfulness, vitality and longevity (Bhandari and Chandrodaya, 2006). It is an annual or perennial Ayurvedic plant which is used in several traditional medicines to cure various diseases. The plant is sometimes cultured for ornamental value and is proliferated by cuttings. Almost all the parts of the plant are useful in ethnobotanical surveys showed by ethnobotanists. The medicinal properties conveyed are antidiabetic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-leprotic, anti-malarial, antipyretic, hepatoprotective, immunomodulatory and antineoplastic activities (Ikram et al., 1987;

Khosa and Prasad,1971). The chemical constituents described from this shrub belong to different classes such as alkaloids, terpenoids lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. The starch attained from the stem known as guduchi satva is highly nutritive and digestive and used in many diseases. Starch is present throughout the parenchyma of the stem (Zhao et al., 1991). It probably acts by refining the production of platelets from bone marrow due to its antioxidant (Eberly et al., 2008) and immunomodulatory properties. This beneficial role of *T. cordifolia* can be ascribed to the presence of various phytochemical constituents like alkaloids (berberine, palmatine D, choline D, tinosporine, magnoflorine, tetrahydropalmatine), sesquiterpenoid (tinocordifolin) and glycosides (furanoidditerpene glycoside, tinocordiside, syringing and sysingine-apiosyglycoside) present in the stem (Kapil and Sharma, 1997). The leaf extract significant anti-oxidant and hydroxyl radical scavenging activities an alpha-glucosidase inhibitor characterized as saponarin (Joshi and Kaur, 2016). Phenylpropanoid glycosides such as cordifolioside A, cordifolioside B and syringin have been stated to be main immunomodulatory active components. D-glucan, isolated from the stem of the plant has been revealed to stimulate the immune system (Maurya et al., 1996). Alkaloids present in stem of *T. Cordifolia* have been confirmed effective against anti-platelet macrophages in patients suffering from idiopathic thrombocytopenic purpura (Chintalwar et al., 1999) indicating the role of *T. cordifolia* in protecting the platelets from destruction. Leaves comprise various phytoconstituents like saponins, tannins, cardiac glycosides and alkaloids. Its chemical constituents are rich in phenolic compounds and Vitamin-c (Rana et al., 2012).

Withania somnifera / Ashwagandha is belongs to the family Solanaceae. It exhibits antimicrobial, anti-inflammatory, immunomodulation, anti-oxidative, anti-anxiety, aphrodisiac, anti-diabetic, anticancer, central nervous system depressant, hepatoprotective, energy-enhancing, rejuvenated and life prolonging properties. Several reports have demonstrated immunomodulator and antitumor effects of ashwagandha (Uddin et al., 2012). Ashwagandha acts as an anti-inflammatory agent through inhibition of complement, lymphocyte proliferation and delayed-type hypersensitivity (Rasool and Varalakshmi, 2006). Ashwagandha display an immuno-potentiating and myeloprotective effects by enhancing the levels of interleukin (IL)-2,

interferon (IFN)-γ and granulocyte macrophage colony stimulating factor in normal and cyclophosphamide-treated mice (Davis and Kuttan, 1998). A sequence of animal studies shows ashwagandha to have thoughtful effects on the hematopoietic system, acting as an immunoregulator and a chemoprotective agent. It increases vital body fluids, blood, lymph, semen and cell production. As the plant is rich in iron, it contributes to red blood cell count (Kuttan, 1996). The roots of *W. somnifera* contains active compound (Withanolide A) which specifically increases the expression levels of T-helper 1 (Th1) cytokines as well as CD4 and CD8 counts. It also enhances natural killer (NK) cell activity in a dose dependent manner with a faster recovery of CD4+ T cells in immune suppressed animals (Davis and Kuttan, 2002).

Asparagus racemosus belongs to family Asparagaceae. Asparagus is the Greek word for "stalk" or "shoot". About 300 species of Asparagus are known to occur in the world (Shao et al., 1997). Out of several species of `Asparagus' grown in India, *A. racemosus, A. gonaclades* and *A. adsendens* are most commonly used in indigenous medicine (Rao, 1952). *A. racemosus* is commonly mentioned as a rasayana in the Ayurveda. Rasayanas are those plant drugs which promote general wellbeing of an individual by increasing cellular vitality or resistance. It possesses anthelmintic and antiseptic properties (Galvez et al., 1995). It also possesses much more important biological properties such as immunostimulants, anti-inflammatory, anti-hepatotoxic, antimicrobial and reproductive agent (Sinha and Biswas, 2011). *A. racemosus* is also used in the treatments of various disorders including stomach ulcers, lung abscess, herpes and chronic fevers investigated by Hayes et al., 2006. In addition to this, roots of this plant can be used in nervous disorders, dyspepsia, diarrhoea, dysentery, tumour's, inflammations, neuropathy, hyperdipsia, cough, bronchitis, hyperacidity and infectious diseases. Generally, root of this plant is bitter in taste, emollients, cooling, nervine tonic, constipating, diuretic and rejuvenating (Sharma et al., 2000). The root extract of *Asparagus racemosus* possess anti-anaemic and anti- thrombocytopenic properties and these could be associated to the phytoconstituents such as alkaloids, saponins, phytosterols, triterpenoids, polyphenols, carbohydrate, flavonoids, glycosides, phenolic compounds and tannins present in the extract (Chaudhary et al., 2016). Other primary chemical constituents of *Asparagus spp.* are essential oils, asparagine, arginine, tyrosine, flavonoids (kaempferol and quercetin), resin, and tannin (Joshi, 2016). The major active

constituents of *Asparagus racemosus* are steroidal saponins. Isoflavones, asparagamine, racemosol, polysaccharides, mucilage, vitamins A, B1, B2, C, E, Mg, P, Ca, Fe and folic acid present in roots (Joshi, 2016). The revitalization of macrophage chemotaxis and interleukin-I (IL-I) and tumor necrosis factor a (TNFa) production by the oral treatment of *A. racemosus* root extract in ochratoxin A treated mice (Dhuley et al., 1997). The alcoholic extract has been found to enhance both, humoral and cell mediated immunity of albino mice injected with sheep red blood cells as particulate antigen. Alcoholic extract of root of *A. racemosus* has been shown to significantly reduce the enhanced levels of alanine transaminase, aspartate transaminase and alkaline phosphatase in $CC1_4$-induced hepatic damage in rats indicating antihepatotoxic potential of *A. racemosus* (Muruganadan et al., 2000).

Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

3.1 Screening of dogs for study

Screening of dogs suspected for Ehrlichia was done on the basis of observation of classical signs of Ehrlichia in dogs. Based on results of previous studies, clinical signs considered for screening includes mucosal pallor, high fever, lymph adenomegaly, epistaxis, splenomegaly, hyphema, depression, ocular abnormalities, vomiting, hematuria, melena, hematemesis, hind limb /or scrotal oedema, bleeding diathesis, icterus, central nervous system signs, ascites and weight loss. Dogs with above mentioned 2-3 classical signs of ehrlichiosis listed above were included in screening procedure.

3.2. Inclusive and Exclusive criteria.

Dogs, irrespective of age, sex and breed were included in the study falling within inclusive criteria designed below.

3.2.1 Inclusive criteria for animals.

1) Dogs should not have any concurrent disease.

2) Dogs must have given preventive health care (Vaccination and Deworming).

3) Dogs should have 2-3 classical signs of ehrlichiosis.

4) Complete blood count test with either pancytopenia or decrease in at least two parameters namely haemoglobin and platelet count.

5) Dogs exhibiting classical clinical signs with either pancytopenia or anaemia with thrombocytopenia and positive for ehrlichiosis on blood smear and buffy coat smear examination test.

6) Dogs exhibiting classical clinical signs with either pancytopenia or anaemia with thrombocytopenia but negative for blood smear and buffy coat smear examination test but further confirmed positive by PCR Test.

3.2.2 Exclusive criteria

1) Dogs positive for ehrlichiosis with no regular history of deworming and vaccination.

2) Dogs positive for ehrlichiosis with a concurrent disease.

3.3 Selection criteria of dogs

A total Sixty-two (62) dogs were screened in the present investigation, which were presented to TVCC, DUVASU, Mathura between August, 2020 to June, 2021. Out of Sixty-two (62) screened samples, 42 samples were found positive for ehrlichiosis on the basis of Nested PCR. The primary confirmation of canine monocytic ehrlichiosis was done by detection of morulae in monocytes in the blood smear which was taken from ear tip and stained by Leishman stain also buffy coat smear for detection of morulae in monocytes stained by Leishman stain examination was done. Dogs having symptoms of CME or having hematological changes signifying for CME but negative for blood and buffy coat smear examination were further confirmed by polymerase chain reaction test.

The Forty-two(42) dogs confirmed for ehrlichiosis were used for clinical and epidemiological studies. For the purpose, detailed clinical symptoms and animal related information were recorded for each dog. Vital physiological parameters like rectal temperature, pulse rate, respiration rate and capillary refill time were recorded. Hematological parameters like total erythrocyte count, hemoglobin, packed cell volume, total leucocyte count, differential leucocyte count, platelet count, platelet indices (MPV, PDW, PCT) and erythrocyte indices (MCV, MCH, MCHC) were done by fully automated hematology analyzer (Celltac Alpha VET MEK-6550) and biochemical parameters (Serum creatinine, blood urea nitrogen, alanine amino transferase, aspartate amino transferase, alkaline phosphatase, total protein, albumin) were analyzed by Biochemical analyzer (AKRAY Healthcare Pvt. Ltd.). Globulin was calculated by subtracting albumin from total protein. Coagulation profile (PT and APTT) were analyzed by micro-coagulometer. Assay of serum Canine Platelet migration inhibition factor (C-PMIF) were done using compatible ELISA kits.

3.4. Diagnostic Studies

In present study all the suspected cases of dogs were examined by following methods.

3.4.1 Parasitological diagnosis of *E.canis* infection in canines

Blood samples were taken from dogs by technique of collection from ear tips and cephalic or saphenous vein aseptically with a sterilized disposable syringe and needle. The sample from cephalic or saphenous vein was collected in three clean and dry blood collection vials one containing EDTA as anticoagulant (For Hematology) and one containing trisodium citrate as anticoagulant (For coagulation profile) and another plain without any anticoagulant (For biochemical).

3.4.1.1 Blood smear examination:

The blood sample which was collected from ear tip was analyzed for hemprotozoan by staining the blood film using Leishman stain. A drop of blood (about 5 µl) was placed 20 mm from one end of a clean microscopic slide and a thin smear was drawn. Then film was air dried very briefly. Fully covered the smear with Leishman's stain solution and kept it for 2 minutes. Add twice the amount of distilled water and mixed by swirling and then incubated it for 10 min. Rinsed thoroughly with distilled water. Dried the slide using blotting paper and air dried as well. Slide was visualized at 100x using oil immersion objective of microscope.

3.4.1.2 Buffy coat smear examination

The 2 ml of blood sample was collected from saphenous vein in EDTA vial containing anticoagulant. Filling of a microhematocrit tube to 75% of its length was done followed by sealing the one end with paraffin. Then after tube was centrifuge at 3000 rpm for 10-20 min till layer separation occur from down to up (erythrocyte layer, buffy coat and plasma). Hematocrit tube was break along the line between the buffy coat and top of the packed RBCs column. A drop of buffy coat was placed on clean glass slide and buffy coat was spreaded by another slide followed by staining with Leishman stain. Slide was visualized at 100x using oil immersion objective of microscope.

3.4.2 Molecular diagnostic technique (Polymerase chain reaction)

3.4.2.1 Experimental dogs and sample collection

Blood samples from suspected dogs were utilized in the present analysis. Approximately 2 ml of blood from representative dogs were collected from cephalic or saphenous vein in vacutainer tubes containing EDTA as anticoagulant under aseptic situation.

3.4.2.2 Isolation of genomic DNA

1) Gently rocked EDTA vial containing blood until thoroughly mixed.

2) Pipette 25 µl of Proteinase K solution into the bottom of autoclaved micro centrifuge tube (1.5ml) to it add 200 µl whole blood (equilibrated at room temp).

3) Add 200 µl of Lysis buffer Solution (GB3) into the tube containing sample and mixed it by vortexing (15 Sec) to obtain a uniform suspension. Incubated it for 10-15 minutes at 70°C temperature.

4) Add 210 µl of ethanol (96-100%) to each sample and vortex again.

5) Transferred the prepared mixture to mini column placed collection tube. Centrifuged it for 1 minute at 11000 rpm. Discard the collection tube containing the flow-through. If the samples are not drawn through matrix completely repeat the centrifugation at higher force 15000×g. Place the column into a new 2 ml collection tube.

6) Added 500 µl of Wash Buffer GBW. Centrifuged it for 1 minute at 11000 rpm. Discarded the flow through and place the column back into fresh collection tube.

7) Added 600 µl of Wash Buffer GB5 to the column. Centrifuged for 1minute at 11000 rpm. Empty the collection tube. Placed the purification column back into the tube and re-spined the column for 1 minute at 11000 rpm.

8) Discard the collection tube containing the flow-through solution and transfer the column to a new sterile 1.5 ml microcentrifuge tube.

9) Added 100 µl of elusion buffer GBE (preheated at 70°C) on silica membrane to elute genomic DNA. Incubated for 1minute at room temperature and centrifuged it for 1 minute at 11000 rpm. Discard the purification column.

10) Stored the DNA at about -20 °C.

3.4.2.3 Checking of concentration and purity of DNA

The concentration of DNA was calculated on Nanodrop (Bio photometer) (BioRad, USA). The OD value at 260 nm gives the amount of nucleic acids present in DNA which is present in given samples whereas OD value at 280 nm gives the amount of protein present in the sample. Based on this, purity of DNA was checked using Nanodrop by taking the ratio of optical density values at 260 and 280 nm. The samples having OD ratio (260/280) between 1.75 to 9 were used in this experiment.

3.4.2.4 Quality of DNA

Horizontal submarine agarose gel electrophoresis was performed to check the quality of DNA. Agarose of 1.2 % w/v was dissolved in 1x TAE buffer by heating in microwave oven. The agarose solution was cooled to 40-50^{o} C and poured into casting gel tray after adding Ethidium Bromide (0.5 µg /ml). Care was taken while placing the comb parallel to the platform and having at least 0.5 mm above the floor of gel casting tray. After half an hour on gel solidification comb was removed. The gel casting tray was submerged in gel tank having 1xTAE buffer. For loading 10 µl of DNA samples were taken and then loaded in the well. Electrophoresis was performed at 70 volts for 120 minutes. Then gel was visualized under iBright 750 imaging system. The genomic DNA having good quality (intact without smearing) was used for further analysis.

3.4.2.5 Amplification of *Ehrlichia* specific genes

Specific primer pairs were used for *E. canis* gene (16 S rRNA) for their identification (Nakaghi et al., 2008).

3.4.2.5.1 Primer sequences used to amplify *E.canis* gene:

The sequences of the primers which were used for the amplification of *E. canis* is as follows (Waner et al., 1997).

Primary PCR assay:

ECC: 5' AGAACGAACGCTGGCGGCAAGC 3'

ECB: 5' CGTATTACCGCGGCTGCTGGCA 3'

Nested PCR assay:

ECAN5: 5'- CAATTATTTATAGCCTCTGGCTCTGGCTATAGGA-3'

HE3: 5'- TATAGGTACCGTCATTATCTTCCCTAT- 3'

3.4.2.5.2 Setting up PCR reaction:

3.4.2.5.2.1 Simple PCR

An aliquot of 10µl genomic DNA was taken in 200 µl PCR tubes and kept on ice. ECC and ECB primers were added amounting 1.5µl each and finally PCR master mix 2X of 12µl was added in the reaction mixture. The reaction mixture was carried out in a final volume of 25µl.

3.4.2.5.2.2 Nested PCR

5 µl of amplified product of simple PCR was taken in a PCR tube kept on ice. 5µl of nuclease free water was added. ECAN5 and HE3 primers were added amounting 1.5 µl each as reverse and forward primers and finally PCR master mix 12 µl was added into the PCR reaction mixture. The reaction mixture was carried out in a final volume of 25µl. The reaction mixture comprises of following components:

Table 1: PCR reaction mixtures for *E. canis*

S. No.	Reaction components	Simple PCR	Nested PCR
1	Nuclease free water	0µl	5µl
2	PCR master mix 2X	12µl	12µl
3	Primer F (15 pmol/µl)	1.5µl	1.5µl
4	Primer R (15 pmol/µl)	1.5µl	1.5µl
5	Template	10µl	5µl
	Final volume	25 µl	25µl

All the above procedure was carried out on ice and master mix were mixed properly by tapping and followed by short spinning.

3.4.2.5.2.3 Amplification profiles

PCR tubes were kept in preprogrammed thermal cycler for DNA amplifications. The cycling conditions were as follows:

SIMPLE PCR: Initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min, and the final extension was performed at 72°C for 8 min.

NESTED PCR: Initial denaturation at 94°C for 3 min, first step consisted of three cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 1.5 min while the second step consisted of 37 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 1.5 min and the final extension was performed at 72°C for 8 min. The amplifications were done in Thermocycler (Bio-Rad, USA). The agarose gels were visualized on UV transilluminator.

3.5. Different clinical signs and their frequency distribution in dogs

Frequency distribution of different clinical signs in ehrlichiosis were evaluated as % frequency of each and every clinical signs

$$\text{Frequency} = \frac{\text{No. of positive cases showing specific clinical signs (n)}}{\text{Total no. of positive cases (N)}} \times 100$$

3.6. Percentage positivity on the basis of sex

$$\% \text{ Positivity} = \frac{\text{No. of positive cases given by nested PCR in different sex (n)}}{\text{Total no. of Ehrlichia positive cases given by nested PCR (N)}} \times 100$$

3.7. Percentage positivity on the basis of age group

$$\% \text{ Positivity} = \frac{\text{No. of positive cases by nested PCR in different age groups (n)}}{\text{Total no. of Ehrlichia positive cases given by nested PCR (N)}} \times 100$$

3.8. Percentage positivity on the basis of breed

$$\% \text{ Positivity} = \frac{\text{No. of positive cases given by nested PCR in different breed (n)}}{\text{Total no. of Ehrlichia positive cases given by nested PCR (N)}} \times 100$$

3.9. Percentage positivity on the basis of season

$$\% \text{ Positivity} = \frac{\text{No. of positive cases given by nested PCR in different season (n)}}{\text{Total no. of Ehrlichia positive cases given by nested PCR (N)}} \times 100$$

3.10. Comparison of Diagnostic efficacy of molecular diagnostic technique with traditional techniques for *E.canis*

Evaluation of PCR based molecular diagnostic technique with conventional diagnostic method (Leishmann stained thin blood smear) for *E.canis* were done in this study, 62 samples of dogs were taken and calculated as.

$$\% \text{ Positivity} = \frac{\text{No. of positive cases given by a particular diagnostic test (n)}}{\text{Total no. of suspected cases (N)}} \times 100$$

3.11. Therapeutic study

Forty-two CME confirmed dogs were taken for therapeutic studies excluding others apparently healthy dogs. The dogs were divided in following three groups having six dogs in the control (healthy) group (G0) and fourteen (14) dogs in each treatment groups i.e. Group 1, Group 2 and Group 3 which shown in Table 2

Conventional treatment used in the study

- Tab. Doxycycline @ 5mg/kg bwt. twice a day for 21-28 days.
- Tab. Pantoprazole @ 1 mg/kg bwt. once a day (if required).
- Tab. Carprofen @ 2.2 mg/kg bwt. twice a day in case of fever (if required).
- Syp. Sucralfate @ 1 gm, thrice a day (if required).
- Other need based supportive treatment and fluid therapy (if required).

Table 2: Grouping of animals for Therapeutic study

Group 0 (Control/Healthy)	Comprises of six healthy dogs of any age, breed and sex. (Their healthy status was confirmed by thorough clinical examination and application of same laboratory test, which were utilized for both the treatment groups).
Group 1	Comprises of 14 dogs suffering from ehrlichiosis and treated with conventional treatment
Group 2	Comprises of 14 dogs suffering from ehrlichiosis and treated with conventional treatment plus Tab Wysolone (prednisolone) @ 2mg/kg with tapering doses

Group 3	Comprises of 14 dogs suffering from ehrlichiosis and treated with conventional treatment along with self-made polyherbal platelet booster formulated capsule containing: Content of capsule 500 mg of capsule containing: Extract of *Carica papaya*-200mg Extract of *Tinospora cordifolia* -200mg Extract of *Withania somnifera*-50mg Extract of *Asparagus racemosus*-50mg Dose rate of capsule: Up to 15 kg Body weight : 1 capsule (500mg) For 15-30 kg Body weight : 2 capsule (1000mg) Above 30 kg body weight : 3 capsule (1500mg)

3.11 Polyherbal Platelet booster formulation preparation

3.11.1. Hot Water Aqueous extracts of *Carica papaya*

- Hot Water Aqueous extracts of *Carica papaya* were prepared using Soxhlet apparatus by hot percolation method.
- The dried leaves were grinded in an electrical grinder to a coarse powder and sieved.
- About 35-45 g of coarsely ground powder of *Carica papaya* was placed in a porous cellulose thimble in Soxhlet apparatus (capacity 500 ml).
- Thimble was placed in the extraction chamber above the flask containing 500 ml of distilled water.
- The flask was heated and the solvent was allowed to evaporate. The temperature (70^0C) was adjusted according to the boiling temperature of solvent in the flask.
- Extraction process was allowed to continue for 48-72 hours involving 20-22 cycles till the solvent in siphon tube of extractor became completely colourless.
- The extract obtained was concentrated in incubator at 37^0C to obtain semisolid mass and per cent yield was calculated.

- The harvested extract was transferred into air tight containers and stored in refrigerator (4^0C) till used.

3.11.2Methanolic extract of *Tinospora cordifolia*

- 100% Methanolic extracts of *Tinospora cordifolia* were prepared using Soxhlet apparatus by hot percolation method.
- The dried stem was grinded in an electrical grinder to a coarse powder and sieved.
- About 35-45 g of coarsely ground powder of *Tinospora cordifolia* was placed in a porous cellulose thimble in Soxhlet apparatus (capacity 500 ml).
- Thimble was placed in the extraction chamber above the flask containing 500 ml of 100% Methanol for extracts.
- The flask was heated and the solvent was allowed to evaporate. The temperature (40^0C) was adjusted according to the boiling temperature of solvent in the flask.
- Extraction process was allowed to continue for 48 hours involving 20-22 cycles till the solvent in siphon tube of extractor became completely colourless.
- Thereafter, the extract obtained was concentrated in incubator at 37^0C to obtain semisolid mass and per cent yield was calculated.
- The harvested extract was transferred into air tight containers and stored in refrigerator (4^0C) till used.

3.11.3. Methanolic extract of *Withania somnifera*:

- 80% Methanolic extracts (Roots) of *Withania somnifera* were prepared using Soxhlet apparatus by hot percolation method.
- The dried root was grinded in an electrical grinder to a coarse powder and sieved.
- About 35-45 g of coarsely ground powder of *Withania somnifera* was placed in a porous cellulose thimble in Soxhlet apparatus (capacity 500 ml).
- Thimble was placed in the extraction chamber above the flask containing 500 ml of 80% Methanol for extracts.

- The flask was heated and the solvent was allowed to evaporate. The temperature (40^0C) was adjusted according to the boiling temperature of solvent in the flask.
- Extraction process was allowed to continue for 48 hours involving 20-22 cycles till the solvent in siphon tube of extractor became completely colourless.
- Thereafter, the extract obtained was concentrated in incubator at 37^0C to obtain semisolid mass and per cent yield was calculated.
- The harvested extract was transferred into air tight containers and stored in refrigerator (4^0C) till used.

3.11.4. Methanolic extract of *Asparagus racemosus*

- 100% Methanolic extracts of *Asparagus racemosus* were prepared using Soxhlet apparatus by hot percolation method.
- The dried stem was grinded in an electrical grinder to a coarse powder and sieved.
- About 35-45 g of coarsely ground powder of *Asparagus racemosus* was placed in a porous cellulose thimble in Soxhlet apparatus (capacity 500 ml).
- Thimble was placed in the extraction chamber above the flask containing 500 ml of 100% Methanol for extracts.
- The flask was heated and the solvent was allowed to evaporate. The temperature (40^0C) was adjusted according to the boiling temperature of solvent in the flask.
- Extraction process was allowed to continue for 48 hours involving 20-22 cycles till the solvent in siphon tube of extractor became completely colourless.
- Thereafter, the extract obtained was concentrated in incubator at 37^0C to obtain semisolid mass and per cent yield was calculated.
- The harvested extract was transferred into air tight containers and stored in refrigerator (4^0C) till used.

3.12. Assay of Canine Platelet Migration Inhibition Factor

3.12.1. Reagent preparation

All reagents should be brought to room temperature before use.

1) Standard preparation:

Standard reconstitute the 120µl of the standard (960ng/L) with 120µl of standard diluent to generate a 480ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (480ng/L) 1:2 with standard diluent to produce 240ng/L, 120ng/L, 60ng/L and 30ng/L solutions. Standard diluent serves as the zero standard (0 ng/L). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

Table 3: Standard preparation for Canine Platelet Migration Inhibition Factor

480ng/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
240ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
120ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
60ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
30ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent

2) Wash buffer: Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

3.12.2. Assay procedure

1) Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.

2) Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.

3) Add 50µl standard to standard well. Note: Don’t add antibody to standard well because the standard solution contains biotinylated antibody.

4) Add 40µl sample to sample wells and then add 10µl anti-P-MIF antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.

5) Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.

6) Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.

7) Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.

8) Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

3.12.3. Calculation of Result

1. Average the duplicate readings for each standard and samples. It is recommended that all standards and samples should run at least in duplicates. A standard curve must be run with each assay every time.

2. Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis.

3.13. Coagulation Profile (PT and APTT) and platelet index

3.13.1 Platelet index

The blood samples were collected from selected dogs on the basis of clinical signs and history of owner of different groups on day 0 (Before), day 7^{th} (After) and day 14^{th} (After) of institution of the medicinal therapy. The blood samples were aseptically collected from cephalic vein or saphenous vein using 24-gauge needle for each dog separately in blood collection vials containing Ethylene Diamine Tetra

acetate (EDTA) as an anticoagulant @1mg/ml blood for platelet index estimation viz. PLT (10^5/µl), MPV (fL), PDW (%) and PCT (%).

3.13.2 Coagulation Profile (PT and APTT)

The blood samples were collected from selected dogs on the basis of clinical signs and history of owner of different groups on day 0 (Before), day 7th (After) and day 14th (After) of institution of the medicinal therapy. The blood samples were aseptically collected from cephalic vein or saphenous vein using 24 gauge needle for each dog separately in blood collection vials containing 3.8% Trisodium citrate as an anticoagulant. PT (second) and APTT (second) estimation was done with the help of microcoagulometer.

Table 4: Interpretation of PT and APTT

Abnormality	Interpretation
Isolated Prolonged APTT	Deficiencies of XII, XI, IX and VIII. However, the APTT can be normal with mild deficiencies of these clotting factors. In general, the deficient factor has to be less than 20-40% of normal before the APTT prolonged. Contact factor deficiency e.g., pre kallikrein deficiency (In multiple clotting factor deficiencies the APTT become prolonged with less severe reduction in factor levels). Acquired clotting factor inhibitors are most commonly directed against F VIII and may occur either as autoantibodies (e.g., acquired HaemophiliaA) or alloantibodies (In individuals with severe Haemophilia A following exposure to exogenous Factor VIII e.g., the use of other factor VIII are rare but do occur e.g. Factor V). Lupus anticoagulant (LA) a LA target phospholipid and so can result in an isolated prolongation of the APTT. Although the PT requires phospholipid the concentration of PL in the PT reagent is high and so it frequently neutralises the LA and so the PT is not prolonged.

Prolonged APTT+ Prolonged PT	Vitamin K deficiency, liver disease due to malabsorption of vitamin K and which leads to decreased gamma carboxylation of the vitamin K dependent clotting factors, Decreased the synthesis of clotting factor, DIC due to the consumption of clotting factor. Massive blood transfusion leading to dilution coagulopathy. In patient receiving thrombolytic therapy, the APTT may be prolonged due to reduction in fibrinogen levels. In multiple clotting factor deficiencies, the APTT becomes prolonged with less severe reduction in factor levels. Combined deficiency of clotting factors e. g. F V and F VIII. Common pathway clotting factor deficiency - F V, Prothrombin and Fibrinogen.
Increased APTT ± Prolonged PT	Unfractionated heparin (UFH): UFH significantly prolongs the APTT but the PT usually shows little prolongation. In cases significant over anticoagulation with UFH the PT will be prolonged. Antiphospholipid antibodies (In some cases the PT can be prolonged although this is unusual due to the high PL concentration in PT reagents) Acquired clotting factor inhibitors e.g., F V, F X.
Prolonged APTT± Prolonged PT	Warfarin – the APTT may be only prolonged by few second in patients who are stably anticoagulated on warfarin – but in patients who are over dosed the APTT may be significantly prolonged.
Short APTT	An acute phase response leading to high F VIII levels. Difficulties in the collection of samples leading to activation of coagulation within the collection tube.

3.15. Hematology

The blood samples were collected from selected dogs on the basis of clinical signs and history of owner of different groups on day 0 (Before), day 7^{th} (after) and day 14^{th} (after) of institution of the medicinal therapy. Five ml blood samples were aseptically collected from cephalic vein or saphenous vein in vials containing Ethylene diamine tetra acetate (EDTA) as an anticoagulant.

Hematology of all blood samples were executed by using fully automated hematology analyzer (Celltac Alpha VET MEK-6550). Total erythrocyte counts, hemoglobin, hematocrit (HCT), MCV, MCH, MCHC, platelets, total leukocyte count, granulocytes (%), monocytes (%), lymphocytes (%) and eosinophils (%) and platelet indices PDW (%), MPV(fL), PCT (%) were evaluated.

3.16. Serum biochemistry

The blood for serum required for biochemical parameters were collected in a 5 ml capacity test tubes with no anticoagulant and were allowed to stand undisturbed in a slant position for about 1 hour. The clots were retracted and the serum separated after rapid centrifugation (3000 rpm for 10 minutes). Extreme care was taken to prevent hemolysis of the serum sample. The obtained serum was stored in a deep freeze at $-80^{o}C$ in serum collection tubes which were properly capped and labeled with full details till analysis. Batch analysis was done to avoid repeated thaw and freeze cycles, which might lead to changes in biochemical values of the serum samples.

Various biochemical parameter like serum total proteins (g/dl), albumin (g/dl), creatinine (mg/dl), BUN (mg/dl), alkaline phosphatase (IU/L), ALT (IU/L), AST (IU/L) were done with the help of Semi-automated biochemical analyzer (ARKRAY Healthcare Pvt. Ltd.) using Autospan diagnostic kits (ARKRAY Healthcare Pvt. Ltd).

Serum globulin was estimated by subtracting the values of serum albumin from the estimated value of serum total proteins (Total Protein-Albumin).

3.17. Statistical Analysis

The values of various parameters were expressed as mean ± S.E. and Data were analyzed by one-way (ANOVA) Analysis of Variance followed by the Post-Hoc Tukey HSD test using Statistical Package for the Social Sciences (SPSS 20.0). The level of statistical significance for all comparisons was established at ($P<0.05$).

Fig. 1: Automated hematology analyzer

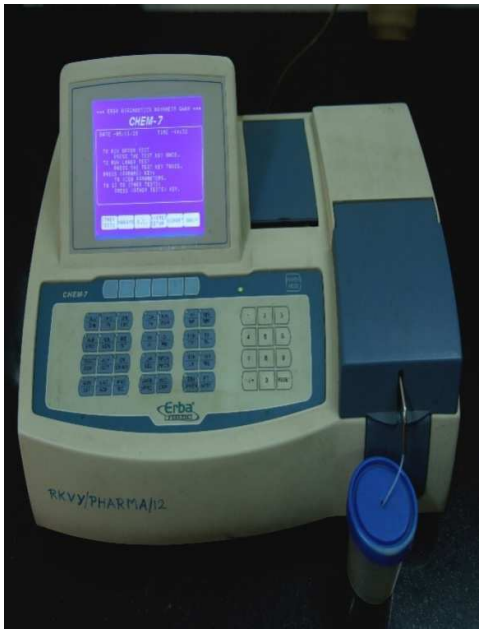


Fig. 2: Biochemical Analyzer

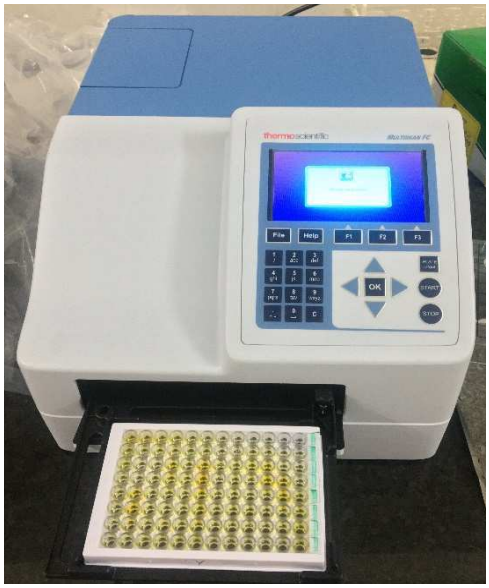


Fig. 3: ELISA reader with ELISA Plate

Fig. 4: iBright 750 Imaging system

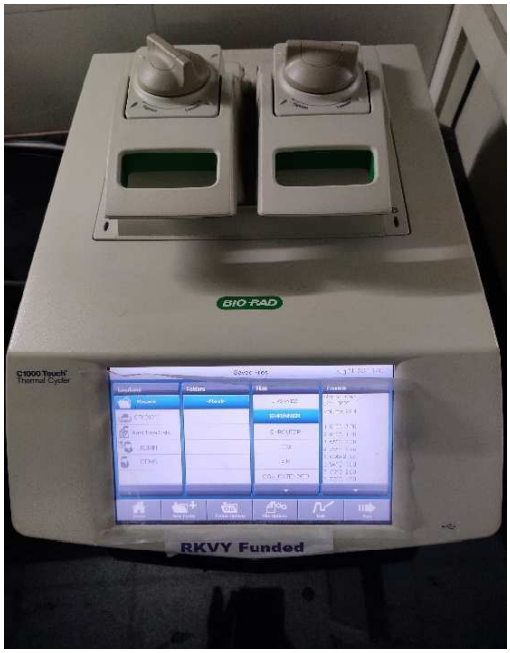


Fig. 5: Thermocycler for DNA amplification

Fig. 6: Soxhlet Extractor for plant extraction

Fig. 7: Lab incubator

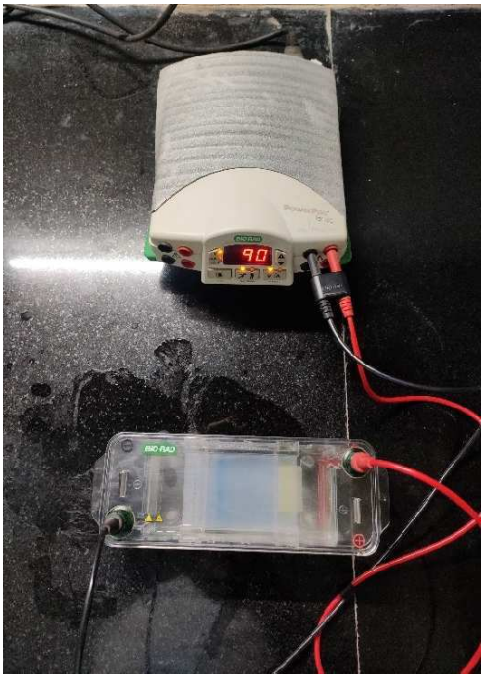


Fig. 8: Gel electrophoresis apparatus

Results

CHAPTER-4

RESULTS

Present investigation entitled "Ameliorative Potential of Polyherbal Formulation on Thrombocytopenia in Dogs with Ehrlichiosis'' has recorded ehrlichiosis associated clinical, haematological, biochemical alterations, coagulation profile (prothrombin time and activated partial thromboplastin time), platelet indices and Canine specific platelet migration inhibition factor (C-PMIF) in dogs with clinical disease.

In present investigation total 62 dogs were screened for ehrlichiosis which was based on the clinical diagnosis criteria as stated earlier in materials and methods section of the thesis. All the screened dogs underwent for blood smear and buffy coat smear examination and haematology to confirm the state of clinical ehrlichiosis. Blood smear examination results showed 3 dogs positive for ehrlichiosis, while buffy coat smear examination results showed 6 dogs positive for ehrlichiosis. In the same cases, primary PCR revealed 18 positive dogs, however nested PCR confirmed 42 dogs positives for ehrlichiosis of all screened dogs. All the 42 nested PCR positive dogs were included for the therapeutic studies. Following parameters were investigated during study:

4.1. Clinician- intensive parameters

4.2. Diagnostic relevance of molecular diagnostic technique with traditional methods

4.3. Percentage positivity according to age, breed, sex and season

4.4. Haematological study

4.5. Biochemical study

4.6. Platelet indices

4.7. Coagulation profile

4.8. Canine specific platelet migration inhibition factor

4.1 Clinician intensive parameters

The clinical study comprised of dogs presented to TVCC, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura, which were found positive for ehrlichiosis during the study period.

4.1.1 Clinical signs & symptoms and their frequency distribution in dogs

Observed clinical symptoms in ehrlichiosis affected dogs and their frequency distribution were presented in Table -5. Important clinical symptoms exhibited by the Ehrlichia positive dogs in order of decreasing frequency were fever (92.85%) and mucosal pallor (88.02%) followed by lymph adenomegaly (88.02%), melena (83.33%), depression (78.57%), tick infestation (42.85%) and weight loss (42.85%). Clinical symptoms with moderate frequency were epistaxis (21.42%), ascites (16.66%), ecchymotic and petechial haemorrhages (14.58%), ocular abnormalities (11.90%), vomiting (9.52%), hind limb/scrotal oedema/facial oedema (7.14 %), icterus (4.76%). Symptoms with least frequency were haematuria (2.38%), hematemesis (2.38%) and CNS signs (2.38%).

Table 5: Clinical signs and their frequency distribution in dogs

S. No.	Parameters	No. of positive cases (n=42)	Frequency (%)
1	Fever	39	92.85
2	Mucosal pallor	37	88.02
3	Lymph adenomegaly	37	88.02
4	Melena	35	83.33
5	Depression	33	78.57
6	Weight loss	18	42.85
7	Tick infestation	18	42.85
8	Epistaxis	9	21.42
9	Ascites	7	16.66
10	Ecchymotic and Petechial hemorrhages	6	14.58
11	Ocular abnormalities	5	11.90
12	Vomiting	4	9.52
13	Hind limb/scrotal edema / facial edema	3	7.14

14	Icterus	2	4.76
15	Hematemesis	1	2.38
16	Hematuria	1	2.38
17	CNS signs	1	2.38

4.1.2. Physiological parameters

Different vital physiological parameters were recorded by using standard method of examination and data is tabulated in the Table -6. Average value (Mean±SE) of rectal temperature (°F), heart rate (per min), respiration rate (per min) and CRT (seconds) in healthy dogs was 101.85±0.54 (°F), 82.97±0.24 (per min), 19.13±0.14 (per min) and 1.73±0.23 sec, respectively. Average value (Mean±SE) of rectal temperature (°F), heart rate (per min), respiration rate (per min) and CRT (seconds) in ehrlichial dogs at day 0 was 104.57±0.44 (°F); 99.74±0.92 (per min), 33.98±1.74 (per min) and 2.303±0.32 sec, respectively.

Table 6: Physiological parameters:

Sr. No.	Parameter	Control (n=6)	Dogs with ehrlichiosis (n=42) on day 0	Dogs with ehrlichiosis (n=42) on day 7	Dogs with ehrlichiosis (n=42) on day 14
1	Rectal temperature (°F)	101.85 ± 0.54	104.7 ±0.44	103.98 ±0.84	102.54 ± 0.60
2	Heart rate/(min)	82.97 ± 0.24	99.74 ±0.92	95.88 ± 0.27	86.26 ± 0.55
3	Respiration rate/(min)	19.13 ± 0.14	33.98 ±1.74	29.54 ± 0.95	24.21 ± 0.54
4	CRT (seconds)	1.73 ± 0.23	2.30 ±0.32	2.12 ±0.17	2.05 ±0.26

4.2. Diagnostic relevance of traditional technique with molecular diagnostic technique

4.2.1. Diagnostic relevance of PCR (simple and nested) with blood smear in *E. canis*.

Diagnostic relevance of traditional technique was compared with molecular diagnostic technique and results are shown in Table 7.

Nested PCR was considered as standard test to compare other diagnostics. Diagnostic relevance of various test was compared on 62 samples suspected for ehrlichiosis based on clinical examination. Leishman-stained thin blood smear examination of all 62 samples revealed the presence of *E.canis* in 3 samples, showing a percentage positivity of 4.8 %. Leishman-stained buffy coat smear examination of all 62 samples revealed the presence of *E. canis* in 6 sample showing a percentage positivity of 14.28 %. Simple PCR of 62 blood samples shows positivity in 18 samples, for which the 42.85% positivity was calculated. All the 62 samples were finally underwent for confirmation of disease by nested PCR, which yield 42 samples positive for the ehrlichiosis with a percentage positivity of 66.74 %.

Table 7: Diagnostic relevance of molecular diagnostic technique with traditional technique

Methods	Clinically Suspected dogs of *E.canis*	Positive Cases	Negative Cases	Positivity (%)
Blood smear	62	3	59	4.8
Buffycoat smear	62	6	56	14.28
Simple PCR	62	18	44	42.85
Nested PCR	62	42	20	67.74

4.3. Percentage positivity according to age, breed, sex and season (Risk Factor Analysis)

Percentage positivity according to age, breed, sex and season are shown in Table 8.

4.3.1 Percent positivity according to age:

Percentage positivity of ehrlichiosis was recorded on the basis of age. Dogs were divided into 3 categories, age group with highest positivity was category 3 (> 1 year) with a positivity of (61.90 %) followed by category 2 (> 6 months-1year) with a positivity of (23.80 %) and lowest positivity was recorded in category 1 (0-6 months) with a positivity of (14.28 %).

4.3.2 Percent positivity according to breed:

In the present study percentage positivity of ehrlichiosis was recorded on the basis of Breed. For that dog were categorized into 8 breeds. Breed with highest positivity was German shepherd with a positivity of 38.09 % followed by Rottweiler (14.28%), Labrador (14.28 %), Non-descript (11.90 %), Pomeranian (9.52 %), Bull mastiff (4.76 %), Spitz (4.76%) and Pug (2.38 %). Most of the time, the breed wise positivity depends on popularity of breed in the region therefore it could neither be considered as breed based prevalence nor the breed predisposition for the disease.

4.3.3 Percent positivity according to sex:

In the present study percent positivity in dogs was recorded on the basis of sex. In present study none of the dog was either spayed/ neutered, therefore, dogs were categorized in 2 categories male and female and positivity of male and female was (54.76%) and (45.23 %), respectively.

4.3.4 Percent positivity according to season:

In the present study percent positivity in dogs was recorded on the basis of season. For that dog were categorized in 2 categories summer and winter, for which the positivity was recorded (78.57 %) and (21.42 %), respectively. Season seems to play an important role in transmission of disease as proliferation of ticks occurs mostly in summer/rainy months.

Table 8: Percentage positivity of CME according to age, breed, sex and season.

Risk factors	Parameters	Positive cases given by (Nested PCR) n=42	Percentage (%) Positivity
Age	0-6 month	3	14.28
	6 month-1year	8	23.80
	More than 1 year	19	61.90
Breed	German Shepherd	16	38.09
	Rottweiler	6	14.28
	Labrador	6	14.28
	Non-Descript	5	11.90
	Pomeranian	4	9.52
	Bull mastiff	2	4.76
	Sptiz	2	4.76
	Pug	1	2.38
Sex	Male	23	54.76
	Female	19	45.23
Season	Summer	33	78.57
	Winter	9	21.42

4.4. Haematology

Haematological parameters of control group (Group 0) and treatment groups (group 1, group 2, group 3) on the day of presentation i.e. pre-treatment (day 0) and post treatment (day 14) have been summarized in the Tables 9, 10, 11, 12, 13 and 14.

4.5.1. Total leucocyte count ($\times 10^3/\mu L$)

The Mean ± SE values of TLC ($\times 10^3/\mu L$) of healthy dogs (G0) at day 0, day 7 and 14 were 9.61±1.07, 9.81±1.07 and 10.25±1.17, respectively. The Mean ± SE values of TLC ($\times 10^3/\mu L$) at day 0, day 7 and day 14 of Group 1 (G1) were 5.12±0.32, 6.92±0.45 and 7.56±0.29, respectively. The Mean ± SE values of TLC ($\times 10^3/\mu L$) at day 0, day 7 and day 14 of Group 2 (G2) were 5.45±0.35, 7.37±0.62 and 8.69±0.41, respectively. The Mean ± SE values of TLC ($\times 10^3/\mu L$) at day 0, day7 and day 14 in dogs of Group 3 (G3) were 5.80±0.38, 8.33±0.61 and 9.83±0.36, respectively. Test of significance show that TLC ($\times 10^3/\mu L$) in all treatment groups

were significantly lower than that of control group on the day of presentation. There was significant increase in the mean TLC ($\times 10^3/\mu L$) on day 7 followed by day14 post-treatment in all treatment groups.

4.4.2. Total erythrocyte count ($\times 10^6/\mu l$)

The Mean±SE values of TEC ($\times 10^6/\mu l$) of healthy dogs (G0) at day 0, day 7 and day 14 were 6.59±0.30, 6.83±0.29 and 7.10±0.16, respectively. The Mean± SE values of TEC ($\times 10^6/\mu l$) at day 0, day 7 and day 14 of Group 1 (G1) were 2.58±0.12, 3.47±0.13and 4.38 ±0.08, respectively. The Mean ± SE values of TEC ($\times 10^6/\mu l$) at day 0, day 7 and day 14 of Group 2 (G2) were 3.16±0.18, 4.90±0.20 and 5.38±0.45, respectively. The Mean ± SE values of TEC ($\times 10^6/\mu l$) at day 0, day7 and day 14 in dogs of Group 3 (G3) were 3.77±0.32, 5.99±0.24 and 6.31±0.28, respectively. Test of significance showed that TEC ($\times 10^6/\mu l$) in all treatment groups were significantly lower than that of control group on the day of presentation. There was significant increase in the mean TEC ($\times 10^6/\mu l$) on day 7 followed by on day14 post-treatment in all treatment groups.

4.4.3. Haemoglobin (Hb) (gm/dl)

The Mean ± SE values of Hb (gm/dl) of healthy dogs (G0) at day 0, day 7 and day 14 were 15.06±0.67, 15.32±0.68 and 15.84±0.63, respectively. The Mean ± SE values of Hb (gm/dl) at day 0, day 7 and day 14 of Group 1 (G1) were 5.57±0.54, 8.06±0.42 and 10.28± 0.21, respectively. The Mean ± SE values of Hb (gm/dl) at day 0, day 7 and day 14 of Group 2 (G2) were 6.51±0.41, 9.60±0.38 and 11.12±0.22, respectively. The Mean ± SE values of Hb (gm/dl) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 7.14±0.53, 10.46±0.20 and 13.15±0.48, respectively. Stastical analysis revealed that the haemoglobin concentration (gm/dl) in the treatment groups were significantly lower than that of control group on the day of presentation (day 0). There was significant increase in the mean haemoglobin concentration (gm/dl) on day 7 followed by on day14 post-treatment in all treatment groups. Treatment groups with highest recovery in 3rd group followed by 2nd group.

4.4.4. Packed cell volume (PCV) (%)

The Mean ± SE values of (PCV) (%) of healthy dogs (G0) at day 0, day 7 and day 14 were 42.76±2.64, 43.11±2.65 and 43.46±2.66, respectively. The Mean ± SE values of (PCV) (%) at day 0, day 7 and day 14 of Group 1 (G1) were 22.97±0.60,

28.71±0.43 and 33.17±0.82, respectively. The Mean ± SE values of (PCV) (%) at day 0, day 7 and day 14 of Group 2 (G2) were 23.96±0.51, 31.46±0.88 and 34.84± 0.42, respectively. The Mean ± SE values of (PCV) (%) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 24.67± 0.36, 32.32±0.35 and 38.80±0.49, respectively. Test of significance showed that the PCV values in the treatment groups were significantly lower than that of control group on the day of presentation. There was significant increase in the mean (PCV) (%) on day 7 followed by on day 14 post-treatment in all treatment groups.

4.4.5. MCV (fL)

The Mean ± SE values of MCV (fL) of healthy dogs (G0) at day 0, day 7 and day 14 were 67.65±1.67, 68.18±1.64 and 68.70±1.57, respectively. The Mean ± SE values of MCV (fL) at day 0, day 7 and day 14 of Group 1 (G1) were 67.60 ±0.35, 67.54±0.25 and 67.88±0.29, respectively. The Mean ± SE values of MCV (fL) at day 0, day 7 and day 14 of Group 2 (G2) were 67.52±0.52, 67.96±0.37 and 68.23±0.33, respectively. The Mean ± SE values of MCV (fL) at day 0, day7 and day 14 in dogs of Group 3 (G3) were 67.84±0.70, 68.09±0.61 and 68.11±0.91, respectively. Test of significance showed that the MCV (fL) of treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in the MCV (fL) on day 7 followed by on day 14 post-treatment in all treatment group.

4.4.6. MCH (pg)

The Mean ± SE values of MCH (pg) of healthy dogs (G0) at day 0, day 7 and day 14 were 33.80±0.59, 34.01±0.61 and 34.05±0.58, respectively. The Mean ± SE values of MCH (pg) at day 0, day 7 and day 14 of Group 1 (G1) were 33.78 ±0.29, 34.28±0.48 and 34.48±0.96, respectively. The Mean ± SE values of MCH (pg) at day 0, day 7 and day 14 of Group 2 (G2) were 34.05±0.68, 34.57±0.81 and 35.08±0.98, respectively. The Mean ± SE values of MCH (pg) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 34.58±0.71, 34.83±0.29 and 34.95±1.10, respectively. The test of significance showed that MCH (pg) of treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in the MCH (pg) on day 7 followed by on day 14 post-treatment in all treatment groups.

4.4.7. MCHC (gm/dl)

The Mean ± SE values of MCHC (gm/dl) of healthy dogs (G0) at day 0, day 7 and day 14 were 34.30±0.59, 34.02±0.61and 34.05±0.58, respectively. The Mean ± SE values of MCHC (gm/dl) at day 0, day 7 and day 14 of Group 1 (G1) were 33.64±0.59, 34.08±0.99 and 33.96±0.88, respectively. The Mean ± SE values of MCHC (gm/dl) at day 0, day 7 and day 14 of Group 2 (G2) were 34.39±1.04, 34.12±0.88 and 34.63±1.18, respectively. The Mean ± SE values of MCHC (gm/dl) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 34.46±0.92, 34.81±1.36 and 34.95±1.56, respectively. The test of significance showed that MCHC (gm/dl) of all the treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in the MCHC (gm/dl) on day 7 followed by on day14 post-treatment in all treatment groups.

4.4.8. Neutrophil (%)

The Mean ± SE values of neutrophil (%) of healthy dogs (G0) at day 0, day 7 and day 14 were 78.83±1.47, 79.51±1.52 and 81.58±1.18, respectively. The Mean ± SE values of neutrophil (%) at day 0, day 7 and day 14 of Group 1 (G1) were 82.64± 0.40, 81.05±0.48 and 80.30±0.86, respectively. The Mean ± SE values of neutrophil (%) at day 0, day 7 and day 14 of Group 2 (G2) were 83.36±0.52, 80.90±1.02 and 78.92±0.96, respectively. The Mean ± SE values of neutrophil (%) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 86.17±0.43, 79.71±1.71 and 76.76±0.57, respectively. Test of significance showed that neutrophils (%) of all treatment group were significantly higher than that of control group on the day of presentation. There was significant decrease in the mean neutrophils (%) on day 7 followed by on day 14 post-treatment in all treatment groups.

4.4.9. Lymphocyte (%)

The Mean ± SE values of lymphocyte (%) of healthy dogs (G0) at day 0, day 7 and day 14 were 14.71±1.87, 15.10±1.89 and 15.45±1.88, respectively. The Mean ± SE values of lymphocyte (%) at day 0, day 7 and day 14 of Group 1 (G1) were 5.58±0.30, 8.21±0.41 and 10.94±0.37, respectively. The Mean ± SE values of lymphocyte (%) at day 0, day 7 and day 14 of Group 2 (G2) were 6.03±0.20, 9.84±0.18 and 12.41±0.54, respectively. The Mean ± SE values of lymphocyte (%) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 6.79±0.35, 11.91±0.56 and

14.24±0.97, respectively. Stastical analysis revealed that lymphocytes % in all the treatment groups were significantly lower than that of control group on the day of presentation. There was significant increase in the mean lymphocytes % on day 7 and day 14 post-treatment in all treatment groups.

4.4.10. Monocyte (%)

The Mean ± SE values of monocyte (%) of healthy dogs (G0) at day 0, day 7 and day 14 were 4.70±0.55, 4.89±0.55 and 4.97±0.54, respectively. The Mean ± SE values of monocyte (%) at day 0, day 7 and day 14 of Group 1 (G1) were 4.56±0.23, 4.94±0.33 and 5.03±0.37, respectively. The Mean ± SE values of monocyte (%) at day 0, day 7 and day 14 of Group 2 (G2) were 4.67±0.26, 4.65±0.17 and 4.74±0.17, respectively. The Mean ± SE values of monocyte (%) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 4.82±0.45, 4.60±0.43, 4.78±0.43, respectively. Test of significance showed that monocyte (%) in all treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in the monocytes (%) on day 7 followed by on day14 post-treatment in all treatment group.

4.4.11. Eosinophil (%)

The Mean ± SE values of eosinophil (%) of healthy dogs (G0) at day 0, day 7 and day 14 were 2.75±0.35, 2.70±0.33 and 2.63±0.48, respectively. The Mean ± SE values of eosinophil (%) at day 0, day 7 and day 14 of Group 1 (G1) were 2.74±0.03, 2.75±0.19 and 2.67±0.20, respectively. The Mean ± SE values of eosinophil (%) at day 0, day 7 and day 14 of Group 2 (G2) were 2.77±0.16, 2.74±0.20 and 2.43±0.19, respectively. The Mean ± SE values of eosinophil (%) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 2.76±0.40, 2.58±0.27 and 2.59±0.42, respectively. Test of significance showed that eosinophil (%) of treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in the eosinophil (%) on day 7 followed by on day 14 post-treatment in the treatment groups.

4.5. Serum Biochemistry

Serum biochemical parameters of control group (Group 0) and treatment groups (group 1, group 2, and group 3) on the day of presentation i.e., pre-treatment

(day 0) and post treatment (day 14) have been summarized in the Tables 18, 19, 20 and 21.

4.5.1. Alanine transaminase (ALT) (IU/L)

The Mean ± SE values of ALT (IU/L) of healthy dogs (G0) at day 0, day 7 and day 14 were 32.44±2.65, 33.55±2.70 and 34.06±2.69, respectively. The Mean ± SE values of ALT (IU/L) at day 0, day 7 and day 14 of Group 1 (G1) were 79.95±0.48, 54.97±0.46 and 47.55±1.72, respectively. The Mean ± SE values of ALT (IU/L) at day 0, day 7 and day 14 of Group 2 (G2) were 82.58±0.36, 49.49±1.65 and 40.13±0.73, respectively. The Mean ± SE values of ALT (IU/L) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 83.79±0.22, 38.98±1.53 and 30.74±1.17, respectively. Test of significance showed that ALT (IU/L) of all treatment group was significantly higher than that of control group on the day of presentation. There was significant decrease in the mean ALT (IU/L) on day 7 and on day 14 on post-treatment in all treatment groups.

4.5.2. Aspartate transaminase (AST) (IU/L)

The Mean ± SE values of AST (IU/L) of healthy dogs (G0) at day 0, day 7 and 14 were 18.91±1.29, 19.86±1.27 and 20.36±1.35, respectively. The Mean ± SE values of AST (IU/L) at day 0, day 7 and day 14 of Group 1 (G1) were 78.01±1.91, 65.50±1.54 and 49.01±2.42, respectively. The Mean ± SE values of AST (IU/L) at day 0, day 7 and day 14 of Group 2 (G2) were 80.01±1.44, 60.83±3.13 and 42.33±2.06, respectively. The Mean ± SE values of AST (IU/L) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 77.50±0.43, 52.16±3.36 and 30.67±1.20, respectively. Test of significance showed that AST (IU/L) of treatment groups were significantly higher than that of control group on the day of presentation. There was significant decrease in the mean AST (IU/L) on day 7 and on day 14 post-treatment in all treatment groups.

4.5.3. Alkaline phosphatase (IU/L)

The Mean ± SE values of alkaline phosphatase (ALP) (IU/L) of healthy dogs (G0) at day 0, day 7 and 14 were 34.45±0.93, 36.43±0.96 and 37.15±0.97, respectively. The Mean ± SE values of alkaline phosphatase (ALP) (IU/L) at day 0, day 7 and day 14 of Group 1 (G1) were 88.00±4.53, 68.01±4.53 and 48.16±2.02, respectively. The Mean ± SE values of alkaline phosphatase (ALP) (IU/L) at day 0,

day 7 and day 14 of Group 2 (G2) were 89.33±2.87, 59.33±2.87 and 44.67±2.65, respectively. The Mean ± SE values of alkaline phosphatase (ALP) (IU/L) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 92.16±3.54, 53.66±4.56 and 39.83±4.31, respectively. Test of significance showed that ALP (IU/L) of treatment groups were significantly higher than that of control group on the day of presentation. There was significant decrease in the mean ALP (IU/L) on day 7 followed on day 14 post-treatment in all treatment groups.

4.5.4. Total protein (gm/dl)

The Mean ± SE values of total protein (gm/dl) of healthy dogs (G0) at day 0, day 7 and day 14 were 6.58±0.17, 6.59±0.18 and 6.60±0.19, respectively. The Mean ± SE values of total protein (gm/dl) at day 0, day 7 and day 14 of Group 1 (G1) were 4.68±0.21, 5.03±0.49 and 6.02±0.33, respectively. The Mean ± SE values of total protein (gm/dl) at day 0, day 7 and day 14 of Group 2 (G2) were 4.85±0.22, 6.09±0.20 and 6.61±0.20, respectively. The Mean ± SE values of total protein (gm/dl) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 4.07±0.30, 6.48±0.42 and 7.02±0.31, respectively. Test of significance showed that total protein (gm/dl) of treatment groups were significantly lower than that of control group on the day of presentation. There was significant increase in the mean total protein (gm/dl) on day7 followed by on day 14 on post-treatment in all treatment groups.

4.5.5. Albumin (gm/dl)

The Mean ± SE values of albumin (gm/dl) of healthy dogs (G0) at day 0, day 7 and day 14 were 2.82±0.14, 2.92±0.14 and 3.01±0.14, respectively. The Mean ± SE values of albumin (gm/dl) at day 0, day 7 and day 14 of Group 1 (G1) were 1.39±0.10, 1.48±0.10 and 1.58±0.12, respectively. The Mean ± SE values of albumin (gm/dl) at day 0, day 7 and day 14 of Group 2 (G2) were 1.50±0.08, 2.47±0.08 and 2.55±0.07, respectively. The Mean ± SE values of albumin (gm/dl) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 1.63±0.04, 2.55±0.07 and 2.69±0.06, respectively. Test of significance showed that the albumin (gm/dl) of all treatment groups were significantly lower than that of control group on the day of presentation. There was significant increase in the mean albumin (gm/dl) on day 7 followed by on day 14 post-treatment in all treatment groups.

4.5.6. Globulin (gm/dl)

The Mean ± SE values of globulin (gm/dl) of healthy dogs (G0) at day 0, day 7 and 14 were 3.35±0.24, 3.55±0.24 and 3.75±0.24, respectively. The Mean ± SE values of globulin (gm/dl) at day 0, day 7 and day 14 of Group 1 (G1) were 2.61±0.05, 3.17±0.11 and 3.46±0.19, respectively. The Mean ± SE values of globulin (gm/dl) at day 0, day 7 and day 14 of Group 2 (G2) were 3.10±0.35, 3.29±0.27 and 3.61±0.27, respectively. The Mean ± SE values of globulin (gm/dl) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 2.70±0.15, 3.38±0.15 and 3.73±0.10, respectively. Test of significance showed that the globulin (gm/dl) of all treatment groups were significantly lower than that of control group on the day of presentation. However, there was no significant change in the serum globulin (gm/dl) on post-treatment in all the treatment groups.

4.5.7. Blood urea nitrogen (BUN) (mg/dl):

The Mean ± SE values of blood urea nitrogen (BUN) (mg/dl) of healthy dogs (G0) at day 0, day 7 and day 14 were 17.58±0.99, 18.46±1.02 and 19.24±1.00, respectively. The Mean ± SE values of blood urea nitrogen (BUN) (mg/dl) at day 0, day 7 and day 14 of Group 1 (G1) were 42.16±1.13, 35.16±1.14 and 30.04±1.29, respectively. The Mean ± SE values of blood urea nitrogen (BUN) (mg/dl) at day 0, day 7 and day 14 of Group 2 (G2) were 45.00±2.42, 36.17±2.55 and 27.00±1.88, respectively. The Mean ± SE values of blood urea nitrogen (BUN) (mg/dl) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 40.83±1.37, 28.83±1.38 and 22.86±1.56, respectively. Test of significance showed that BUN (mg/dl) of the treatment group were significantly higher than that of control group on the day of presentation. There was significant decrease in the mean BUN (mg/dl) on day 7 followed by on day 14 post-treatment in all the treatment groups.

4.5.8. Serum creatinine (mg/dl)

The Mean ± SE values of serum creatinine (mg/dl) of healthy dogs (G0) at day 0, day 7 and day 14 were 0.68±0.09, 0.66±0.07 and 0.68±0.08, respectively. The Mean ± SE values of serum creatinine (mg/dl) at day 0, day 7 and day 14 of Group 1 (G1) were 0.83±0.10, 0.55±0.07 and 0.47±0.05, respectively. The Mean ± SE values of serum creatinine (mg/dl) at day 0, day 7 and day 14 of Group 2 (G2) were 0.64±0.10, 0.53±0.09 and 0.39±0.10, respectively. The Mean ± SE values of serum

creatinine (mg/dl) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 0.67±0.14, 0.49±0.12 and 0.37±0.10, respectively. The test of significance showed that serum creatinine (mg/dl) of the treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in the serum creatinine (mg/dl) on day 7 followed by on day14 post-treatment in all the treatment groups.

4.6. Platelet indices

Platelet indices parameters of control group (Group 0) and treatment groups (group 1, group 2, group 3) on the day of presentation i.e. pre-treatment (day 0) and post treatment (day 14) have been summarized in the Tables 15 and 16.

4.6.1. Platelets (10^5/µL)

The Mean ± SE values of platelets (10^5/µL) of healthy dogs (G0) at day 0, day 7 and day 14 were 2.93±0.30, 2.97±0.30 and 3.00±0.29, respectively. The Mean ± SE values of platelets (10^5/µL) at day 0, day 7 and day 14 of Group 1 (G1) were 0.48±0.01, 1.00±0.04 and 1.56±0.01, respectively. The Mean ± SE values of platelets (10^5/µL) at day 0, day 7 and day 14 of Group 2 (G2) were 0.54±0.05, 1.30±0.03 and 2.22±0.10, respectively. The Mean ± SE values of platelets (10^5/µL) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 0.67±0.01, 1.99±0.02 and 2.70±0.05, respectively. Stastical analysis revealed that the mean platelets (10^5/µL) concentration in all the treatment groups were significantly lower than that of control group on the day of presentation (day 0). There was significant increase in the mean platelets (10^5/µL) concentration on day 7 followed by on day 14 post-treatment in all the treatment groups with highest recovery in 3rd group followed by 2nd group.

4.6.1. PCT (%)

The Mean ± SE values of PCT % of healthy dogs (G0) at day 0, day 7 and day 14 were 0.29±0.02, 0.30±0.02 and 0.57±0.10, respectively. The Mean ± SE values of PCT % at day 0, day 7 and day 14 of Group 1 (G1) were 0.03±0.00, 0.16±0.05 and 0.26±0.03, respectively. The Mean ± SE values of PCT % at day 0, day 7 and day 14 of Group 2 (G2) were 0.03±0.00, 0.24±0.11 and 0.43±0.05, respectively. The Mean ± SE values of PCT % at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 0.05±0.00, 0.32±0.07 and 0.51±0.08, respectively. Test of significance shows that value of PCT% of treatment group was significantly lower than that of control

group on the day presentation. There was significant increase in the mean PCT% on 7 day then after 14 day post-treatment in all the treatment groups.

4.6.1. PDW (%):

The Mean ± SE values of PDW (%) of healthy dogs (G0) at day 0, day 7 and day 14 were 15.90±0.38, 15.66±0.39 and 15.40±0.41, respectively. The Mean ± SE values of PDW (%) at day 0, day 7 and day 14 of Group 1 (G1) were 14.75±0.77, 15.71±0.64 and 15.48±0.80, respectively. The Mean ± SE values of PDW (%) at day 0, day 7 and day 14 of Group 2 (G2) were 14.95±0.64, 15.40±0.60 and 15.14±0.63, respectively. The Mean ± SE values of PDW (%) at day 0, day 7 and day 14 in dogs of Group 3 (G3) 15.78±0.61, 15.07±0.54 and 15.61±0.67, respectively. The test of significance showed that PDW (%) of all the treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in the PDW (%) on day 7 followed by on day 14 post-treatment in all the treatment groups.

4.6.4. MPV (fL):

The Mean ± SE values of MPV (fL) of healthy dogs (G0) at day 0, day 7 and day 14 were 6.82±0.07, 6.94±0.08 and 6.39±0.21, respectively. The Mean ± SE values of MPV (fL) at day 0, day 7 and day 14 of Group 1 (G1) were 6.83±0.05, 6.61±0.07 and 6.36±0.06, respectively. The Mean ± SE values of MPV (fL) at day 0, day 7 and day 14 of Group 2 (G2) were 6.83±0.28, 6.60±0.21 and 6.07±0.17, respectively. The Mean ± SE values of MPV (fL) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 6.77±0.21, 6.66±0.22 and 6.31±0.14, respectively. The test of significance showed that MPV (fL) of the treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in the MPV (fL) on day 7 and day 14 post-treatment in all the treatment groups

4.7. Coagulation profile

Coagulation profile of control group (Group 0) and treatment groups (group 1, group 2, group 3) on the day of presentation i.e. pre-treatment (day 0) and post treatment (day 14) have been summarized in the Table 17.

4.7.1. Prothrombin time (PT) (Sec)

The Mean ± SE values of prothrombin time (PT) (sec) of healthy dogs (G0) at day 0, day 7 and day 14 were 8.98±0.17, 8.97±0.16 and 8.96±0.16, respectively. The Mean ± SE values of prothrombin time (PT) (sec) at day 0, day 7 and day 14 of Group 1 (G1) were 8.79±0.18, 9.18±0.02 and 8.62±0.27, respectively. The Mean ± SE values of prothrombin time (PT) (sec) at day 0, day 7 and day 14 of Group 2 (G2) were 8.91±0.17, 9.16±0.00 and 8.72±0.19, respectively. The Mean ± SE values of prothrombin time (PT) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 9.15±0.01, 8.92±0.11 and 8.82±0.20, respectively. The test of significance showed that values of the prothrombin time (PT) (sec) of all treatment groups were non-significantly different from the control group on the day of presentation. There was no significant change in prothrombin time (PT) (sec) on day 7 followed by on day 14 post-treatment in all treatment groups.

4.7.2. Activated Partial Thromboplastin Time (aPTT) (Sec)

The Mean ± SE values of aPTT (Sec) of healthy dogs (G0) at day 0, day 7 and day 14 were 12.70±0.07, 12.74±0.06 and 12.86±0.01, respectively. The Mean ± SE values of aPTT (Sec) at day 0, day 7 and day 14 of Group 1 (G1) were 23.79±0.26, 21.23±0.62 and 16.28±0.17, respectively. The Mean ± SE values of aPTT (Sec) at day 0, day 7 and day 14 of Group 2 (G2) were 25.57±0.94, 17.49±0.34 and 14.34±0.19, respectively. The Mean ± SE values of aPTT (Sec) at day 0, day 7 and day 14 in dogs of Group 3 (G3) were 23.98±0.20, 14.05±0.88 and 13.42±0.19, respectively. Stastical analysis showed that values of aPTT (Sec) treatment groups were significantly higher than that of control group on the day of presentation (day 0). There was significant decrease in the values of aPTT (Sec) on day 7 followed by day 14 post-treatment in all the treatment groups.

4.8.1. Canine Platelet Migration Inhibition Factor (C-PMIF)

Canine Platelet Migration Inhibition Factor (C-PMIF) (ng/dl) of control group (Group 0) and treatment groups (group 1, group 2, group 3) on the day of presentation i.e. pre-treatment (day 0) and post treatment (day 14) have been summarized in the Table 22.

The Mean ± SE values C-PMIF (ng/dl) of healthy dogs (G0) at day 0, day 7 and day 14 were 60.81±2.07, 60.61±1.66 and 60.80±2.54, respectively. The

Mean ± SE values of C-PMIF (ng/dl) at day 0, day 7 and day 14 of Group 1 (G1) were 51.28±3.64, 57.85±1.00 and 62.77±2.57, respectively. The Mean ± SE values of C-PMIF (ng/dl) at day 0, day 7 and day 14 of Group 2 (G2) were 48.66±2.13, 58.94±0.53 and 67.09±0.71, respectively. The Mean ± SE values of C-PMIF (ng/dl) at day 0, day 7 and day 14 of Group 3 (G3) were 47.39±2.63, 60.07±0.54 and72.34 ±0.84, respectively. Test of significance shows that value of C-PMIF (ng/dl) of treatment group was significantly lower than that of control group on the day presentation. There was non-significant increase in the mean C-PMIF (ng/dl) on day 7 then followed by on day 14 post-treatment in all the treatment groups.

Table 9: Haematological alterations in various groups of dogs suffering from CME

Groups	TLC ($\times 10^3/\mu L$)			TEC ($\times 10^6/\mu l$)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	9.61 $\pm 1.07^{bA}$	9.81 $\pm 1.07^{bA}$	10.25 $\pm 1.17^{bA}$	6.59 $\pm 0.30^{cA}$	6.83 $\pm 0.29^{dA}$	7.10 $\pm 0.16^{cA}$
G1	5.12 $\pm 0.32^{aA}$	6.92 $\pm 0.45^{aB}$	7.56 $\pm 0.29^{Bb}$	2.58 $\pm 0.12^{aA}$	3.47 $\pm 0.13^{aB}$	4.38 $\pm 0.08^{aC}$
G2	5.45 $\pm 0.35^{aA}$	7.37 $\pm 0.62^{aB}$	8.69 $\pm 0.41^{abB}$	3.16 $\pm 0.18^{abA}$	4.90 $\pm 0.20^{bB}$	5.38 $\pm 0.45^{bB}$
G3	5.80 $\pm 0.38^{aA}$	8.33 $\pm 0.61^{abB}$	9.83 $\pm 0.36^{bC}$	3.77 $\pm 0.32^{bA}$	5.99 $\pm 0.24^{cB}$	6.31 $\pm 0.28^{cB}$

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 10: Haematological alterations in various groups of dogs suffering from CME

Groups	Hb (gm/dl)			PCV (%)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	15.06 ±0.67^{bA}	15.32 ± 0.68^{cA}	15.84 ±0.63^{cA}	42.76 ± 2.64^{bA}	43.11 ± 2.65^{bA}	43.46 ± 2.66^{cA}
G1	5.57 ±0.54^{aA}	8.06 ± 0.42^{aB}	10.28 ± 0.21^{aC}	22.97 ± 0.60^{aA}	28.71 ± 0.43^{aB}	33.17 ± 0.82^{aC}
G2	6.51 ±0.41^{aA}	9.60 ± 0.38^{bB}	11.12 ± 0.22^{aC}	23.96 ± 0.51^{aA}	31.46 ± 0.88^{aB}	34.84 ± 0.42^{abC}
G3	7.14 ±0.53^{Aa}	10.46 ±0.20^{bB}	13.15 ± 0.48^{bC}	24.67 ± 0.36^{aA}	32.32 ± 0.35^{aB}	38.80 ± 0.49^{bC}

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 11: Haematological alterations in various groups of dogs suffering from CME

Groups	MCV (fL)			MCH (pg)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	67.65 ±1.67	68.18 ±1.64	68.70 ±1.57	33.80 ±0.59	34.01 ±0.61	34.05 ± 0.58
G1	67.60 ±0.35	67.54 ± 0.25	67.88 ± 0.29	33.78 ±0.29	34.28 ±0.48	34.48 ± 0.96
G2	67.52 ±0.52	67.96 ± 0.37	68.23 ± 0.33	34.05 ±0.68	34.57 ±0.81	35.08 ± 0.98
G3	67.84 ±0.70	68.09 ±0.61	68.11 ± 0.91	34.58 ± 0.71	34.83 ±0.29	34.95 ±1.10

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 12: Haematological alterations in various groups of dogs suffering from CME

Groups	MCHC (gm/dL)		
	Day 0	Day 7	Day 14
Healthy (Control)	34.30 ±0.59	34.02 ± 0.61	34.05 ±0.58
G1	33.64 ± 0.59	34.08 ± 0.99	33.96 ±0.88
G2	34.39 ± 1.04	34.12 ± 0.88	34.63 ± 1.18
G3	34.46 ± 0.92	34.81 ± 1.36	34.95 ±1.56

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 13: Haematological alterations in various groups of dogs suffering from CME

Groups	Monocytes (%)			Eosinophil (%)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	4.70 ±0.55	4.89 ±0.55	4.97 ± 0.54	2.75 ± 0.35	2.70 ± 0.33	2.63 ± 0.48
G1	4.56 ±0.23	4.94 ± 0.33	5.03 ± 0.37	2.74 ± 0.03	2.75 ± 0.19	2.67 ± 0.20
G2	4.67 ±0.26	4.65 ± 0.17	4.74 ± 0.17	2.77 ± 0.16	2.74 ± 0.20	2.43 ± 0.19
G3	4.82 ±0.45	4.60 ± 0.43	4.78 ± 0.43	2.76 ± 0.40	2.58 ± 0.27	2.59 ± 0.42

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 14: Haematological alterations in various groups of dogs suffering from CME

Groups	Neutrophil (%)			Lymphocyte (%)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	78.83 ±1.47^{aA}	79.51 ± 1.52^{aA}	81.58 ±1.18^{bA}	14.71 ± 1.87^{bA}	15.10 ± 1.89^{cA}	15.45 ± 1.88^{bA}
G1	82.64 ±0.40^{bB}	81.05 ± 0.48^{aAB}	80.30 ± 0.86^{bA}	5.58 ± 0.30^{aA}	8.21 ± 0.41^{aB}	10.94 ± 0.37^{aC}
G2	83.36 ±0.52^{bB}	80.90 ± 1.02^{aAB}	78.92 ± 0.96^{abA}	6.03 ± 0.20^{aA}	9.84 ± 0.18^{abB}	12.41 ± 0.54^{abC}
G3	86.17 ± 0.43^{cB}	79.71 ± 1.71^{aA}	76.76 ± 0.57^{aA}	6.79 ± 0.35^{aA}	11.91 ± 0.56^{bB}	14.24 ± 0.97^{abC}

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 15: Haematological alterations in various groups of dogs suffering from CME

Groups	Platelet (×10^5/µL)			PCT (%)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	2.93 ±0.30^{bA}	2.97 ± 0.30^{cA}	3.00 ±0.29^{cA}	0.29 ± 0.02^{bA}	0.30 ± 0.02^{aA}	0.57 ± 0.10^{Bb}
G1	0.48 ±0.01^{aA}	1.00 ± 0.04^{aB}	1.56 ± 0.01^{aC}	0.03 ± 0.00^{aA}	0.16 ± 0.05^{aB}	0.26 ± 0.03^{aB}
G2	0.54 ±0.05^{aA}	1.30 ± 0.03^{aB}	2.22 ± 0.10^{bC}	0.03 ± 0.00^{aA}	0.24 ± 0.11^{aAB}	0.43 ± 0.05^{abB}
G3	0.67 ±0.01^{aA}	1.99 ± 0.02^{bB}	2.70 ± 0.05^{cC}	0.05 ± 0.00^{aA}	0.32 ± 0.07^{aB}	0.51 ± 0.08^{bC}

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 16: Haematological alterations in various groups of dogs suffering from CME

Groups	MPV(fL)			PDW (%)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	6.82 ±0.07^{aA}	6.94 ± 0.08^{aA}	6.39 ±0.21^{aA}	15.90 ± 0.38	15.66 ± 0.39	15.40 ± 0.41
G1	6.83 ±0.05^{aA}	6.61 ± 0.07^{aB}	6.36 ± 0.06^{aC}	14.75 ± 0.77	15.71 ± 0.64	15.48 ± 0.80
G2	6.83 ±0.28^{aA}	6.60 ± 0.21^{aA}	6.07 ± 0.17^{aA}	14.95 ± 0.64	15.40 ± 0.60	15.14 ± 0.63
G3	6.77 ±0.21^{aA}	6.66 ± 0.22^{aA}	6.31 ± 0.14^{aA}	15.78 ± 0.61	15.07 ± 0.54	15.61 ± 0.67

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 17: Coagulation profile in various groups of dogs suffering from CME

Groups	PT (sec)			aPTT (sec)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	8.98 ± 0.17	8.97 ± 0.16	8.96 ± 0.16	12.70 ± 0.07^{aA}	12.74 ± 0.06^{aA}	12.86 ± 0.01^{aA}
G1	8.79 ± 0.18	9.18 ± 0.02	8.62 ± 0.27	23.79 ± 0.26^{bC}	21.23 ± 0.62^{cB}	16.28 ± 0.17^{dA}
G2	8.91 ± 0.17	9.16 ± 0.00	8.72 ± 0.19	25.57 ± 0.94^{bC}	17.49 ± 0.34^{bB}	14.34 ± 0.19^{cA}
G3	9.15 ± 0.01	8.92 ± 0.11	8.82 ± 0.20	23.98 ± 0.20^{cB}	14.05 ± 0.88^{aA}	13.42 ± 0.19^{bA}

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 18: Biochemical alterations in various groups of dogs suffering from CME

Groups	ALT (IU/L)			AST (IU/L)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	32.44 ±2.65^{aA}	33.55 ± 2.70^{aA}	34.06 ±2.69^{aA}	18.91 ± 1.29^{bA}	19.86 ± 1.27^{bA}	20.36 ± 1.35^{bA}
G1	79.95 ±0.48^{bC}	54.97 ± 0.46^{dB}	47.55 ± 1.72^{cA}	78.01 ± 1.91^{aC}	65.50 ± 1.54^{aB}	49.01 ± 2.42^{aA}
G2	82.58 ±0.36^{bC}	49.49 ± 1.65^{cB}	40.13 ± 0.73^{bA}	80.01 ± 1.44^{aC}	60.83 ± 3.13^{aB}	42.33 ± 2.06^{aA}
G3	83.79 ±0.22^{bC}	38.98 ± 1.53^{bB}	30.74 ± 1.17^{aA}	77.50 ± 0.43^{aC}	52.16 ± 3.36^{aB}	30.67 ± 1.20^{aA}
Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.						

Table 19: Biochemical alterations in various groups of dogs suffering from CME

Groups	BUN (mg/dl)			Creatinine(mg/dl)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	17.58 ±0.99^{aA}	18.46 ± 1.02^{aA}	19.24 ±1.00^{aA}	0.68 ± 0.09^{aA}	0.66 ± 0.07^{aA}	0.685 ± 0.08^{aA}
G1	42.16 ±1.13^{bC}	35.16 ± 1.14^{cB}	30.04 ± 1.29^{cA}	0.83 ± 0.10^{aA}	0.55 ± 0.07^{aA}	0.47 ± 0.05^{aA}
G2	45.00 ±2.42^{bC}	36.17 ± 2.55^{cB}	27.00 ± 1.88^{bcA}	0.64 ± 0.10^{aA}	0.53 ± 0.09^{aA}	0.39 ± 0.10^{aA}
G3	40.83 ±1.37^{bC}	28.83 ±1.38^{bB}	22.86 ±1.56^{abA}	0.67 ± 0.14^{aA}	0.49 ± 0.12^{aA}	0.37 ± 0.10^{aA}
Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.						

Table 20: Biochemical alterations in various groups of dogs suffering from CME

Groups	ALP(IU/L)			Total Protein (gm/dl)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Healthy (Control)	34.45 ±0.93^{aA}	36.43 ± 0.96^{aA}	37.15 ±0.97^{aA}	6.58 ± 0.17^{cA}	6.59 ± 0.18^{bA}	6.60 ± 0.19^{bA}
G1	88.00 ±4.53^{bC}	68.01 ± 4.53^{cB}	48.16 ± 2.02^{bA}	4.68 ±0.21^{abA}	5.03 ± 0.49^{aAB}	6.02 ± 0.33^{aB}
G2	89.33 ±2.87^{bC}	59.33 ± 2.87^{bcB}	44.67 ± 2.65^{abA}	4.85 ± 0.22^{bA}	6.09 ± 0.20^{bB}	6.61 ± 0.20^{abB}
G3	92.16 ±3.54^{bC}	53.66 ± 4.56^{bB}	39.83 ± 4.31^{abA}	4.07 ± 0.30^{aA}	6.48 ± 0.42^{bB}	7.02 ± 0.31^{bB}

Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

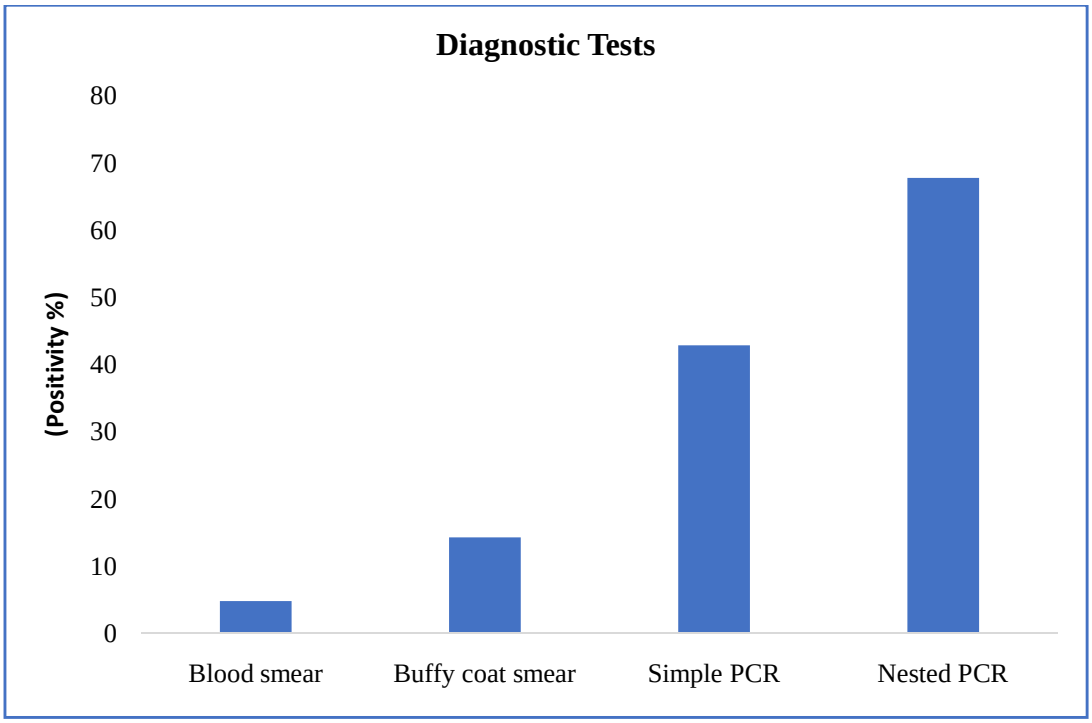
Table 21: Biochemical alterations in various groups of dogs suffering from CME

Groups	Albumin (gm/dl)			Globulin (gm/dl)		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 4
Healthy (Control)	2.82 ±0.14^{bA}	2.92 ± 0.14^{cA}	3.01 ±0.14^{cA}	3.35 ± 0.24^{bA}	3.55 ± 0.24^{aA}	3.75 ± 0.24^{aA}
G1	1.39 ±0.10^{aA}	1.48 ± 0.10^{aA}	1.58 ± 0.12^{aA}	2.61 ± 0.05^{aA}	3.17 ± 0.11^{aB}	3.46 ± 0.19^{aB}
G2	1.50 ±0.08^{aA}	2.47 ± 0.08^{bB}	2.55 ± 0.07^{bB}	3.10 ±0.35^{abA}	3.29 ± 0.27^{aB}	3.61 ± 0.27^{aB}
G3	1.63 ±0.04^{aA}	2.55 ± 0.07^{bB}	2.69 ± 0.06^{bB}	2.70 ±0.15^{abA}	3.38 ± 0.15^{aB}	3.73 ± 0.10^{aB}

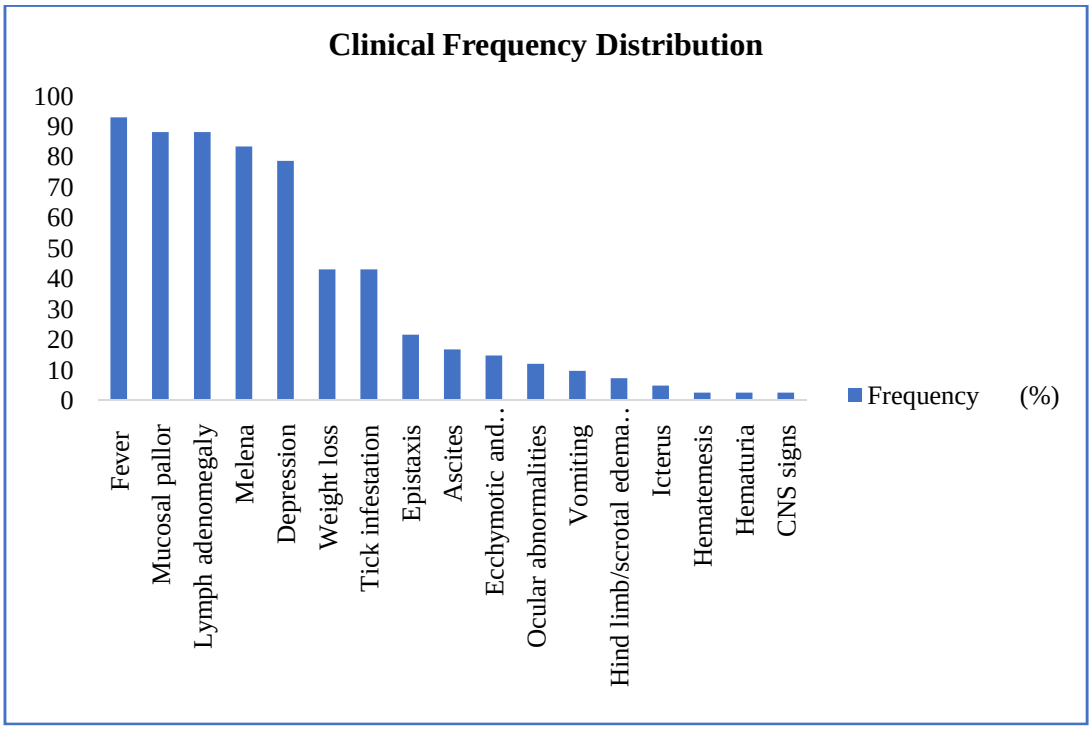
Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.

Table 22: C-PMIF alterations in various groups of dogs suffering from CME

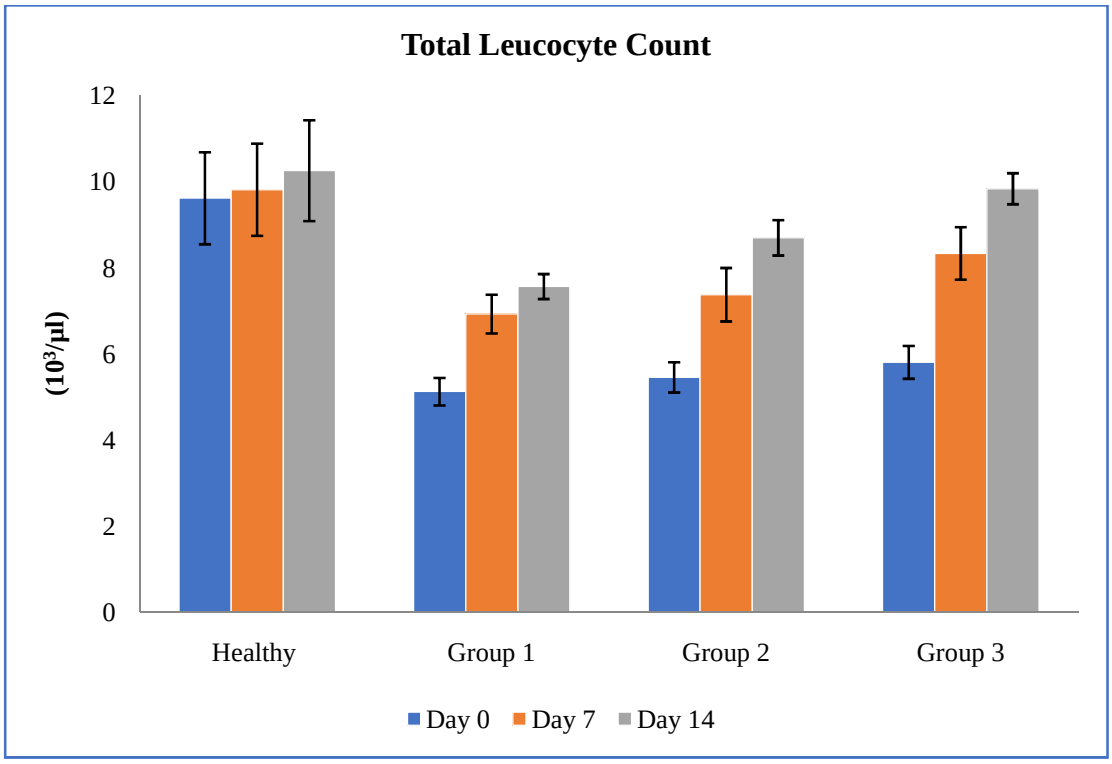
Groups	C-PMIF (ng/dl)		
	Day 0	**Day 7**	**Day 14**
Healthy (Control)	60.81 ±2.07^{bA}	60.61 ± 1.66^{aA}	60.80 ± 2.54^{aA}
G1	51.28 ± 3.64^{aA}	57.85 ± 1.00^{aAB}	62.77 ± 2.57^{abB}
G2	48.66 ± 2.13^{aA}	58.94 ± 0.53^{aB}	67.09 ± 0.71^{bcC}
G3	47.39 ± 2.63^{aA}	60.07 ± 0.54^{aB}	72.34 ±0.84^{Cc}
Mean with different superscript (a, b, c, d) in columns are differing significantly in between the groups, otherwise non-significant. Mean with different superscript (A, B, C, D) in rows are differing significantly in between the intervals, otherwise non-significant.			



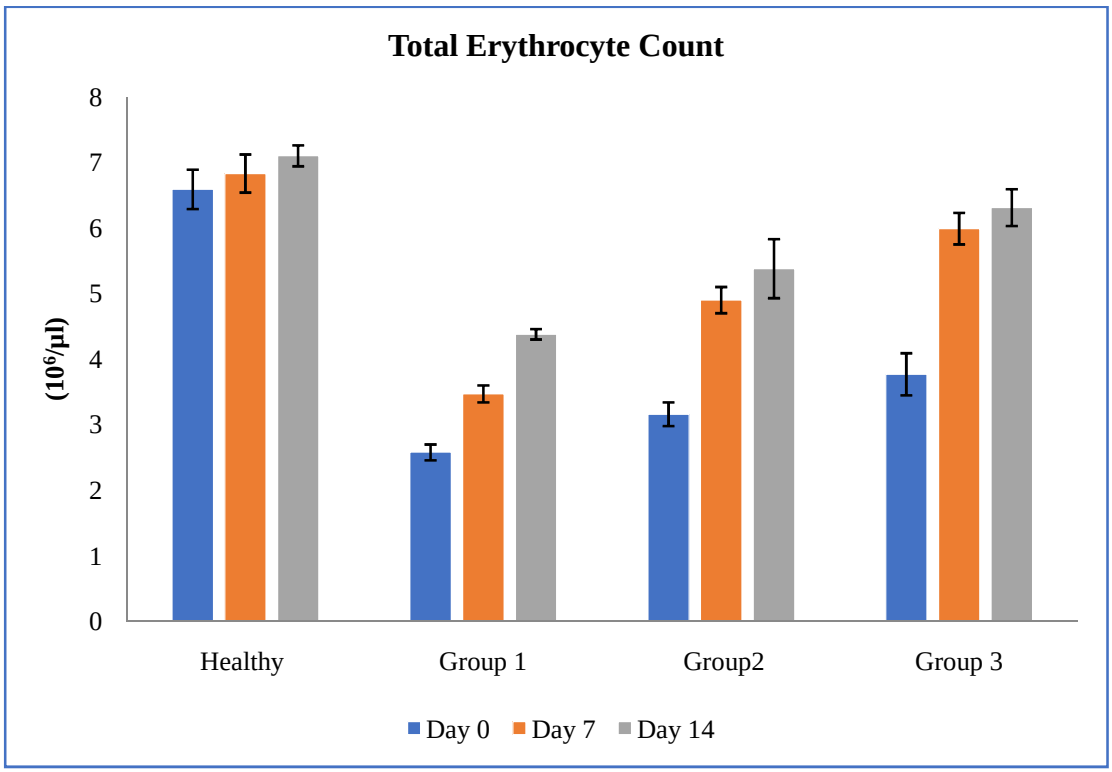
Graph 1: Percentage (%) positivity of diagnostic tests



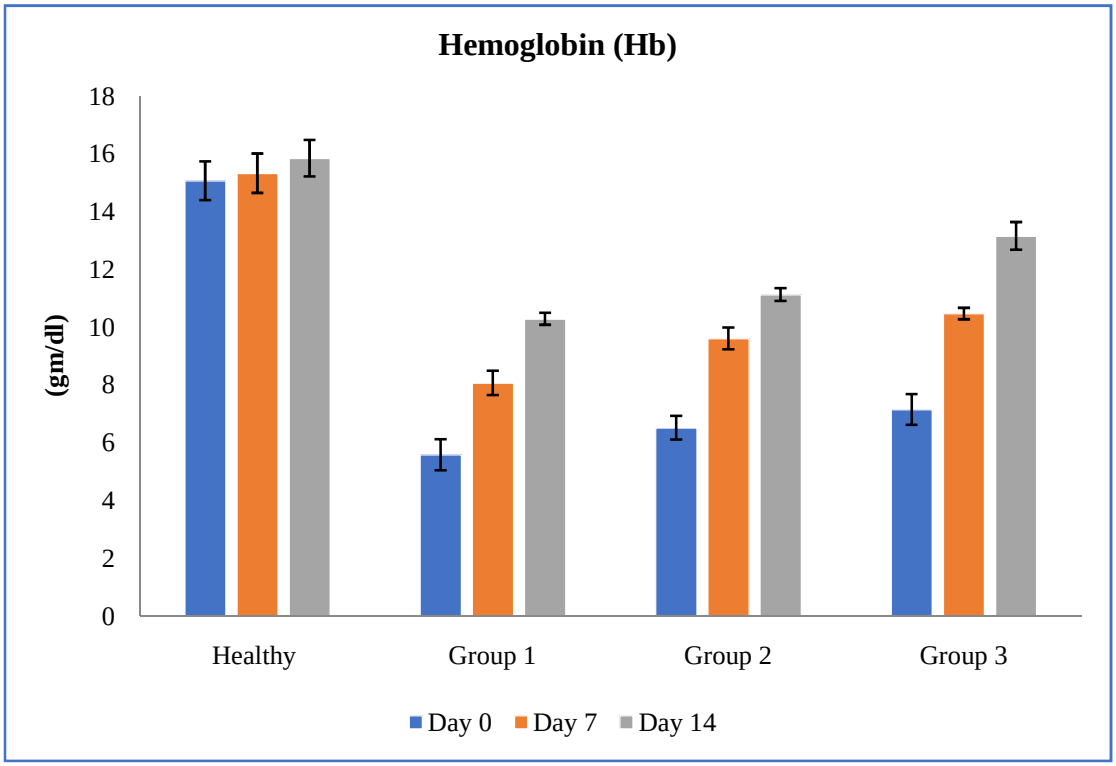
Graph 2: Percentage (%) positivity of Clinical Frequency Distribution



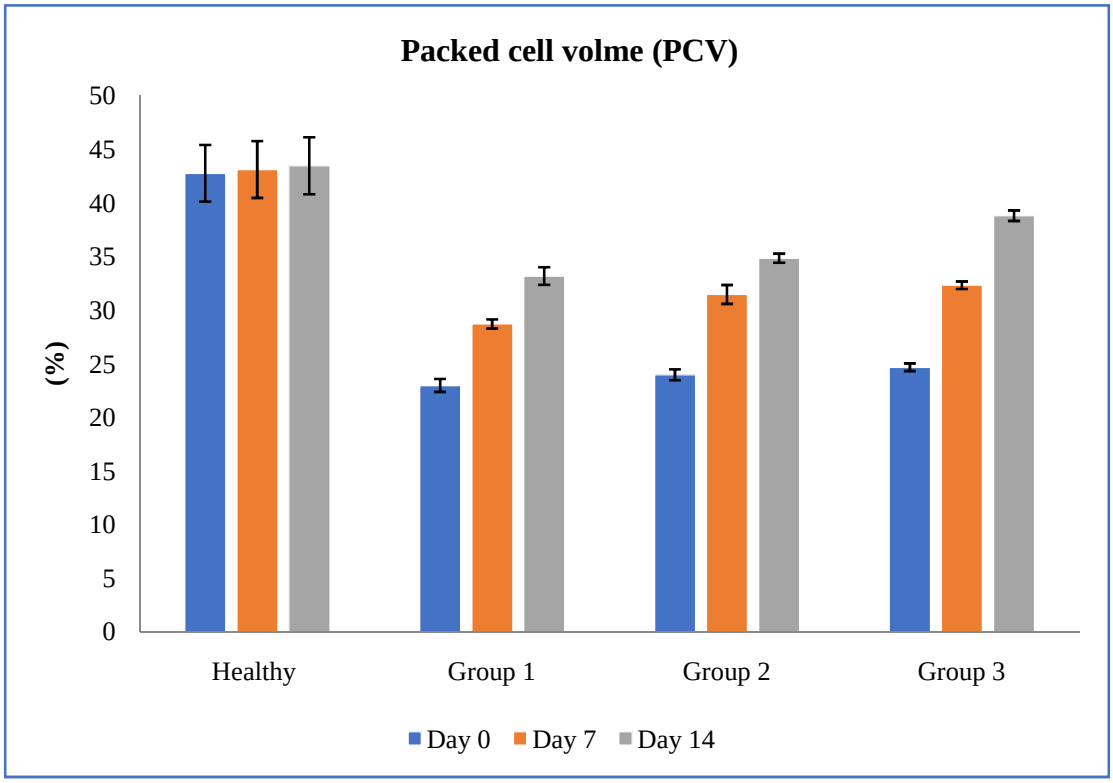
Graph 3: Showing levels of TLC in healthy dogs and dogs with CME



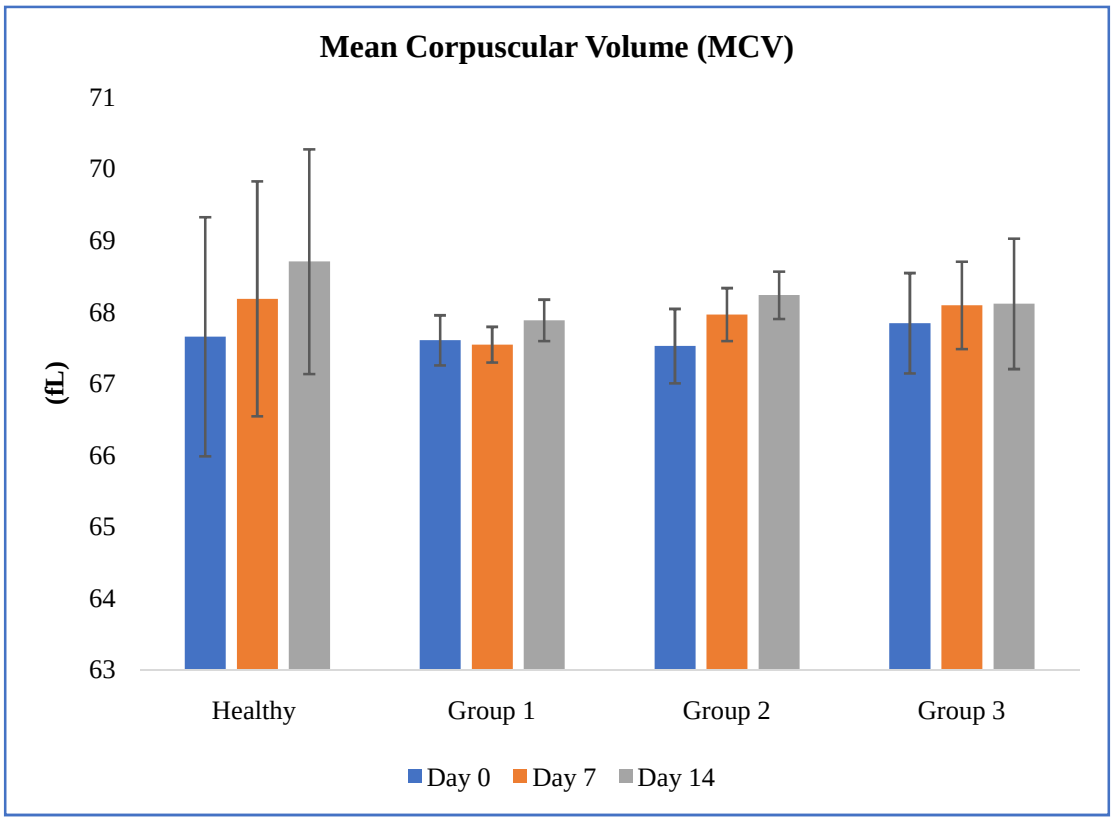
Graph 4: Showing levels of TEC in healthy dogs and dogs with CME



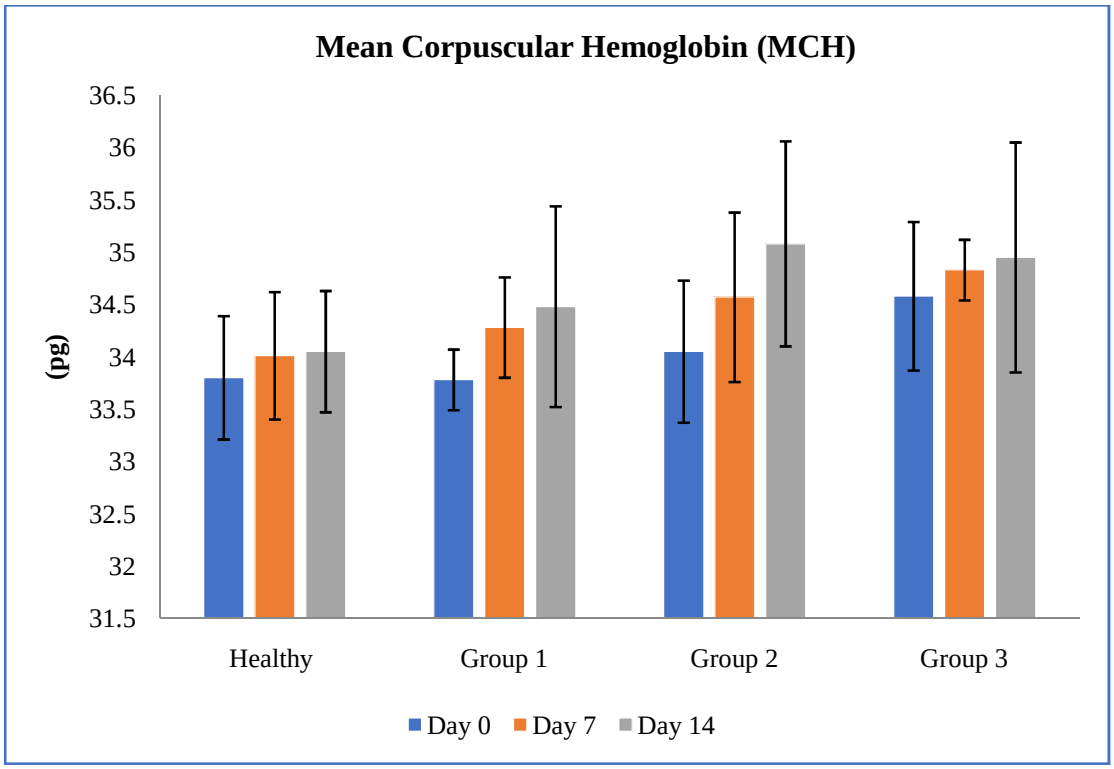
Graph 5: Showing levels of Hemoglobin in healthy dogs and dogs with CME



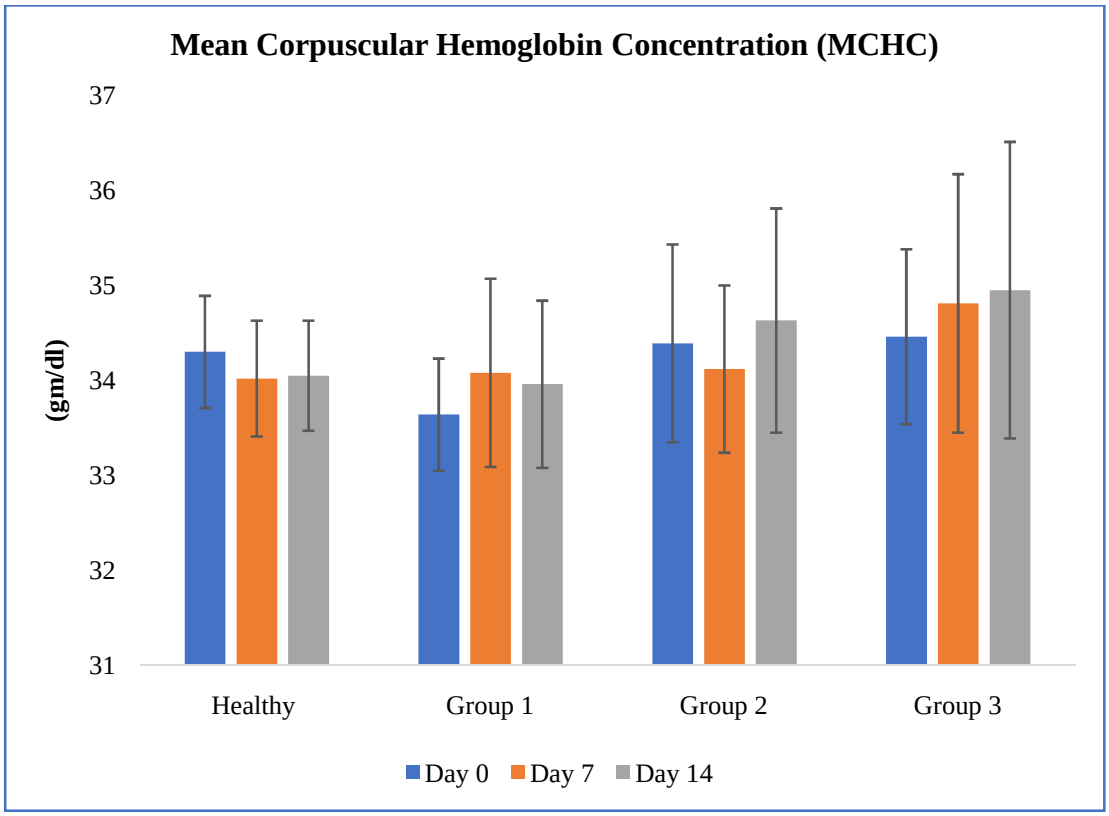
Graph 6: Showing levels of PCV in healthy dogs and dogs with CME



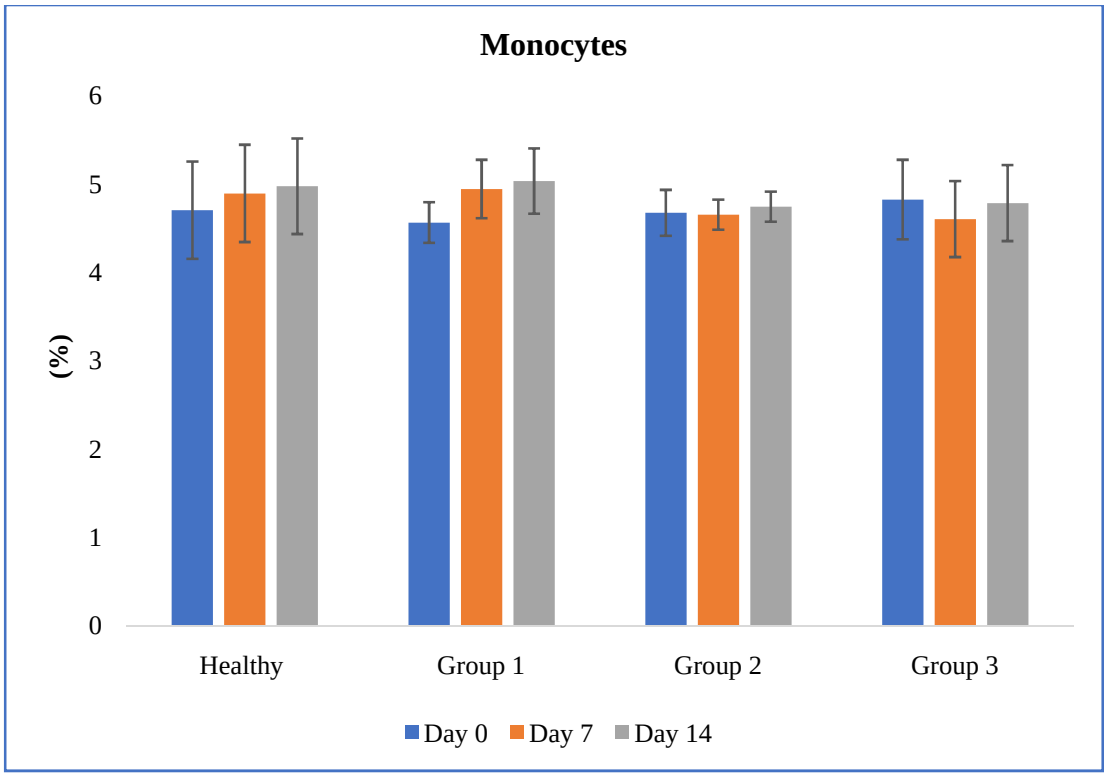
Graph 7: Showing levels of MCV in healthy dogs and dogs with CME



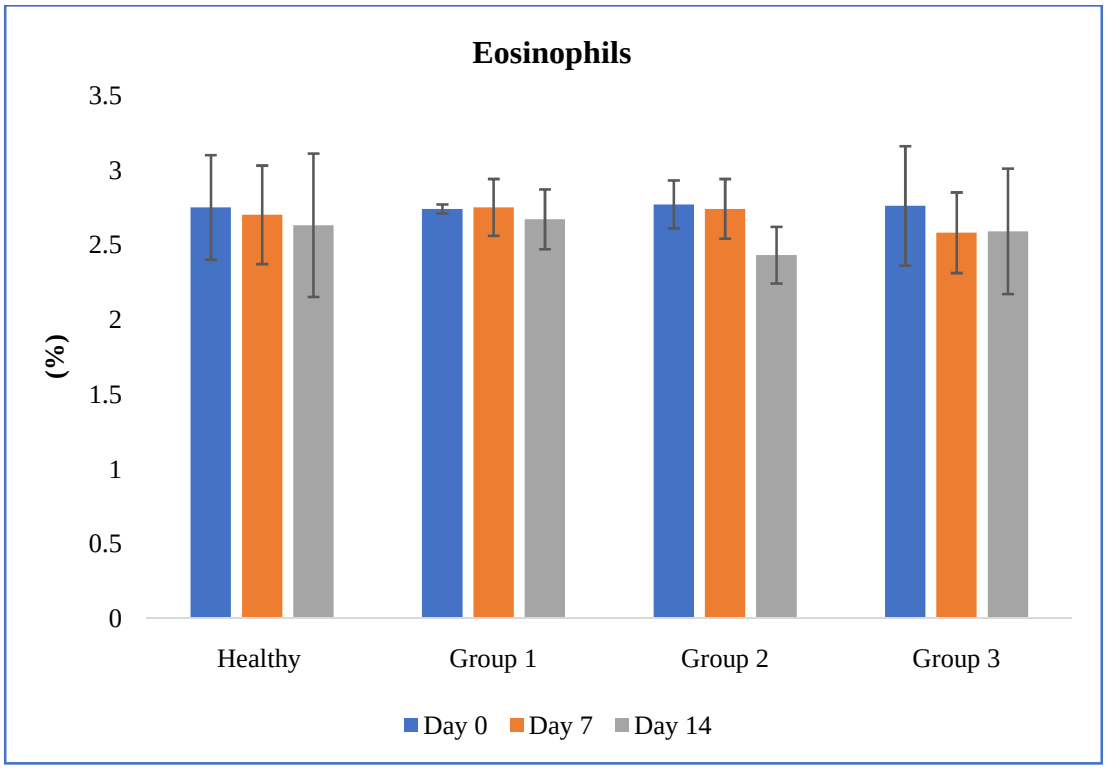
Graph 8: Showing levels of MCH in healthy dogs and dogs with CME



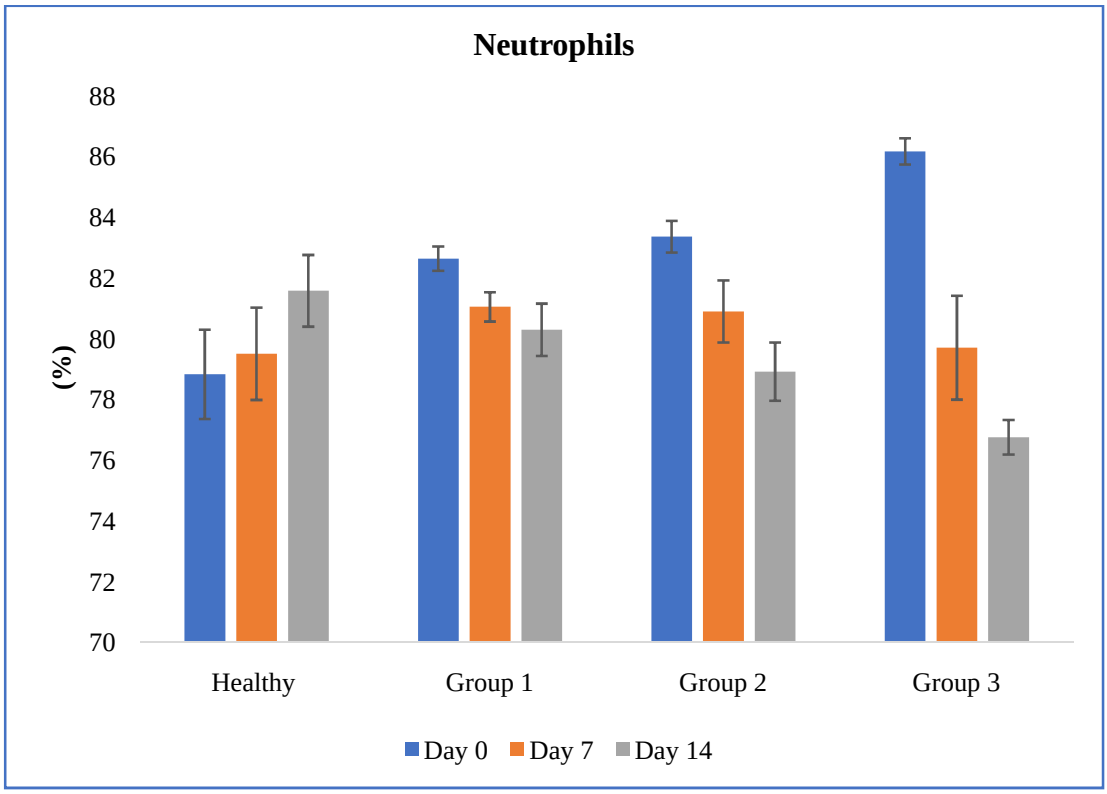
Graph 9: Showing levels of MCHC in healthy dogs and dogs with CME



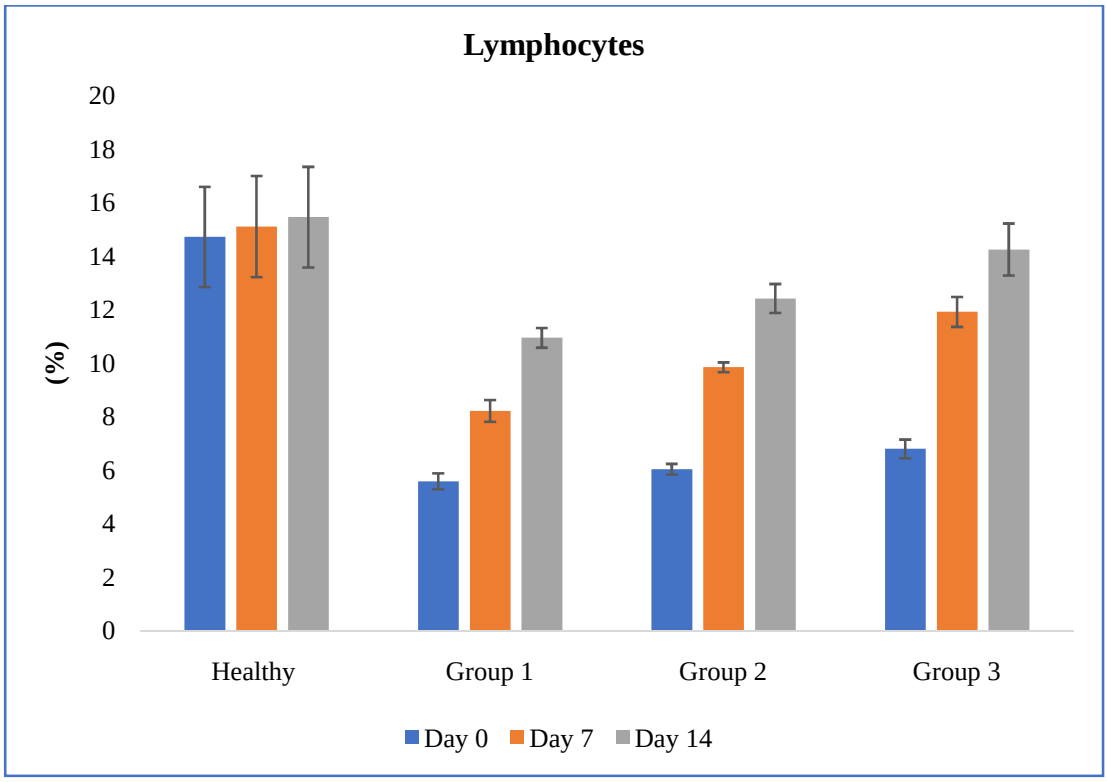
Graph 10: Showing levels of Monocytes in healthy dogs and dogs with CME



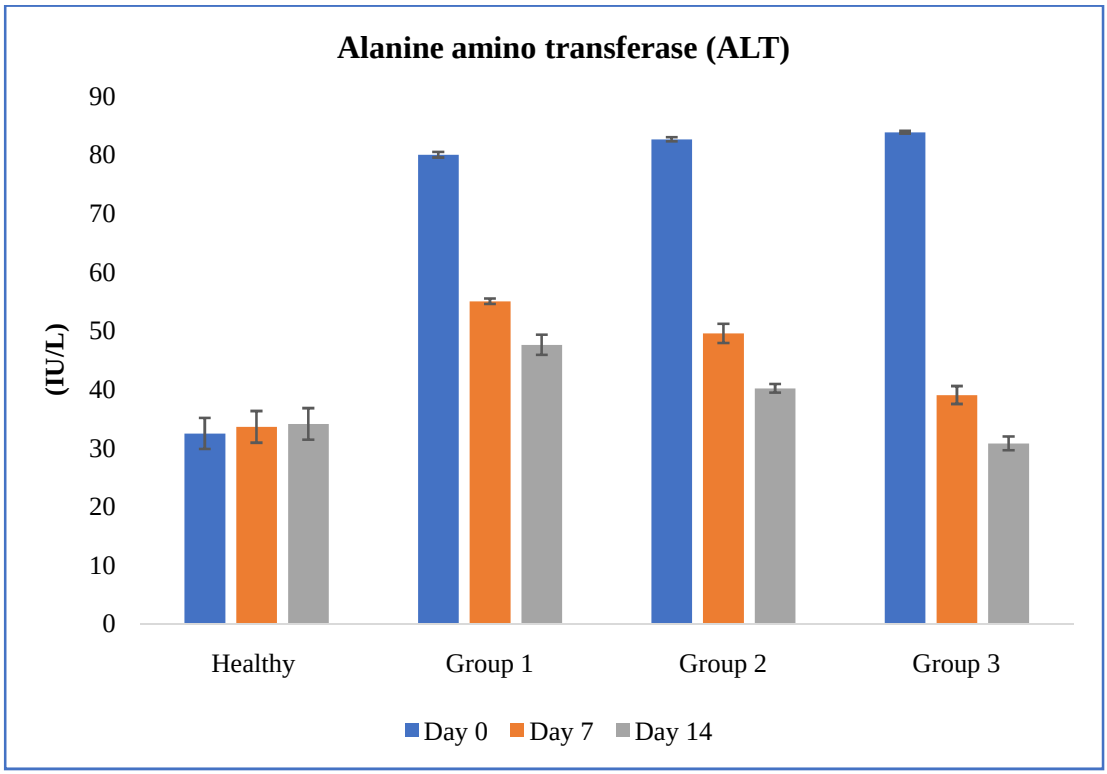
Graph 11: Showing levels of Eosinophils in healthy dogs and dogs with CME



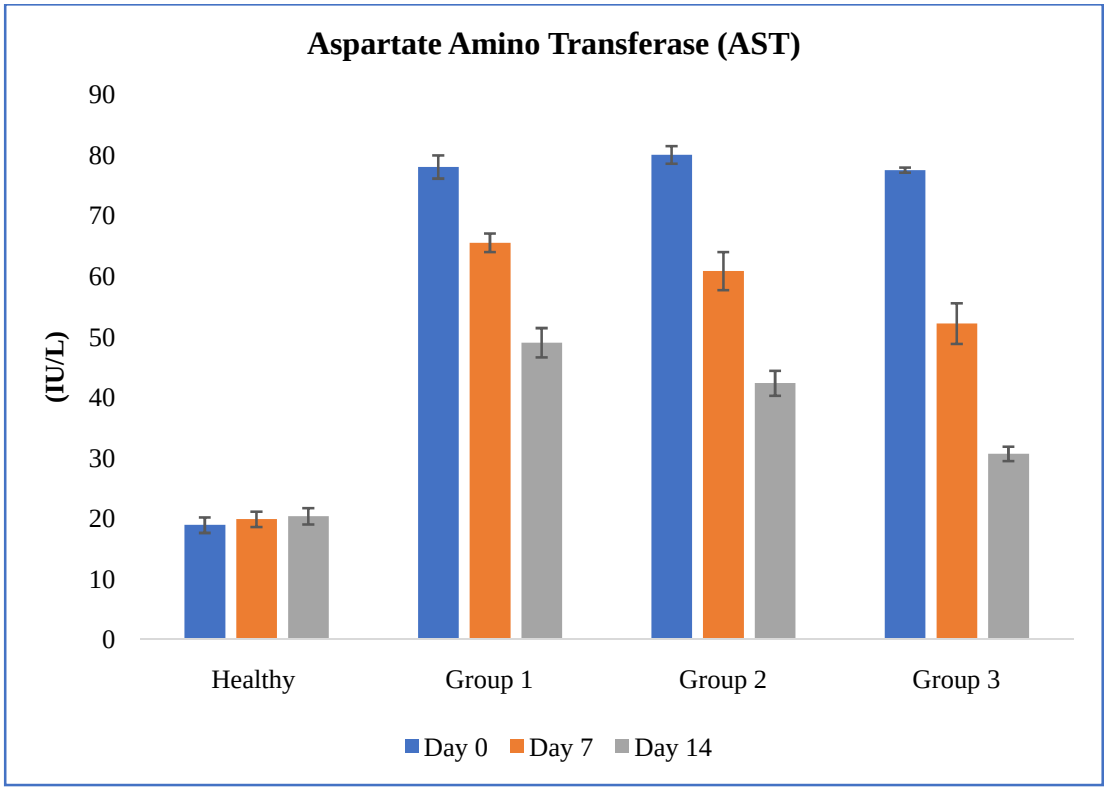
Graph 12: Showing levels of Neutrophils in healthy dogs and dogs with CME



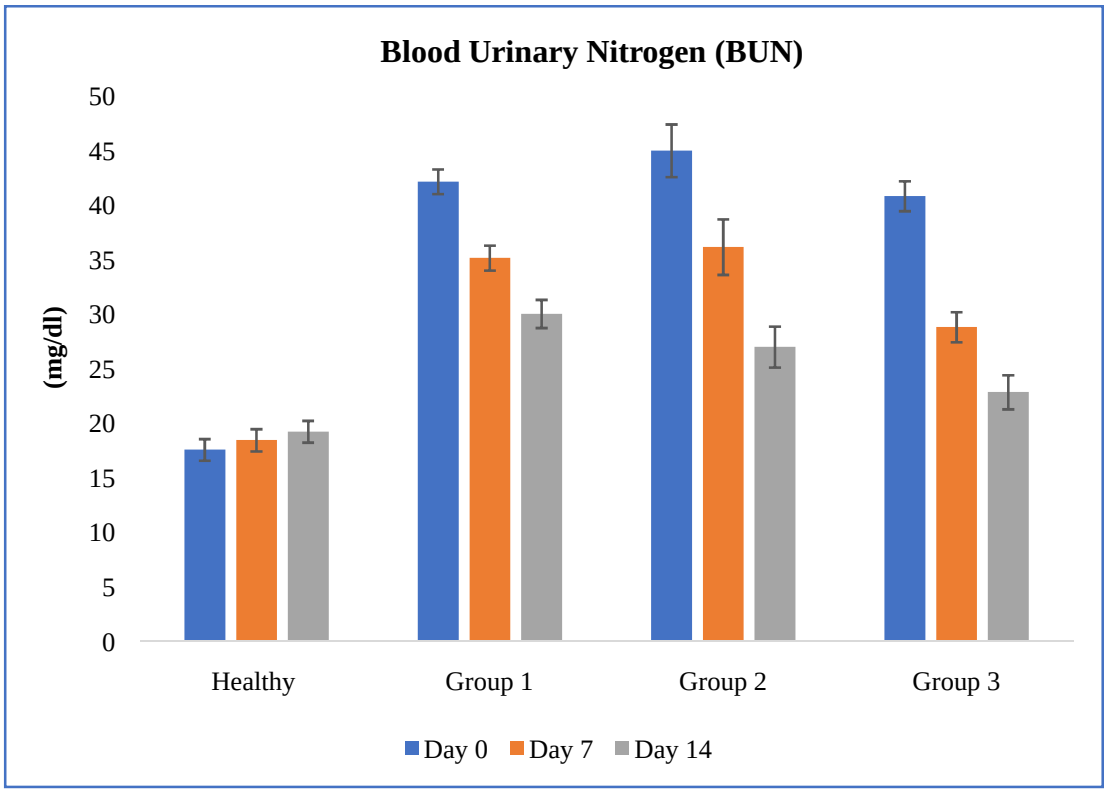
Graph 13: Showing levels of lymphocytes in healthy dogs and dogs with CME



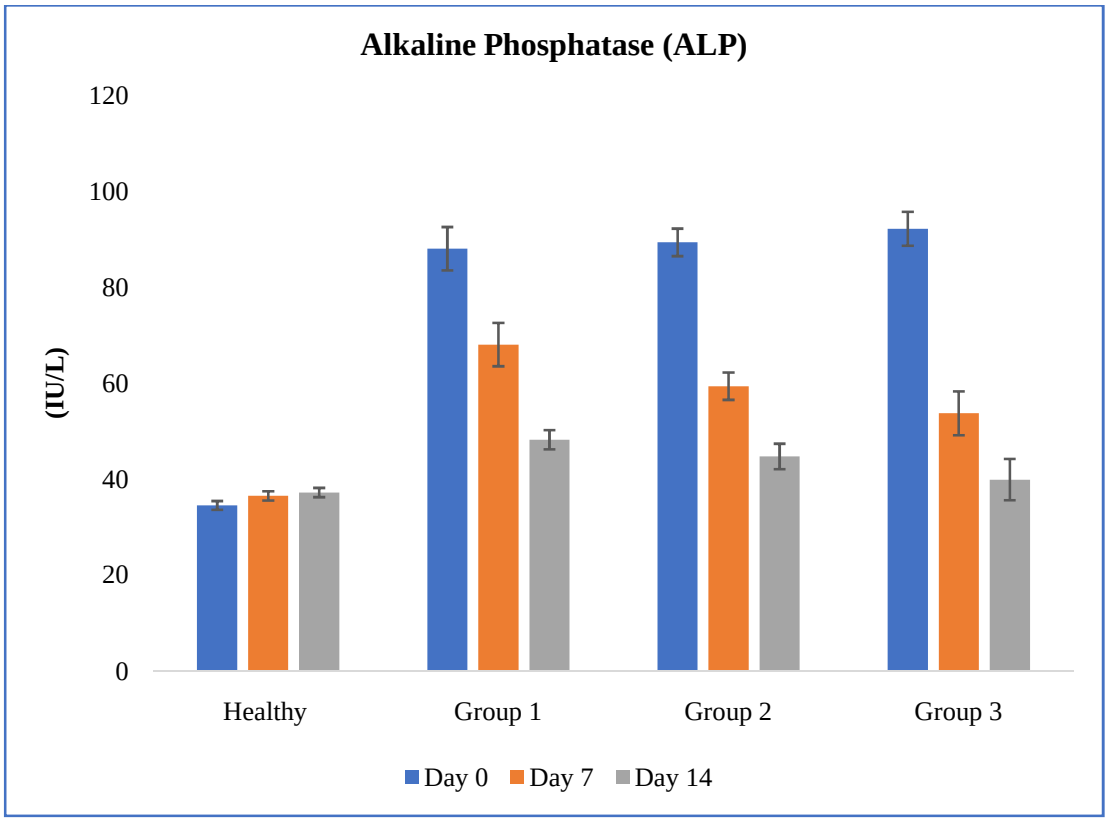
Graph 14: Showing levels of ALT in healthy dogs and dogs with CME



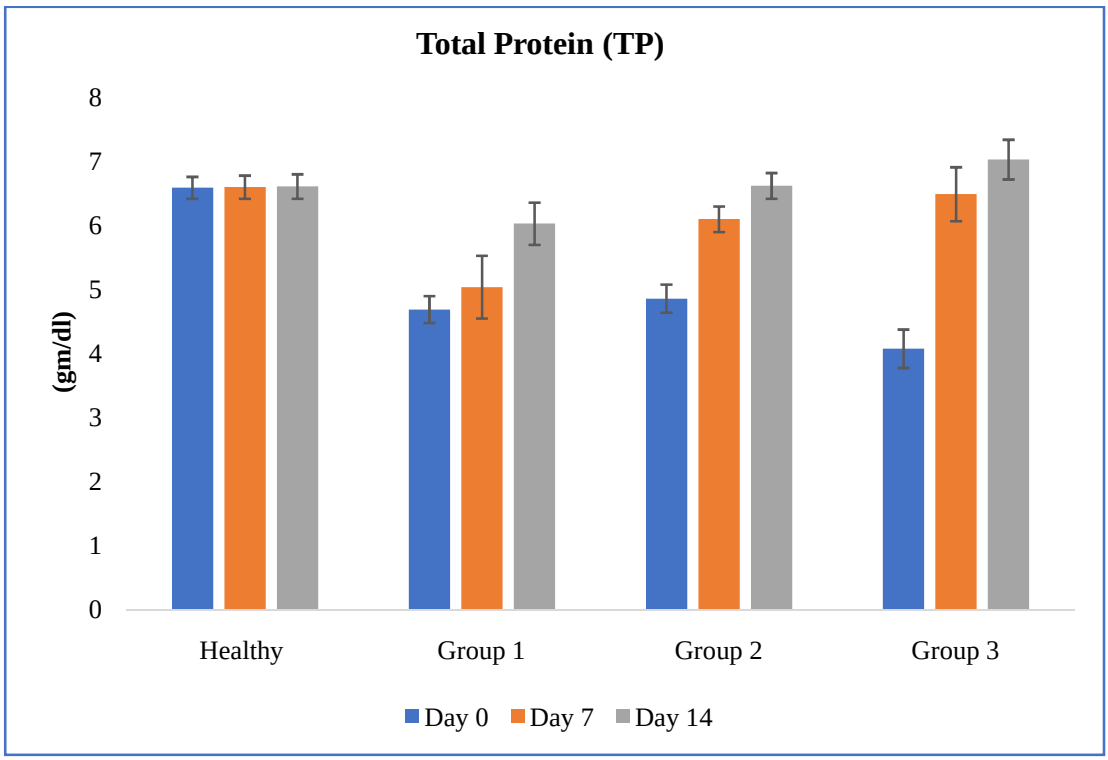
Graph 15: Showing levels of AST in healthy dogs and dogs with CME



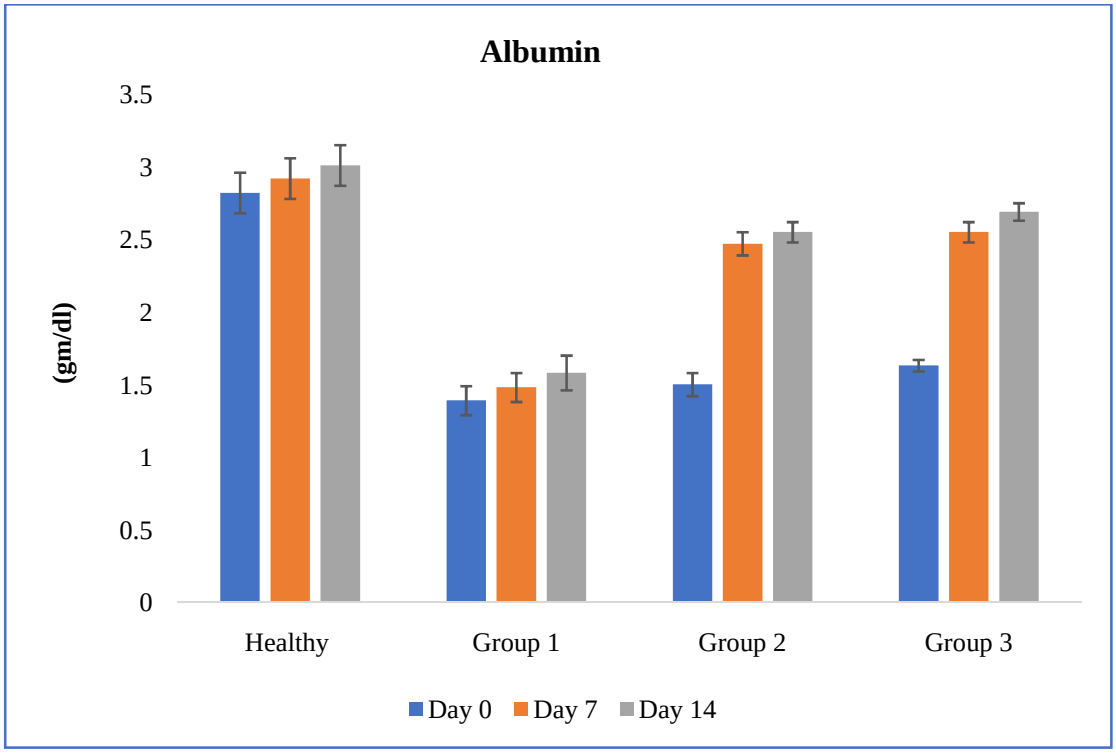
Graph 16: Showing levels of BUN in healthy dogs and dogs with CME



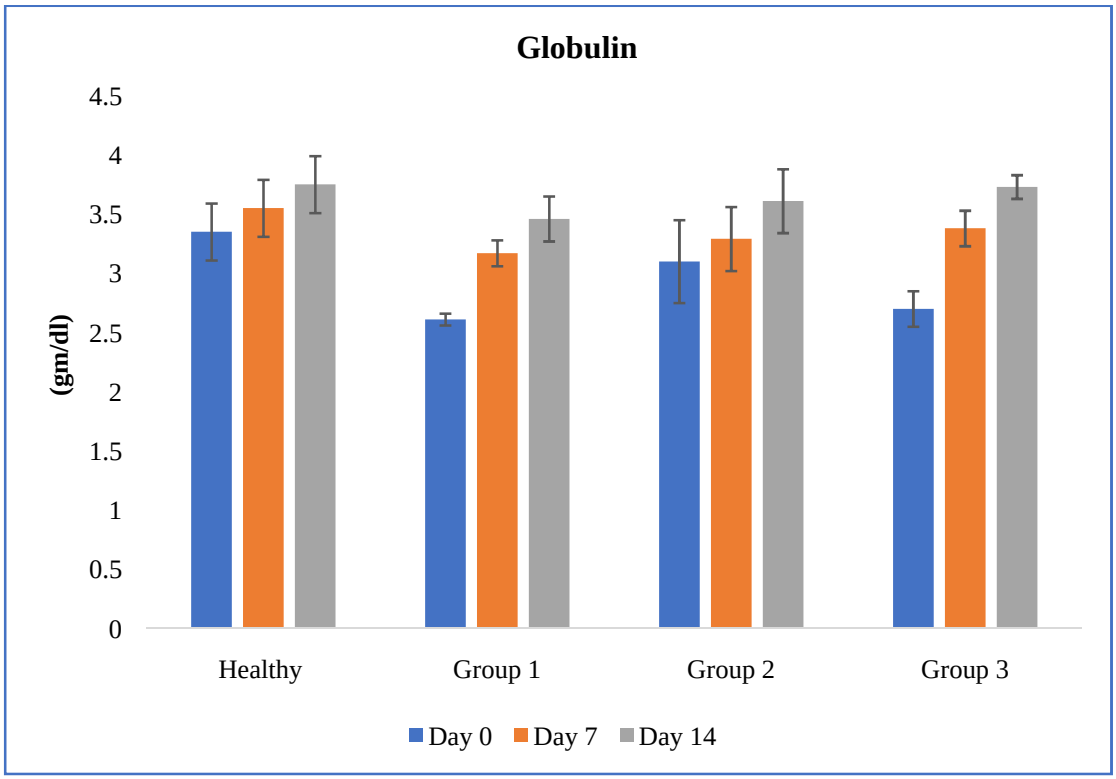
Graph 17: Showing levels of ALP in healthy dogs and dogs with CME



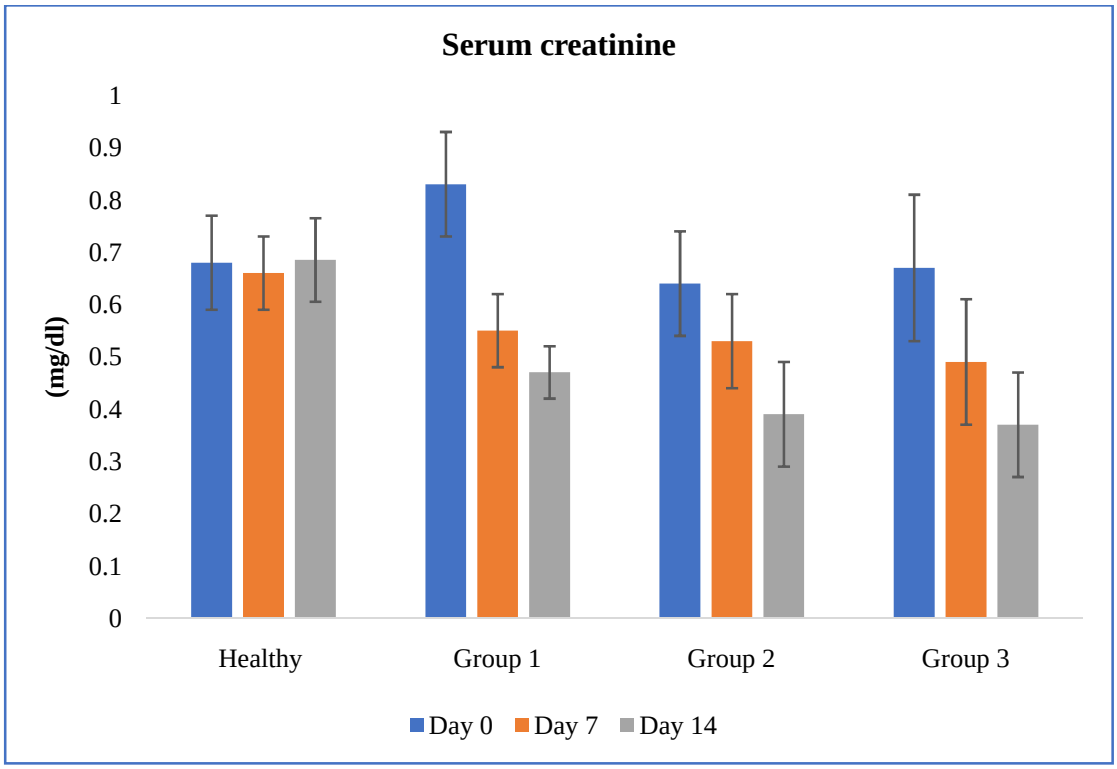
Graph 18: Showing levels of TP in healthy dogs and dogs with CME



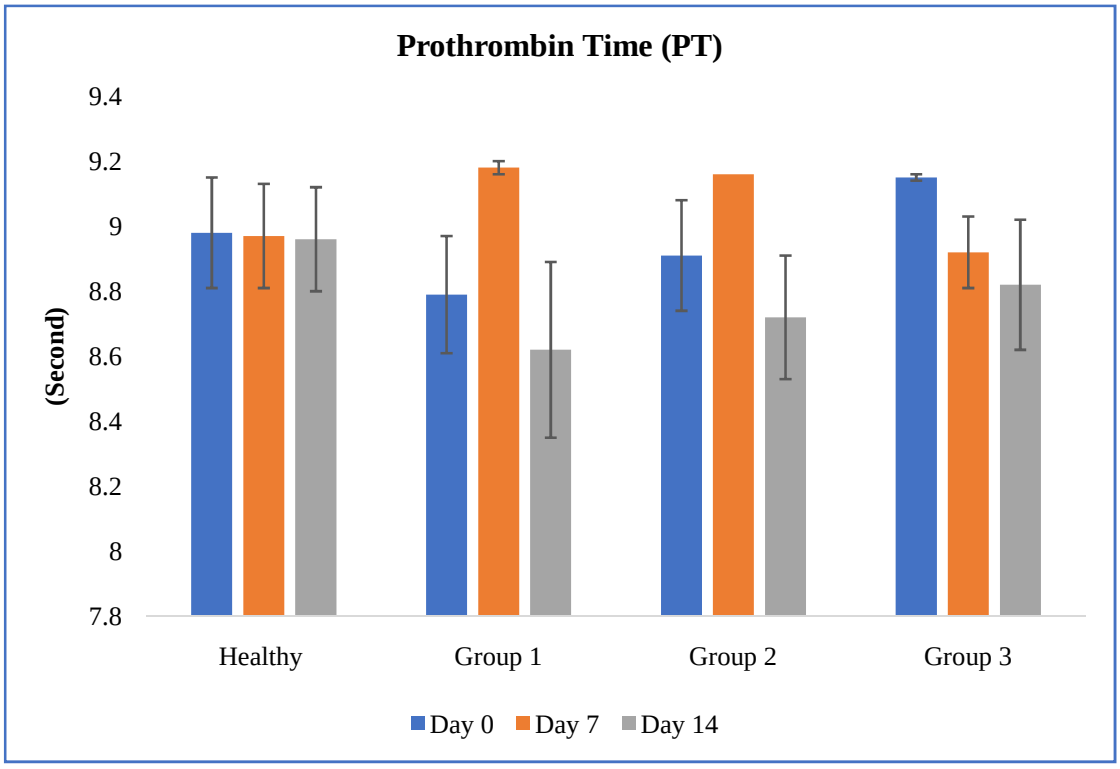
Graph 19: Showing levels of Albumin in healthy dogs and dogs with CME



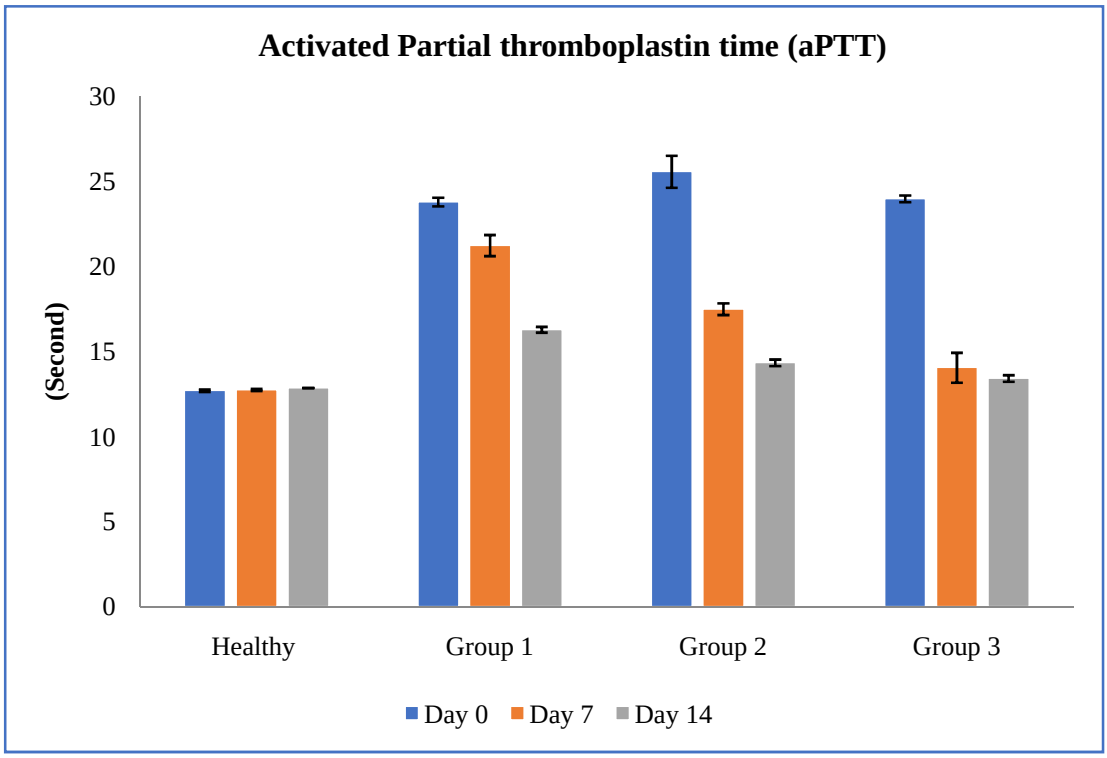
Graph 20: Showing levels of Globulin in healthy dogs and dogs with CME



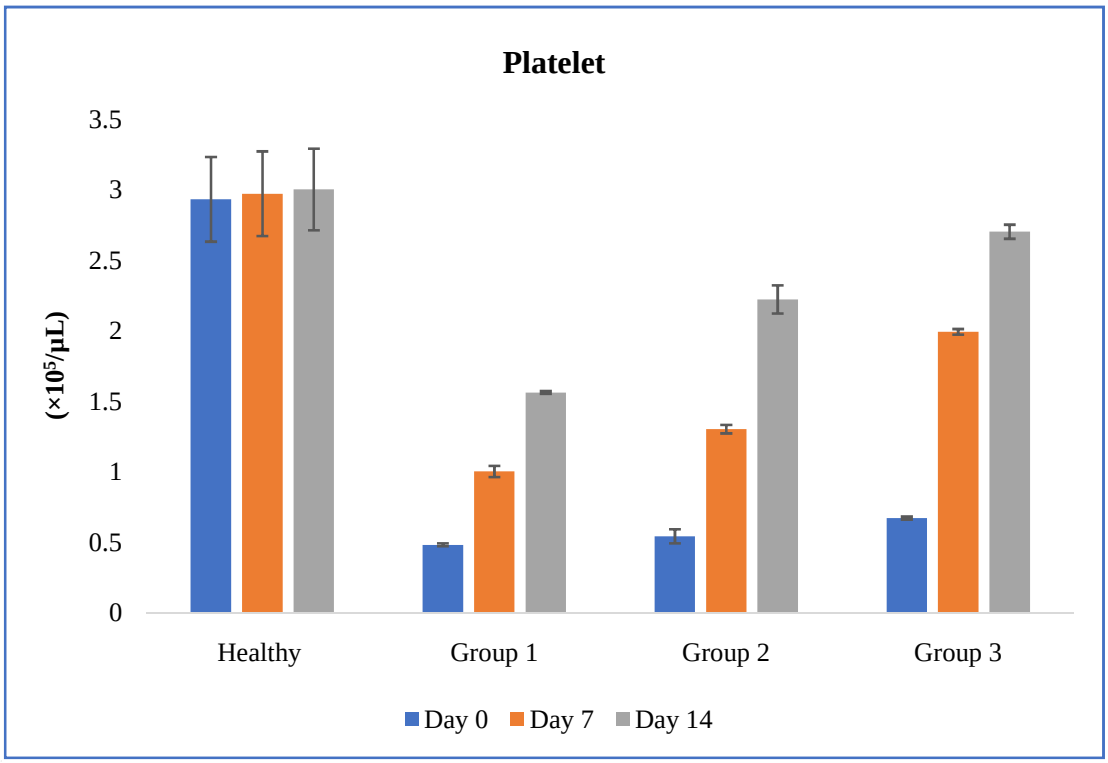
Graph 21: Showing levels of Serum creatinine in healthy dogs and dogs with CME



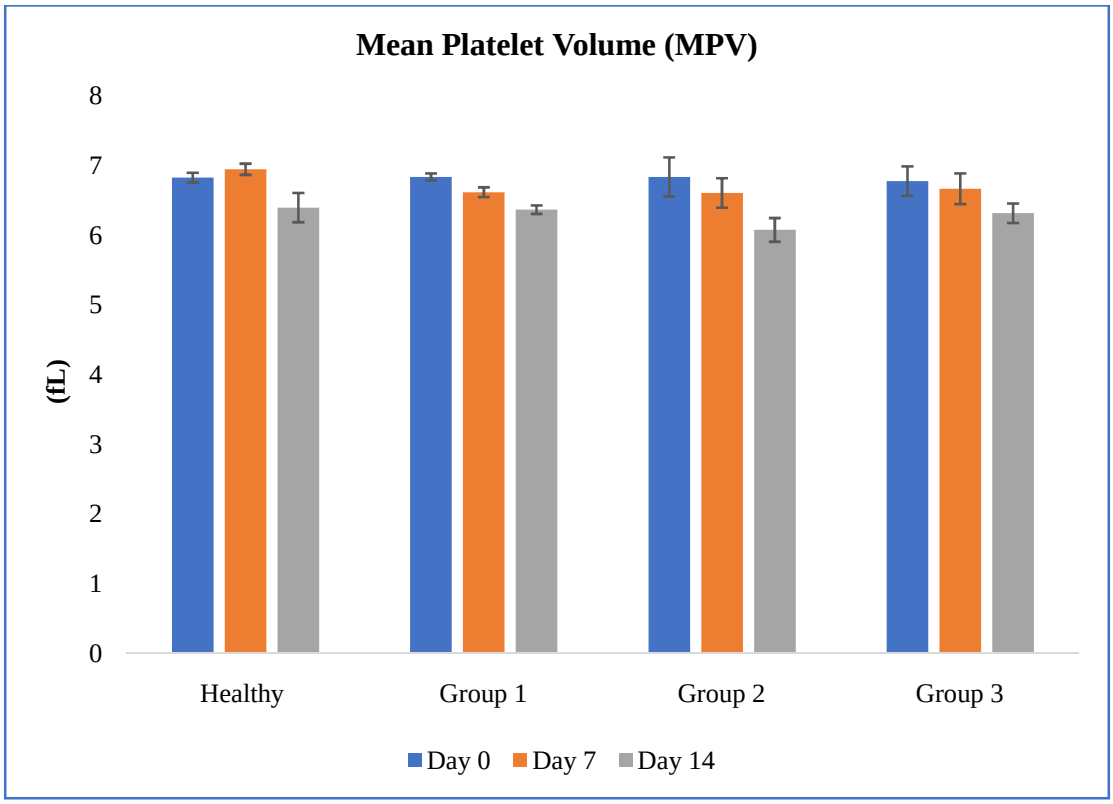
Graph 22: Showing levels of PT in healthy dogs and dogs with CME



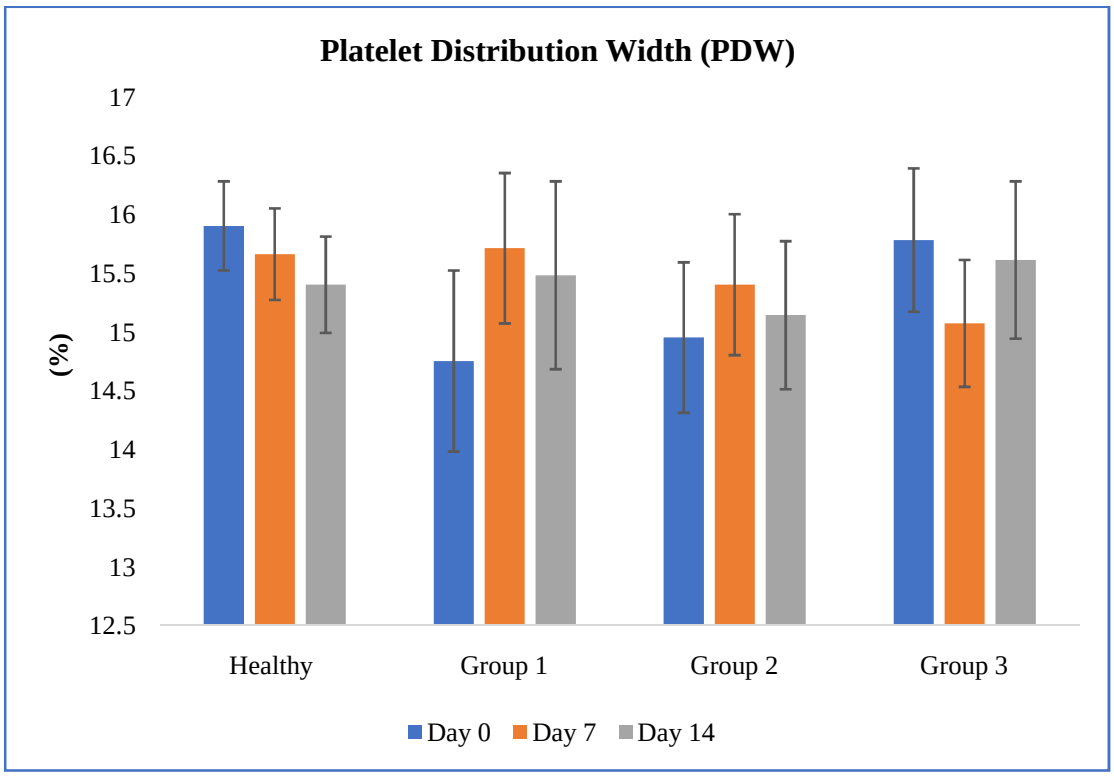
Graph 23: Showing levels of aPTT in healthy dogs and dogs with CME



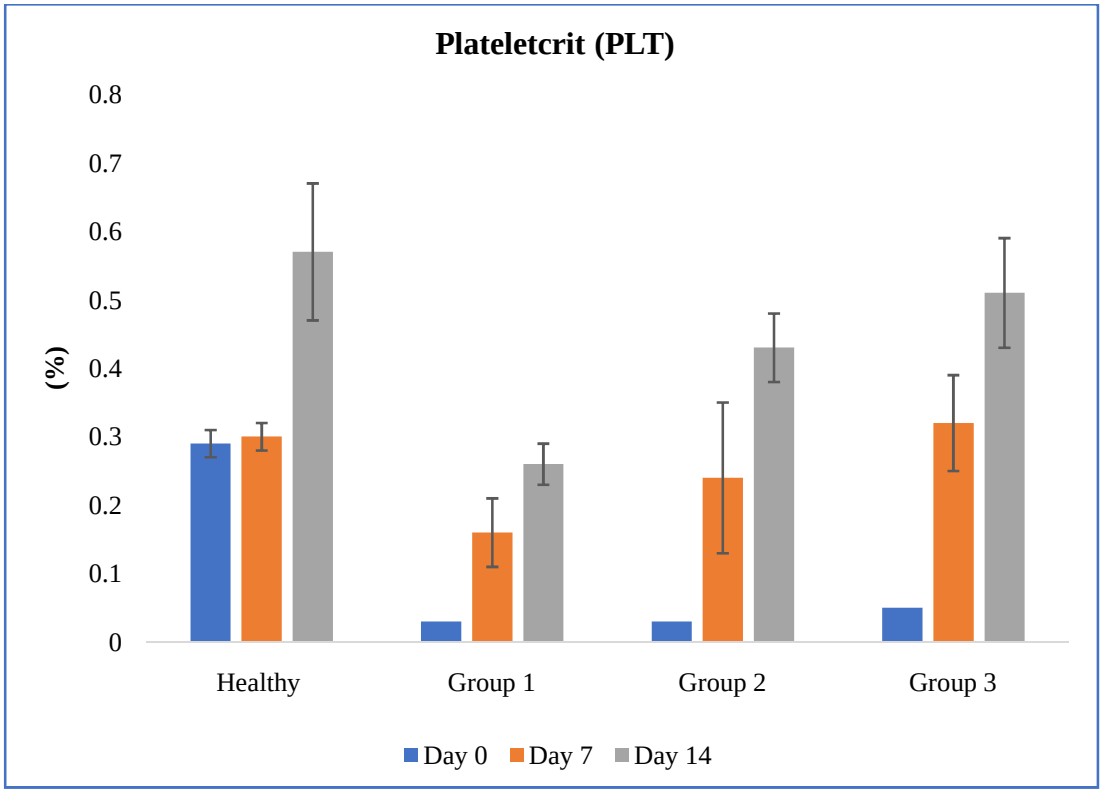
Graph 24: Showing levels of Platelets in healthy dogs and dogs with CME



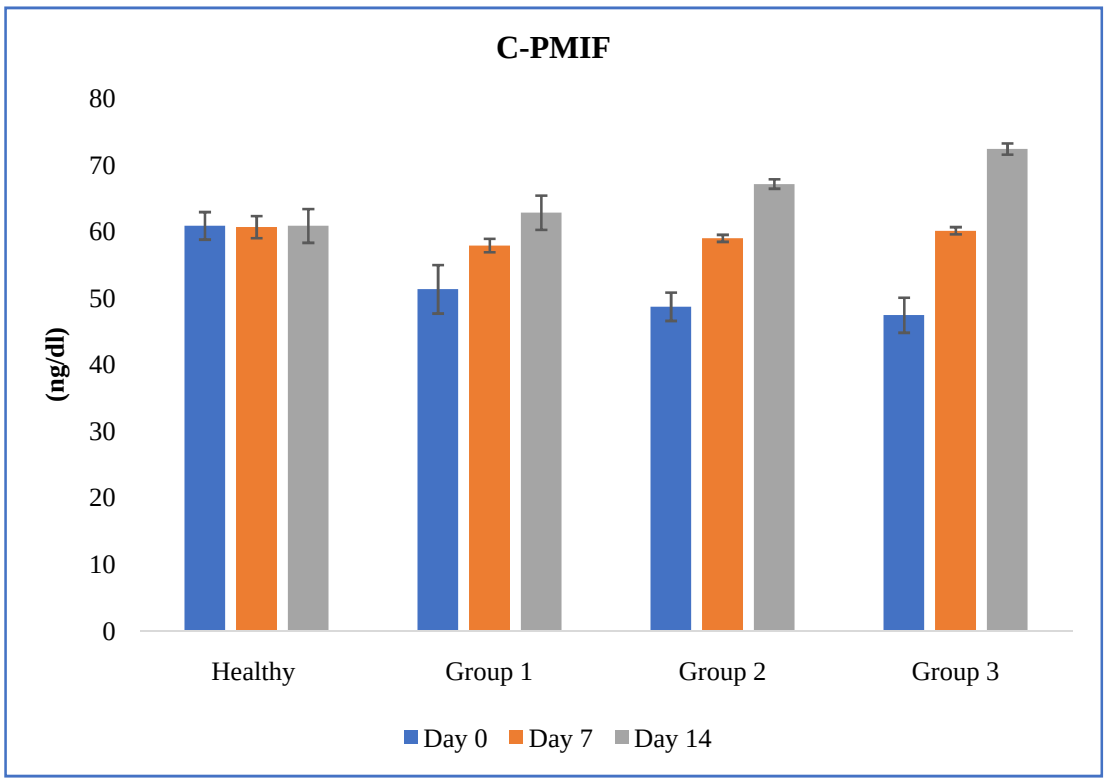
Graph 25: Showing levels of MPV in healthy dogs and dogs with CME



Graph 26: Showing levels of PDW in healthy dogs and dogs with CME



Graph 27: Showing levels of PCT in healthy dogs and dogs with CME



Graph 28: Showing levels of C-PMIF in healthy dogs and dogs with CME

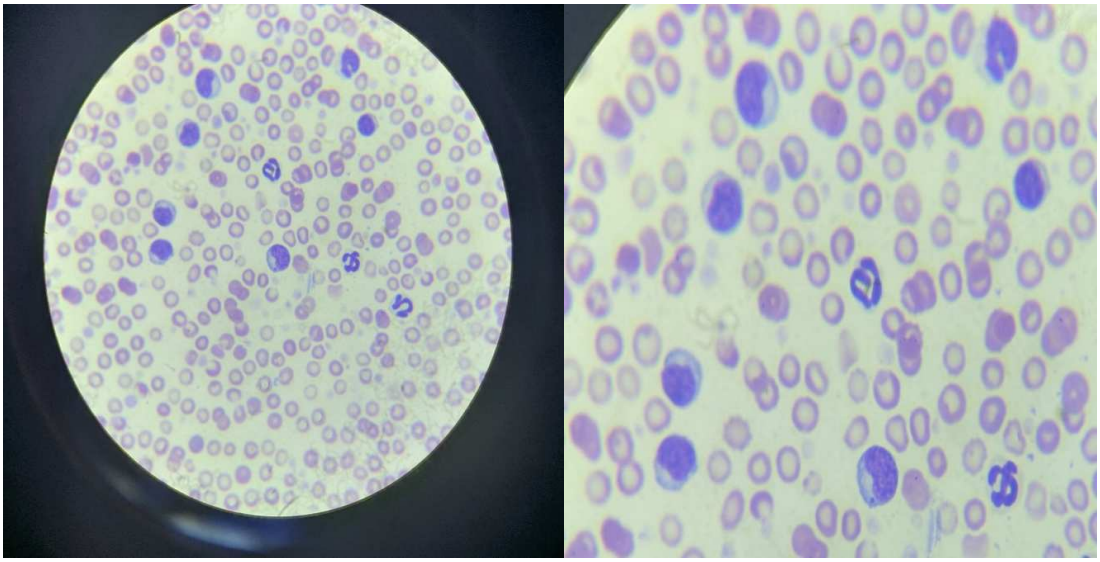


Fig. 9: Morulae in CME

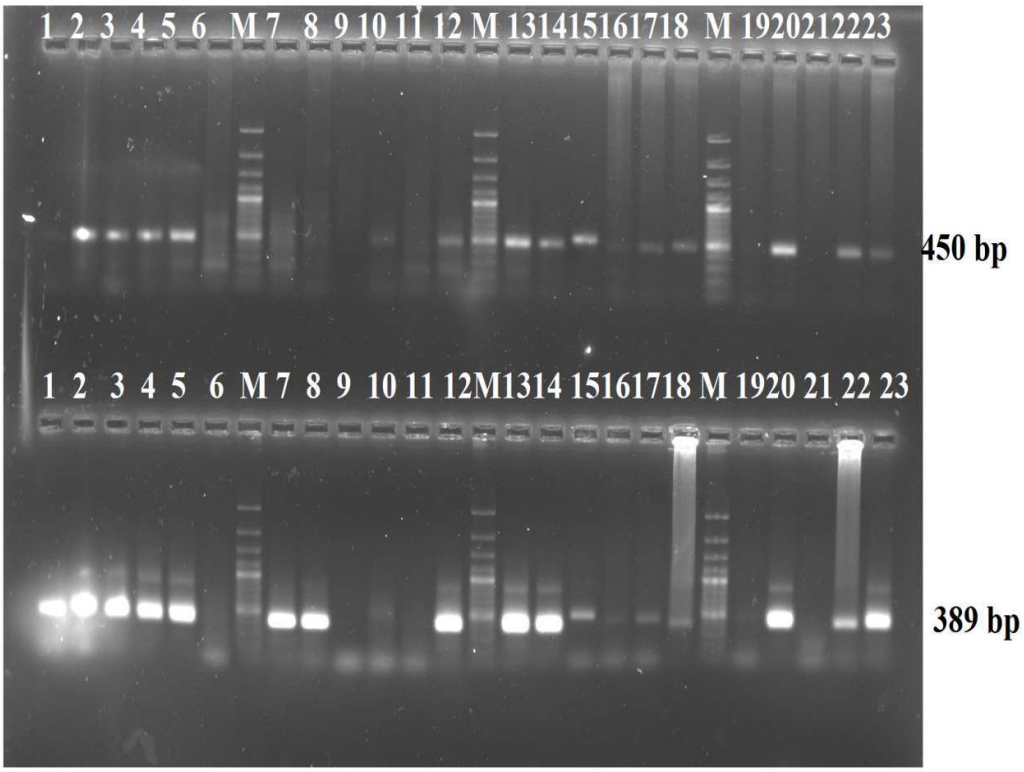


Fig. 10: PCR analysis on iBright 750 Imaging system

Fig. 11: Ascites in Labrador dog

Fig. 12: Pale gum mucosa in Labrador dog

Fig. 13: Epistaxis in GSD dog

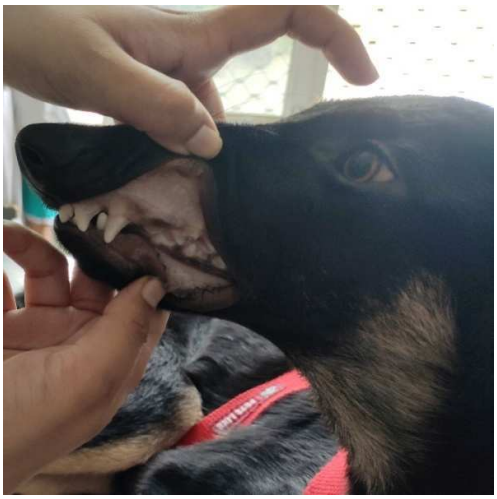


Fig. 14: Pale gum mucosa in non-descript dog

Fig. 15: Popliteal lymph adenomegaly in GSD dog

Fig. 16: Scrotal Oedema in Labrador

Fig. 17: Corneal opacity in Pug

Fig. 18: Hyphema in Pomerian

Fig. 19: Scleral bleeding in Pomerian

Fig. 20: Corneal ulcer in Pomerian

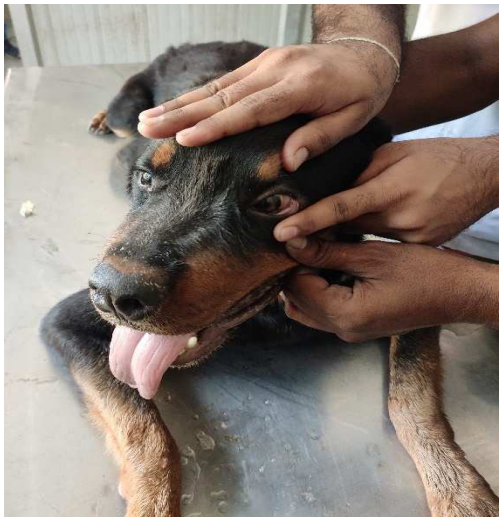


Fig. 21: Pale Mucus membrane in Rottweiler

Fig. 22: Pale Mucus membrane in non-descript dog

Discussion

CHAPTER-5

DISCUSSION

Present investigation entitled "Ameliorative Potential of Polyherbal Formulation on Thrombocytopenia in Dogs with Ehrlichiosis'' has recorded ehrlichiosis associated clinical, hematological, biochemical alterations, coagulation profile (prothrombin time and activated partial thromboplastin time), platelet indices and Canine specific platelet migration inhibition factor (C-PMIF) in dogs with clinical disease.

In present investigation total 62 dogs were screened for ehrlichiosis which was based on the clinical diagnosis criteria as stated earlier in materials and methods section of the thesis. All the screened dogs underwent for blood smear and buffy coat smear examination and hematology to confirm the state of clinical ehrlichiosis. Blood smear examination results showed 3 dogs positive for ehrlichiosis, while buffy coat smear examination results showed 6 dogs positive for ehrlichiosis. In the same cases, primary PCR revealed 18 positive dogs, however nested PCR confirmed 42 dogs positives for ehrlichiosis of all screened dogs. All the 42 nested PCR positive dogs were included for the therapeutic studies. Following parameters were investigated during study:

5.1. Clinician- intensive parameters

5.2. Diagnostic relevance of molecular diagnostic technique with traditional methods

5.3. Percentage positivity according to age, breed, sex and season

5.4. Hematological study

5.5. Biochemical study

5.6. Platelet indices

5.7. Coagulation profile

5.8. Canine specific platelet migration inhibition factor (C-PMIF)

5.1 Clinician-intensive parameters

5.1.1 Clinical signs and their frequency distribution in dogs

Clinical findings of high fever or pyrexia in 92.85% cases of ehrlichiosis affected dogs in the present investigation is in harmony with Ramakant et al. (2020), Petrov et al. (2018), Kottadamane et al. (2017), Chethan. (2016), Bhadesiya and Raval, (2015), Sainz et al. (2015), Chaudhary et al. (2015), Moonarmart. (2014), Das and Konar, (2013), Procajlo et al. (2011), Nakaghi et al. (2008). Pyrexia might be due to release of cytokine IL-1 in response to *E. canis* (Morais et al., 2010), immunological components like CD4 and CD8 T cells, natural killer (NK) cells, interferon-γ (IFN-γ), IL-12 and antibodies promote to control of *E. canis* infection (Saito and Walker, 2016). Even if the exact mechanism of pyrexia not studied in dogs in detail but obligate intracellular bacteria like Rickettsia and Chlamydia spp. have been carried to persuade production of inflammatory intermediaries such as TNF-a, interleukin-1 (IL-1) and/or prostaglandins by macrophages which might be responsible for fever accompanying with the disease.

Clinical findings of pallor of mucosal membranes in (88.02%) cases of ehrlichiosis affected dogs in present study is in agreement with Dhavalagi et al. (2021), Ramakant et al. (2020), Jubril et al. (2020), Tuna et al. (2019); Malik et al. (2018); Bhardwaj, 2013; Nakaghi et al. (2008); Castro et al. (2004); McQuiston et al. (2003). The paleness of the mucosal layers can be in straight relation with reduced levels of PCV, Hb and RBCs. The diversity of the prevalence of anemia may be allied with changes in strain pathogenicity or the phase of the disease (Tuna et al., 2019). An immunological mechanism may be intricate through production of antibodies and their consecutive binding on the membranes of erythrocytes which may result in devastation of these cells may leads to anemia (Taylor et al., 2007). Anemia in ehrlichial dogs might be due to bone marrow suppression and aplasia (Weiss, 2005), blood loss by ticks, extensive hemorrhages all over the body due to thrombocytopenia, immune mediated demolition of erythrocytes and platelet dysfunction (Harrus et al., 1996).

Clinical findings of lymph adenomegaly in 88.02% cases of ehrlichiosis affected dogs in present study is in agreement with Dhavalagi et al. (2021), Ramakant et al. (2020), Bai et al. (2017), Elitok and Ungur, (2016), Kitaa et al. (2014), Dixitet

al. (2012), Aysul et al. (2012) and Sacchini et al. (2007). During the acute phase of the disease, size of the lymph nodes increases in mainly due to hyperplastic activity of both B- and T- lymphocytes in response to stimulation by ehrlichial antigens (Harrus et al., 2011). The altered architecture of lymphopoietic tissue with plasmacytosis and a generalized perivascular lymphoid and plasma cell accumulation which seen histologically is a characteristic of CME infection (Nakaghi et al., 2008; Reardon and Pierce, 1981).

Clinical findings of melena in 83.33% cases of dogs with ehrlichiosis in present investigation is analogous to Mylonakis et al. (2019), Petrov et al. (2018), Parashar et al. (2016), Chaudhary et al. (2015), Parmar et al. (2013), Mathews and Ewing, (2006), Frank and Breitschwerdt, (1999). Ecchymotic and petechial hemorrhages in dogs with ehrlichiosis has been earlier noted by Arulanandam et al. (2020), Gupta et al. (2017), Yadav et al. (2017), Bhadesiya and Raval, (2015), Frank and Breitschwerdt, (1999).Observation of epistaxis in 21.42% dogs with ehrlichiosis is in agreement with Prabhavathy and Joseph, (2019), Dilipkumar (2018), Bai et al. (2017), Sivajothi et al. (2016), Pat-Nah et al. (2015), Bhardwaj, (2013), Baba et al. (2012), Nakaghi et al. (2008) and Mathews and Ewing, (2006). Findings like hematuria in (21.42 %) and hematemesis in 2.38% dogs with ehrlichiosis under present investigation are in accordance with Malik et al. (2018), Dilipkumar (2018), Moonarmart (2014), Parmar et al. (2013), Dhankar et al. (2011) and Mylonakis et al., (2004). Bleeding diathesis is more severe in the chronic phase of CME. It is presented typically as cutaneous and mucosal petechiae and ecchymoses, hyphema, epistaxis, hematuria, melena, prolonged bleeding from venipuncture sites or intraoperative bleeding (Elitok and Ungur, 2016, Lanza perea et al., 2014, Shaw et al., 2001). Rapid decrease in platelet count might be the reason for presence of petechial hemorrhages in dogs with ehrlichiosis. Bleeding disorders in canine ehrlichiosis is multifactorial in etiology and may include thrombocytopathia, thrombocytopenia and decrease in concentration of clotting factor in affected dogs. Bleeding tendencies namely epistaxis, melena, hematemesis, petechial and ecchymotic hemorrhages on oral gums and ventral abdomen are attributed to thrombocytopenia and damage to vascular endothelium due deposition of immune complexes on the vascular wall (Dhankar et al., 2011).

Clinical findings in ehrlichiosis affected dogs having scrotal edema/hind limb edema/facial edema in 7.14% of cases of ehrlichiosis in present study is in agreement with findings of Barrantes-González. (2016), Sarma, et al. (2010), Leiva et al. (2005), Mathews and Ewing, (2006). In the similar pattern, clinical finding of ascites in 16.66% dogs with ehrlichiosis in present study is almost similar to previous studies of Ramakant et al. (2020), Dilipkumar (2018) and Petrov et al. (2018). Due to hypoalbuminemia, increased plasma hydrostatic pressure and decrease in plasma oncotic pressure might be the reason of hind limb/scrotal edema. The hypoalbuminemia might be the consequence of peripheral loss of albumin to edematous inflammatory fluids as a result of increased vascular permeability (Woody and Hoskins, 1991), blood loss or reduced protein production due to coexisting liver disease as indicated by elevated liver enzymes. The pathological changes in the liver associated with disease embraces congestion of the sinusoids, severe steatosis, mild to moderate perivascular and periportal mononuclear cell infiltration which is suggestive of hepatic dysfunction leading to hypoproteinemia in ehrlichiosis (Agnihotri et al., 2012).

Clinical findings of tick infestation in 42.85% cases of ehrlichiosis affected dogs in present investigation is in accordance with Mylonakis et al. (2019), Bai et al. (2019), Yadav et al. (2017), Dantas Torres, (2010) and Trapp (2006). Clinical findings of Weight loss in 42.85% cases of ehrlichiosis affected dogs in present investigation is in accordance with Yadav et al. (2017), Barman et al. (2014), Agnihotri et al. (2012), Mathews and Ewing, (2006), Mylonakis et al. (2004), Dagnone et al. (2003) and McQuiston et al. (2003). Due to anorexia or inappetence during the whole disease process leads to catabolism of fats and protein fats which leads to weight loss and also severe dehydration might be responsible for weight loss in dogs with ehrlichiosis. Additionally, poor functioning of liver also reduces many biosynthetic processes, which may promote to weight loss. Some reports have suggested the role of increase level of inflammatory mediators such as TNF alpha may be causative of inappetence (Freeman, 2012).

Clinical findings of presence of ocular abnormalities like corneal opacity, corneal ulcer, hyphema and scleral bleeding in 11.90% cases of dogs with ehrlichiosis in present study is in harmony with Piso et al. (2021), Singh, 2019, Chochlios. (2019), Mylonakis. (2019), Saeng-Chuto. (2016), Walser-Reinhardt (2012), Leiva et al.

(2005), Gould et al. (2000). In their study, Panciera et al. (2001) recorded that dog mostly in the acute stage developed ocular lesions, however some dogs in the subclinical phase may also show ocular lesions. Singh, (2019) reported unilateral corneal opacity in 25%, bilateral corneal opacity in 8.33%, uveitis with corneal ulcer in 16.66%, conjunctivitis in 16.66%, corneal ulcer in 8.33%, scleral bleeding in 8.3%, dermoid growth over cornea in 8.33% and corneal opacity with glaucoma in 8.33% of the affected cases. The reason for most of the ocular abnormalities in Ehrlichia positive dogs is commonly related with thrombocytopenia and widespread immunecomplex formation/deposition in the eye (Komnenou et al., 2007). In the chronic form of the disease not only due to severe thrombocytopenia, retinal hemorrhages, hyphema and conjunctival (Glaze and Gaunt, 1986) but also frequent hemorrhages may induce secondary retinal detachment may be seen in affected dogs (Evermann, 1998). Ocular lesions described to occur in almost each structure of the eye. The common ocular lesions which were earlier recognized consist of corneal ulceration, decreased tear production, necrotic scleritis and orbital cellulitis. Ocular discharge, conjunctivitis, blepharitis, corneal ulceration, painful necrotic scleritis, secondary glaucoma and retinal hemorrhage and/or detachment leading to blindness have also been identified (Komnenou et al., 2007; Leiva et al., 2005). Hypergammaglobulinemia observed with *E. canis* infected dogs which in most cases is polyclonal, however in some dogs developed advance paraproteinemia characterized by monoclonal gammopathy (Harrus et al., 1998a). Monoclonal gammopathy and hypoalbuminemia have been seen in canine ehrlichiosis, even though polyclonal gammopathy followed by hypoalbuminemia are more commonly found (Michels et al., 1995). Platelet dysfunction can also subsidize to ocular hemorrhages, when a high concentration of paraproteins is present. The clinical manifestations of monoclonal gammopathy such as hemorrhages and hyper viscosity syndrome are most common. The hyper viscosity, secondary ischemic alterations and elevation of oncotic pressure could cause a hypertensive retinopathy and hyphema. The deposition of immune complexes on the vascular wall and vasculitis which is seen as a lymphoplasmacytic perivascular cuffing. Due to increase oncotic pressure, vasculitis as well as thrombocytopenia and platelet dysfunction can cause an ocular hemorrhage in affected dogs (Harrus et al., 1998a).

5.1.2. Physiological parameters

Rectal temperature, heart rate and respiration rate were noted in healthy and Ehrlichial dogs. Test of significance presented that substantial increase in rectal temperature, heart rate and respiration rate in Ehrlichia positive dogs as related to dogs of control group on the day of presentation.

Similar findings of increase in rectal temperature in ehrlichiosis affected dogs were earlier noted by Ramakant et al. (2020), Petrov et al. (2018), Chethan et al. (2016), Chaudhary et al. (2015), Moonarmart (2014), Das and Konar, (2013), Procajlo et al. (2011), Nakaghi et al. (2008). Similar to present findings of increase in respiration rate has been previously documented by Singh (2019), Petrov et al. (2018) and Yadav et al. (2017). Findings of increase heart rate is in pact with findings of Chandran et al. (2021), Maheshwarappa et al. (2020), Gianfranchesco Filippi et al. (2019). In contrast to findings of present search non-significant alteration in heart rate and respiration rate in Ehrlichia positive dogs were earlier recorded by Kottadamane et al. (2017).

Obligate intracellular bacteria such as Chlamydia and Rickettsia spp. have been reported to uplift the production of inflammatory mediators such as interleukin-1(IL-1), TNF-a and/or prostaglandins by macrophages which might be responsible for tachycardia, fever and respiratory distress correlated with CME (Saito and Walker, 2016; Van Heeckeren et al., 1993). Fever detected in all diseased dogs may have been stimulated by increased production of interleukin-1 (IL-1) by antigen presenting cells and B cells or exogenous pyrogen products of the parasite (Gershwin et al., 1995).

5.2. Diagnostic relevance of traditional technique with molecular diagnostic technique

Diagnostic significance of traditional technique was associated with molecular diagnostic technique on 62 samples suspected for ehrlichiosis investigated under present investigation. Leishman-stained thin blood smear examination reported presence of *E.canis* in 3 samples showing a positivity of 4.8 %. Leishman-stained buffy coat smear examination reported the presence of *E. canis* in 6 samples showing a positivity of 14.28 %. Analysis of 62 samples reported the presence of *E.canis* infection in 18 samples in primary PCR or simple PCR showing diagnostic relevance of 42.85 %, while 42 (67.74%) samples were found positive by nested PCR/secondary

PCR. Due to non-specific clinical signs of *E. canis*, diagnosis was difficult in dogs with ehrlichiosis (Makino et al., 2015). *E. canis* has been notoriously difficult to diagnose based on the traditional methods (Nazari et al., 2013). Hence, the diagnosis of ehrlichiosis is mostly made on the basis of the presenting clinical signs and the presence of a thrombocytopenia (Evermann, 1998) together with recognition of antibodies using serological techniques and molecular study using PCR (Tanikawa et al., 2013).

Demonstration of *Ehrlichia spp.* morulae in monocytes, macrophages and lymphocytes in Romanowsky-type stain smears from buffy coat and less commonly lymph node, spleen, liver, bone marrow and cerebrospinal fluid smears is supportive in establishing ultimate diagnosis of acute CME (Singh., 2019; Mylonakis et al., 2019; Harrus et al., 2012; Faria et al., 2010). Blood smears of infected dogs may encompass reactive monocytes, erythrophagocytosis, thrombophagocytosis, phagocytosis of nuclear material and mega platelets. But red blood cell morphology is unremarkable in most cases (Harrus et al., 1997). Large granular lymphocytosis may be present in progressive stages. A previous report has documented lymphocytosis. Approximately 50% of 200 lymphocytes evaluated in Wright-stained blood smears had prominent azurophilic granules and the cell nuclei were often slashed or indented with mature nuclear chromatin reliable with large granular lymphocytes (Heeb et al., 2003). In a study with dogs naturally infected by *E. canis* (presumptive acute CME), the diagnostic sensitivity of buffy coat (review of 1000 oil immersion fields, 100x objective lens), lymph node (500 oil immersion fields, 100x objective lens) or their combination was 66%, 61% and 74%, respectively. In another study, diagnostic sensitivity of spleen cytology in dogs naturally by *E. canis* was 49% (Faria et al., 2010). Cytology is a labor-intensive examination even in the acute phase of the disease (less than 1% infected mononuclear cells) and/or almost unresponsive in the subclinical and chronic CME and its specificity is unfavorably exaggerated by the inability to detect the intricate Ehrlichia species and the fact that extraneous material such as phagocytosed platelets or nuclear remnants and lymphocytic azurophilic granules may emulate morulae (Mylonakis et al., 2004). The blood sample is easily obtainable and provides good results (Santos et al., 2013; Silva et al., 2012) then introduction of molecular techniques for the detection of *E. canis* has tolerable for the rapid, sensitive and specific diagnosis of acute and chronic phases of the disease.

Serology is currently the key stay for the confirmation of exposure to *E. canis*. Polymerase chain reaction (PCR) may devastate several diagnostic limitations of serology (confirmation of exposure rather than current infection) and cytology (overall low diagnostic sensitivity). PCR and sequencing are delicate methods for sensing and characterizing *E. canis* DNA. False positive outcomes may occur with comparatively low annealing temperatures, when contaminants are present or when non-specific amplification happens. A negative PCR result exemplifies that no target DNA was distinguished, but does not necessarily show that no DNA was present in the sample (false negative result) (Iqbal et al., 1994). It is a highly sensitive method for the early detection (usually 4-10 days post-inoculation, molecular characterization and quantification (real-time PCR) of the ehrlichial organisms. Several assays are created on different target genes (e.g., *16S rRNA, p28, p30, dsb, VirB9*), though the *16S rRNA* and *p30*-based PCR assays are most usually used. The *p30*-based nested PCR assay has been revealed to be more subtle than the *16S rRNA*-based nested PCR assay. The fact that *E. canis* contains several copies of the *p30* gene, but only single copy of the *16S rRNA* gene, which surges the chances for recognition when *p30* is targeted (Stitch et al., 2002). Successful amplification of ehrlichial DNA may be obtained from several tissues with whole blood, lymph nodes, bone marrow, spleen, lungs, liver, kidney and cerebrospinal fluid. If blood or other tissues are not available, PCR may also be applied in residual serum samples (Sainz et al., 2015; Mylonakis et al., 2009; Baneth et al., 2009; Stich et al., 2002).

The nested PCR assay standardized for the recognition of 16S rRNA gene fragment of *Ehrlichia canis* from the blood of dogs for the prime time in India. The genus-specific primers amplified a 450 bp band of Ehrlichia spp. in the first PCR. The nested PCR assay via species-specific primers produced a 389 bp band of *E. canis*. The nested PCR noticed *E. canis* organisms in 50% of samples as in contradiction of the routine blood smear examination which discovered morulae in only 4.8 % of samples while buffy coat smear examination discovered morulae in only 14.28 % of samples. Specificity, sensitivity, rapidity, ease of use and the ability to evaluate a large number of samples at the same time makes nested PCR a superior option for recognition of initial as well as insistent canine ehrlichiosis (Lakshmanan et al., 2007). The nested PCR is highly specific and sensitive for the recognition of *E. canis* and it may be helpful for laboratory diagnosis and assessment of the efficiency of

antibiotic therapy for *E. canis* infection especially in combination with the Indirect florescent antibody test. It can offer a more consistent, yet rapid diagnosis of canine ehrlichiosis (Wen et al., 1997). The nested PCR is extremely sensitive. It could detect as little as 0.2 pg of purified *E. canis* DNA, which was as sensitive as Southern hybridization and 100 times more sensitive than one-step PCR, which could sense up to 20 pg of *E. canis* DNA (Iqbal et al., 1994). The blood samples of 214 dogs estimated for the presence of *Ehrlichia canis* using PCR-based assays detecting a portion of the 16S rRNA gene. Of the total samples subjected to tedious blood smear examination, the morulae of *E. canis* were detected in 2.34% samples and nested PCR assay formed amplicons of expected size (389 bp) exact for *E. canis* in 41.59% (89/214) of samples (Milanjeet et al., 2014). A total of 60 dogs alleged for CME were evaluated for detection of *E. canis* in blood by nested PCR assay targeting a portion of 16S rRNA gene. Consequently, the percent positivity for the recognition of *E. canis* by nested PCR was estimated 30% (18/60) as compared to 8.33% (5/60) by blood smear examination. Five dogs which were positive on peripheral blood smear examination were also found positive by nested PCR (Bai et al., 2017).

5.3. Percent positivity on the basis of season, breed, age and sex

In the present investigation overall occurrence of ehrlichiosis was more in the summers which in concord with Ramakant et al. (2020), Senthil et al. (2020), Selim et al. (2020), Singh et al. (2019), Milanjeet et al. (2014), Eljadar, (2010), Green. (2006). In conflicting to the present findings Kottadamane et al. (2017) detected more prevalence of ehrlichiosis in rainy season. The high ambient temperature might be conducive for breeding and hence augmented activity of the vector tick, *Riphicephalus sanguineous* which increases the incidence of canine ehrlichiosis. The variations in the prevalence could also be due to the geographical region considered. The presence and abundance of the vector *Rhipicephalus sanguineus* in the region which was studied by Soulsby, 2006.

In the present investigation overall occurrence of ehrlichiosis was higher in the German shepherd breed which in concord with the findings of Dhavalagi et al. (2021), Frezoulis et al. (2017), Bhadesiya and Raval, (2015), Milanjeet et al. (2014), Dhankar et al. (2011), Lakshmanan et al. (2007). In conflicting to present finding recorded significantly higher prevalence of ehrlichiosis in Labrador breed of dog, this may be

due to more popularity/habituation of this breed in the region (Bai et al., 2019; Kottadamane et al., 2017). German shepherd breed of dog is reported to be more susceptible for canine ehrlichiosis, which may be due to the imperfect cell-mediated immune response (Dhankar et al., 2011) and/or also due to genetic susceptibility of GSD breed (Salib and Farghali, 2015).

In the present study occurrence of ehrlichiosis was higher in adult dogs with more than 1 years of age. The findings in this regard are in accordance with the findings of Bai et al. (2019), Piratae et al. (2019), Malik et al. (2018), Parmar et al. (2013). In conflicting to present finding, significantly higher prevalence of ehrlichiosis in dogs less than 1 year of age was recorded previously (Milanjeet et al., 2014; Chandrasekar et al., 2002). However, one study showed that there is no correlation between age groups for occurrence of canine ehrlichiosis (Rahman et al., 2010).

In the present study, higher sex-wise occurrence was recorded in male sex as compared to female, which is in contrary with Bhadesiya and Raval (2015). The present findings of sex wise occurrence of ehrlichiosis is in agreement with the findings of Dhavalagi (2021), Bai et al. (2019), Piratae et al. (2019), Guedes et al. (2015), Yuasa et al. (2012) and Manigandan et al. (2003). However, some previous study showed that there is no correlation between occurrence of ehrlichiosis and sex of animal (Selim et al., 2020; Rahman et al., 2010; Bindu et al., 2006 and Rodriguez et al., 2005).

5.4. Haemato-biochemical study

5.4.1. Hematological study

Present study had revealed significant decrease in total erythrocyte count, hemoglobin concentration, packed cell volume and total leucocyte count in all treatment groups (Group 1, Group 2 & Group 3) in comparison with the Control group on day of presentation. There was significant increase in total erythrocyte count, hemoglobin concentration, packed cell volume and total leucocyte count in all treatment groups (Group 1, Group 2 & Group 3) on the day 7^{th} and day 14^{th} after the treatment in all the treatment groups with highest recovery seen in group 3. Therefore, in terms of improvement in all treatment groups of dogs, best recovery was seen in group which was treated with conventional treatment combined with polyherbal

platelet booster containing extracts of *Carica papaya, Tinospora cordiofolia, Withinia somnifera, Asparagus racemosus*.

The present findings of decrease in total erythrocyte count, hemoglobin concentration, packed cell volume and total leucocyte count in cases of ehrlichiosis are in agreement with the findings of Tuna et al. (2019), Mylonakis et al. (2019), Singh, 2019, Petrov et al. (2018), Kasondra et al. (2017), Yadav et al. (2017), Castro et al. (2004) and Mylonakis et al. (2004). Anemia accompanying with ehrlichiosis affected dogs might be due to bone marrow aplasia (Weiss, 2005), extensive hemorrhages throughout the body which is due to thrombocytopenia and platelet dysfunction, blood loss by ticks, immune mediated devastation of erythrocytes through production of antibodies and their succeeding binding on the membranes of erythrocytes and platelets which may result in obliterations of these cells (Taylor et al., 2007). Phagocytosis of antibody-opsonized erythrocytes has been reported by Woody and Hoskins, (1991) in *E. canis* infected animals which might be a factor conducive to anemia.

Bone marrow dysfunctioning in ehrlichial dogs is due to tropism for hematopoietic cells and bone marrow hypoplasia such as suppression in myeloid, erythroid and megakaryocytic cells. Non regenerative anemia is a consequence of liberal replication of *E.canis* in the bone marrow and inhibition of outcome of colony forming units in the process of erythropoiesis and megakaryopoiesis (Abeygunavardena et al., 1990). Generalized vasculitis (Pick et al., 2000) has been testified in *E. canis* infections accompanying to production of IL-1 which may play a vital role in marginalization and adhesion of leucocytes to the vascular wall and subsequently accumulation of cells in inflammation foci (Cotran et al., 1989).

There was a significant increase in neutrophil (%) on day 0 in all treatment groups (Group 1, Group 2 & Group 3) in comparison with the control group. There was significant decrease in the neutrophils (%) on the day 7^{th} and day 14^{th} after the treatment in all the treatment groups with highest recovery seen in all the treatment groups. The present findings of increase in neutrophils (%) in cases of ehrlichiosis are in agreement with the findings of many earlier studies (Yadav et al., 2017; Kottadamane et al., 2016; De Caprariis et al., 2011; Mulla, 2007; Castro et al., 2004). However, Osathanon et al., 2013, Dixit et al., 2012, Mylonakis et al., 2011, Sacchini

et al., 2007 reported significant decrease in neutrophils % in dogs with ehrlichiosis on the day of presentation. Neutrophilia in canine ehrlichiosis may denote underlying co-infection (Agnihotri et al., 2012). Neutrophils produce a trypsin-like enzyme by which they digest dead tissue and bacteria, so phagocytic action of neutrophils may thus be correlated with their increased number (Yadav et al., 2017).

There was a significant decline in lymphocyte (%) on day 0 in all the treatment groups (Group 1, Group 2 & Group 3) in comparison with the Control group. There was significant increase in lymphocyte (%) on the day 7^{th} and day 14^{th} after the treatment in all the treatment groups with highest recovery seen in all the treatment groups. The present findings of decrease in lymphocyte (%) in cases of ehrlichiosis are in pact with the findings of many earlier studies (Aguiar et al., 2020; Kottadamane et al., 2017; Yadav et al., 2017; Gianopoulos et al., 2016; Castro et al., 2004; Mylonakis et al., 2004; Oliveira et al., 2000). However, Parmar et al. (2013) and Dixit et al. (2012), Heeb et al. (2003) reported significant increase in lymphocytes (%) in dogs with ehrlichiosis on day 0. The observed decrease in the lymphocyte (%) could be a result of equivalent increase of neutrophils count during the infection. Lymphocytosis in dogs was from an increase of T lymphocyte counts (Lorente et al., 2008).

A non significant change in monocyte (%) and eosinophil (%) on day 0 in all the treatment groups (Group 1, Group 2 & Group 3) of dogs in comparison with the control. There was non-significant change in the monocytes (%) and eosinophil % on day 7^{th} and day14^{th} after treatment in all the treatment groups. Similar observations of insignificant change in the monocytes were reported by Kottadamane et al. (2017); Yadav et al. (2017), Badesiya and Raval, (2015) and Moonarmart et al. (2014). In conflicting to present findings, Oliveira, (2000) reported significant decrease in levels of monocytes in CME.

A non-significant change in eosinophil (%) on day 0 in all the treatment groups (Group 1, Group 2 & Group 3) of dogs in comparison with the control. There was non-significant change in the eosinophil % on day 7^{th} and day14^{th} after treatment in all the treatment groups. The non-significant change in the eosinophil were reported by Kottadamane et al. (2017); Silva et al. (2016) and Castro et al. (2004). In

conflicting to present findings, Moonarmart et al. (2014) reported significant decrease in levels of eosinophil in CME.

There was a non-significant change in MCV, MCH and MCHC on day 0 in all the treatment group (Group1, Group 2 & Group 3) of dogs in comparison with the control. There was no significant change in the MCV, MCH and MCHC on day 7 and day 14 post-treatment in all the treatment group. The present findings of insignificant change in MCV, MCH and MCHC in cases of ehrlichiosis are in pact with the findings of many prior studies conducted by Thriunavukkarasu et al. (1994), representing normocytic and normochromic anemia. Similar findings of insignificant change in the mean MCV and MCH (Thongsahuan et al., 2020; Badesiya and Raval, 2015; Chaudhary et al., 2015; Seamer and Snape, 1972), have been previously reported. The significant decrease in levels of MCH in dogs with ehrlichiosis (Mulla, 2007). The significant decrease in levels of MCH and MCHC and large MCV in dogs with ehrlichiosis (Tsachev et al., 2013).

5.4.2. Biochemical study

5.4.2.1. Serum ALT, AST and ALP

In present study, there was significant increase in ALT, AST, ALP on day 0 in all treatment groups (Group1, Group 2 & Group 3) of dogs in comparison with control. There was significant decrease in the ALT, AST, ALP on day 7^{th} and 14^{th} after the treatment in all the treatment groups.

The present findings of increase in ALT in cases of ehrlichiosis are in agreement with the findings of many previous studies (Jubrill et al., 2020; Bai et al., 2017; Yadav et al., 2017; Bhadesiya and Raval, 2015; Salem et al., 2014; Srikala et al., 2012; Sasanelli et al., 2009; Niwetpathomwat et al., 2005) while increase in AST in dogs of ehrlichiosis are in agreement with the findings of many previous studies (Jubrill et al., 2020; Bai et al., 2017; Morar et al., 2015; Bhardwaj, 2013; Agnihotri et al., 2012; Shipov et al., 2005). The present findings of increase in ALP in cases of ehrlichiosis are in pact with the findings of (Bai et al., 2017; Mann et al., 2017; Kottadamane et al., 2016; Morar et al., 2015; Agnihotri et al., 2012; Shipov et al., 2005).

ALT is a liver specific enzyme in dogs (Solter et al., 1994); an increase may follow hepatic necrosis or milder revocable impairment in which hepatocytes become

leaky but do not die. Augmented levels of SGPT and SGOT are indicative of hepatic dysfunction leading to hypoproteinemia in dogs with ehrlichiosis (Agnihotri et al., 2012; Niwetpathomwat et al., 2005; Premila, 2005). Liver pathology accompanying with experimental *E. canis* infection without overt clinical disease has been documented as a portal infiltration of lymphocytes, plasma cells and macrophages resulting in marked distortion of the surrounding acinar architecture. In another experimental study, centrilobular fatty degeneration and mild to modest perivascular and periportal mononuclear cell infiltration, severe steatosis and congestion of the sinusoids were described (Reardon. and Pierce, 1981).

5.4.2.2. Serum total protein, albumin and globulin:

In present study there was significant decrease in total protein on day 0 in all treatment groups (Group 1, Group 2 & Group 3) of dogs in comparison with control. There was significant increase in the total protein on day 7^{th} and day 14^{th} after the treatment in all the treatment groups. The present findings of decrease in total protein in cases of ehrlichiosis are in agreement with the findings of many earlier studies (Arulanandam et al., 2020; Syaputra et al., 2020; Bai et al., 2017; Rungsipipat et al., 2014; Srikala et al., 2012; Srivastava and Srivastava, 2011, Castro et al., 2004). In conflicting to our findings (Smitha and Vijayakumar, 2014, Irwin, 2007; Mylonakis et al., 2004; Harrus et al., 1997; Varela et al., 1997; Harrus et al., 1996; Weiser et al., 1991) recorded significant increase in the level of total protein in dogs with CME.

Present study showed significant decrease in albumin on day 0 in all treatment groups (Group1, Group 2 & Group 3) of dogs in comparison with control. There was significant increase in the albumin on day 7^{th} and day14^{th} after the treatment in all the treatment groups. The present findings of decrease in albumin in cases of ehrlichiosis are in pact with the findings of (Maheshwarappa et al., 2020; Kottadamane et al., 2017; Silva et al., 2016; Salem et al., 2014; Smitha and Vijayakumar, 2014; Mylonakis et al., 2004; Codner and Maslin, 1992; Woody and Hoskins, 1991; Breitschwerdt et al., 1987).

Test of significance showed that the globulin of all the treatment groups were significantly lower than that of control group on the day of presentation. However, there was no significant change in the serum globulin on day 7 and day 14 post-treatment in all the treatment groups. On conflicting many previous studies noted

significant increase in globulin levels in dogs with ehrlichiosis (Silva et al., 2016; Parmar et al., 2013; Akhtardanesh et al., 2010; Irwin, 2007; Perille and Matus, 1991; Greene et al., 1985).

The hypoalbuminemia in CME might be the consequence of peripheral loss of albumin to edematous inflammatory fluids as a consequence of increased vascular permeability (Woody and Hoskins 1991), blood loss or diminished protein production due to concurrent liver disease, hyperglobulinemia-related down-regulation of albumin synthesis, intralobular necrosis secondary to hemorrhage, anemic hypoxia or secondary bacterial sepsis (Harrus et al.,1999).

5.4.2.3. Serum blood urea nitrogen (BUN) and Creatinine

Present investigation showed significant increase in BUN in all treatment groups (Group 1, Group 2 & Group 3) in comparison with control. There was significant decrease in the BUN on day 7^{th} and day 14^{th} after treatment in all the treatment groups. The present findings of increase in BUN in cases of ehrlichiosis are in pact with the findings of many earlier investigations (Bai et al., 2020; Priyanka et al., 2019; Behera et al., 2017; Mann et al., 2017; Silva et al., 2016; Agnihotri et al., 2012; Dixit et al., 2012; Mulla, 2007). However, many previous studies recorded insignificant change in BUN levels in dogs with ehrlichiosis (Kottadamane et al., 2017; Kottadamane et al., 2016). BUN increase occurs not only in renal diseases (including prerenal azotemia, renal azotemia and postrenal azotemia) but also in protein catabolism and a high-protein diet (Niwetpathomwat et al., 2005).

In present study, there was insignificant change in serum creatinine in all the treatment groups (Group 1, Group 2 & Group 3) of dogs in comparison with control. There was no substantial change in the serum creatinine (mg/dl) on day 7^{th} and day 14^{th} post treatment in all the treatment groups. These findings were in pact with the Agnihotri et al. (2012), Srivastava and Srivastava, (2011).

The presence of inflammatory infiltrates rich in lymphocytes might be responsible for immuno-pathogenesis of renal lesion in dogs with CME. Due to membrano-proliferative glomerulopathy, interstitial nephritis and may also be due to glomerulonephritis with or without deposition of immune-complexes (Agnihotri et al., 2012; Frank and Breitschwerdt, 1999), which raises the urea and creatinine level in body.

5.5. Platelet indices

Present study shows significant decrease in platelet count in all treatment groups (Group 1, Group 2 & Group 3) in comparison with the control group. There was significant rise in platelet count in all treatment groups (Group 1, Group 2 & Group 3) on day 7^{th} and 14^{th} after the treatment in all the treatment groups with highest retrieval seen in group 3.

Present observations in favor to platelet count reduction in canine ehrlichiosis are in harmony with previous study where thrombocytopenia has been recorded as dominating change (Singh, 2019; Tuna et al., 2019; Mylonakis et al., 2019; Ansari-Mood et al., 2015; Tuna et al., 2009; Kakoma et al., 1978).

Thrombocytopenia is considered to be the most common and reliable hematological abnormality of dogs naturally or experimentally ill with *E. canis* (Waner et al., 1995). The mechanism leading to thrombocytopenia is not rather clear while the main role looks to be played by autoimmunological processes (Herring and Mc Michael, 2012), decreased production (chronic phase) (Harrus et al., 1997; Weisiger et al., 1975), increased platelet consumption (Bulla et al., 2004), splenic sequestration (Lakkawar et al., 2003), intravascular disseminated coagulopathy (Dubie et al., 2014), decreased circulating half-life of platelets during acute phase of infection (Smith et al., 1975), reduced adhesiveness of platelets due to antiplatelet antibody and platelet aggregation (Kuehn and Gaunt, 1985), augmented concentrations of circulating platelet migration-inhibition factor (Abeygunawardena et al., 1990). Throughout, the *E. canis* infection antibodies against glycoprotein of the dog's own platelets are produced which leads to their malfunction (Burghen et al., 1971). Platelet survival time decreased from a mean of 9 days to 4 days, within 2- 4 days of infection with *E. canis* (Smith et al., 1975). A platelet migration inhibition factor is also estimated to play role in enhancing platelet sequestration and stasis, primary to reduced peripheral blood platelet counts (Abeygunawardena et al., 1990).

Recently new indices related to platelet have been provided by hematologic investigates namely plateletcrit (PCT), mean platelet volume (MPV) and platelet distribution width (PDW) to detect platelet abnormalities in dogs affected with thrombocytopenia, although quantification of platelets in peripheral blood is a well-recognized tool in veterinary diagnostics (Baranidharan et al., 2017).

MPV designate a marker of platelet function and activation (Kaito et al., 2005 and Boomer et al., 2008) which is accompanying with indicators of platelet function including aggregation, release of thromboxane A2, platelet factor 4 and beta-thromboglobulin as power exchanges or imitate the level of platelets stimulation or the index platelets production. The (MPV) is the predictable size of platelets and is contrary-wise relative to the number of platelets (Feldman et al., 2000). When there is an increase in turnover and consequent lack of platelet production leads to rise of MPV. Within the normal ranges of platelet counts and MPVs, there is converse correlation between platelet count and MPV and a direct correlation between MPV and megakaryocyte ploidy (i.e., larger megakaryocyte) in dogs (Boudreaux and Ebbe, 1998). Hence an increased MPV could show active thrombopoiesis (Northern and Tvedten, 1992). The MPV has been stated to be increased in some dogs in response to thrombocytopenia due to large immature platelet production during regenerative process (Dircks et al., 2009) but normal or even diminished MPVs had been related with dogs and humans with immune-mediated thrombocytopenia (IMT) due to presence of platelet fragments (Northern and Tvedten, 1992) formed due to amended platelet utilization/destruction and also in inflammatory conditions that consequence in enhanced platelet utilization/demolition (Moritz et al., 2003) and this may be the possible reason for non-significant changes in the MPV in Ehrlichiosis positive dogs in present study. A declined MPV may be linked with early stages of immune-mediated thrombocytopenia. It is supposed that this drop could be presence of small platelets proposed that platelet indices may be of beneficial and related to the underlying causes of thrombocytopenia in dogs (Topper and Welles, 2003).

The present study revealed insignificant change in MPV, PDW in the dogs of treatment group in comparison to control. There was no significant change in the MPV and PDW on day 7^{th} and day 14^{th} after the treatment in all treatment group. These observations were similar to study of Baranidharan et al. (2017) and Bommer et al. (2008) who found non-significant changes in platelet indices recorded in dogs with ehrlichiosis, on the basis of their study they suggested that the platelet indices (MPV, PDW and PCT) did not have any diagnostic role in dogs with Ehrlichiosis.

PCT is the volume percentage that platelets match on a total volume of blood and acts as indicator of platelets mass in the body due to these it is directly related to total number of platelets. As it is linked with number of platelets then significant drop

in PCT in dogs with ehrlichiosis in present study can be elucidated on the basis as of thrombocytopenia (Moraes, 2001).

The platelets distribution width (PDW) is the width of the curve of distribution of platelets linked to the divergent sizes produced by these cells and has been used to discriminate between immune mediated thrombocytopenia and thrombocytopenia in aplastic anemia but it is less understood in veterinary literature. Like MVP, when the PDW is not normal displays the occurrence of some disorder in the production of platelets (Moraes, 2001). Non-significant alteration in PDW can be elucidated on same basis as MPV in present study. PDW can rise due to small variations in platelet morphology before MPV values are different, similar to what occurs with the anisocytosis index RDW showing the greater sensitivity of PDW (Matos at al., 2008)

15.6. Coagulation profile

Present investigation revealed significant increase in APTT in all treatment groups (Group 1, Group 2 & Group 3) in comparison with control. There was significant decrease in the APTT on day 7^{th} and day 14^{th} after treatment in all the treatment groups. Analysis of PT showed non- significant changes in between treatment and control group. Similar to present observation, Pasa et al. (2017) previously recorded PT (sec) 9.1±0.07 (sec) and 9.1±0.20 (sec) in healthy and ehrlichiosis affected dogs, respectively and 12.7±1.1 (sec) and 23.1±9.2 APTT (sec), in healthy and ehrlichiosis affected dogs, respectively.

PT and APTT are frequently used coagulation biomarkers for interpretation of dogs with bleeding tendencies. In this test, term thromboplastin denotes to the foundation of a complex proposed from various plasma clotting factor which modifies prothrombin to thrombin and the uninterrupted formation of the fibrin clot. Partial thromboplastin time (PTT) record the inclusive speed at which blood clots by means of two incessant series of biochemical reactions identified as the intrinsic pathway and common pathway of coagulation. PTT record the VIII (anti-hemophilic factor), X (Stuart-Prower factor), XI (plasma thromboplastin antecedent) and XII (Hageman factor) mandatory in coagulation. Prolonged APTT and normal PT values could be due to shortage of coagulation factors VIII, IX and XI. However, the APTT can be normal with mild deficiencies of clotting factors. In general, deficient factor has to be less than 20-40% of normal before the APTT is prolonged. The prothrombin time is a

measure of the integrity of the extrinsic and final common pathways of the coagulation cascade. Prothrombin or factor II is clotting factors made by the liver. Vitamin K is needed to produce prothrombin and other clotting factors. Prothrombin time is central test because it checks to see if five different blood clotting factors (factors I, II, V, VII, and X) (Sumathi et al., 2012).

Similar to present observation in favor to PT and APTT, in many prior studies the significantly elevated APTT values were reported in dogs with ehrlichiosis and ehrlichiosis-leishmaniasis co infection (Cortese et al., 2006) in particular when escorted by liver dysfunctions lowering the synthesis of one or more coagulation factors. Possible reason for increased APPT in treatment group may be ehrlichiosis induced hepatopathy and consumption of clotting factors. Hence, bleeding disorders in canine ehrlichiosis seems to be multifactorial and may comprise thrombocytopenia, thrombocytopathy and reduction in concentration of clotting factor in affected dogs (Shipov et al., 2008).

The prolonged APTT in the presence of normal PT values could be either due to the lesser sensitivity of the commercial PT tests. When working on canine plasma accompanying with the greater sensitivity of APTT reagents in representing coagulation factor deficiencies or to a faster consumption of one or more of the intrinsic pathways of clotting factors which are working as activators in the formation of inflammatory chemical mediators (Jimenez and Fernandez, 2000).

15.7. Canine specific serum platelet migration inhibition factor:

Platelet synthesis, storage, release and maturation involves complex series of events in which bone marrow, liver and spleen play significant role (Thon and Italiano, 2012). After its synthesis from megakaryocytes series platelet undergo maturation and sequestration event which are dependent on reticuloendothelial system of spleen and splenic sinusoids (Thon et al., 210; Hartwig and Italiano, 2002). Studies have shown involvement of multiple factors in the regulation of platelet release into circulation and their associated terminal events. The involvement of platelet migration element are crucial during platelet release, migration and activation (Abeygunavardena et al., 1990). However, conditions like canine ehrlichiosis exhibit aberrant platelet migration resulting in thrombocytopenia. Previously, the involvement of PMIF has been reported and evidently during canine ehrlichiosis the

affection of B-lymphocyte results in reduction of release of PMIF (Greene, 1995). During, clinical management of Ehrlichia, strategies are planned to enhance activity of platelets by reducing the activity of PMIF.

Present investigation showed significant decrease in PMIF in all treatment groups (Group 1, Group 2 & Group 3) on day 0 comparison with control. However, there was non-significant increase in PMIF in all treatment groups (Group1, Group 2 & Group 3) on day 7^{th} and day 14^{th} after treatment in all the treatment groups comparison with control. However, it was interesting to observe that platelet count was also increased. Conventionally there is contrasting to observe an increase in PMIF and increase in platelet count.

From the available literature it is evident that a platelet migration inhibitory factor produced by lymphocytes of *E. canis* -infected dogs contribute to the thrombocytopathia by inhibiting platelet pseudopod formation. Hyperglobulinemia may also interfere with platelet migration and adhesiveness. Antiplatelet antibodies have been demonstrated in dogs with experimentally induced ehrlichiosis (Greene, 1995).

The polyherbal formulation used during the study was not purified and was used in a definite form and there may be affecting different target system by which platelet count increasing even though PMIF increase. It is suggested that, the polyherbal which was used in the study may not directly affecting B-lymphocyte or PMIF or sequestration event in spleen. Rather, there is possibilities of affection of reticular system of both liver and spleen. The altered permeability and sequestration system of spleen may be affected by polyherbal group and thereby stasis and sequestration events of spleen are alters / increased. It is also possible that the polyherbal may be directly impacting red bone marrow or its associated system leading to enhanced terminal maturation and differentiation of megakaryocyte series into platelets. Platelet release from its terminal are also the targets for platelet circulation and activation; and possibly they may be also targeted by polyherbal. Further studies are warranted to investigate the mechanistic involvement of polyherbal with platelet activation and sequestration events.

5.7. Therapeutic study

The treatment regimen to increase the platelet count include the use of corticosteroids is in current practice but have their own serious side effects (Kohn et al., 2008). All these treatment modalities are costlier and increase the hospital stay and associated with increased risk of mortality and morbidity. In present study effect of self-made polyherbal platelet booster capsule on platelet count have been investigated along with standard treatment and compared with other treatment groups. Analysis showed highest recovery in the Group 3 on day 7^{th} and day 14^{th} after treatment. There was significant increase in the platelet count in all treatment the groups. Therefore, in terms of improvement in all the treatment groups of dogs, best recovery was seen in group 3 which was treated with conventional treatment combined with extract of *Carica papaya, Tinospora cordiofolia, Withinia somnifera and Asparagus racemosus*.

Carica papaya is rich source of flavonoids, papain, chymopapain, cystatin, tocopherol, ascorbic acid, glycosides, alkaloids, glutathione, cyanogenic glucosides and glucosinolates which not only acts as platelets booster by stimulating bone marrow and inhibiting immune mediated platelet destruction but also they have good anti-inflammatory activity which is proved in many research studies. This fastens the natural course of recovery with increasing the platelet count without any side effects (Gadhwal et al., 2016). Papaya leaf contains anti-oxidant, vitamins, minerals, cardiac glycosides and alkaloids which play an important role in curing of thrombocytopenia and also help to increase the hemoglobin, hematocrit, red blood cells and total protein contents (Saraf and Kavimandan, 2018). Papaya leaf extracts possess biological membrane stabilization properties preventing stress-induced destruction of the plasma membrane. Flavonoids and other phenolic compounds present in papaya leaf extracts were responsible for the observed membrane stabilizing property and thereby prevent the internal bleeding in the blood vessels (Ranasinghe et al., 2012). Papaya leaf juice significantly increases the platelet count (Benazir and Abhinayani, 2015. Senthilvel et al., 2013). Aqueous extract of *Carica papaya* leaf at concentrations of 400-800mg/kg body weight/day were originate to significantly increase the platelet count in cyclophosphamide induced rat model. The alkaloids present include carpaine, pseudocarpaine and dehydrocarpaine I and II which act on the bone marrow prevent its destruction and augment its ability to produce platelets (Yogiraj et al., 2014). It can

prevent platelet destruction in the blood and thereby increase the life of the platelet in circulation (Patil et al., 2013). Major gene that stimuli platelet production and platelet aggregation is arachidonate12-lipoxygenase (ALOX 12) also known as the platelet-type lipoxygenase and the platelet-activating factor receptor (PTAFR) (Joshi and Kaur, 2016). The active elements of *Carica papaya* up regulate the ALOX 12 and PTAFR gene thereby leading to an increased production of megakaryocytes and its conversion into platelets. *Carica papaya* leaves juice significantly hastens the rate of increase in platelet count among patients with dengue fever and dengue hemorrhagic fever (Yunita et al. 2012).

The oral administration of *T. cordifolia* extract to mice for 15 days significantly enhanced humoral immune response. *T. cordifolia* water extracts improved cellular immune functions (Saeed et al., 2020; Maurya et al., 1996; Thatte et al., 1994). *T. cordifolia* triggers macrophages to release GM-CSF activity, when given at the rate of 100 mg/ kg/day for 10 days. GM-CSF causes initiation of leukocytosis as well as prevention of cytotoxic chemotherapy tempted neutropenia (Burgess et al., 1977). The stem of *T. cordifolia* contain an alkaloid which is effective against anti-platelet macrophages in patients suffering from idiopathic thrombocytopenic purpura signifying the role of *T. cordifolia* in protecting the platelets from demolition (Chintalwar et al., 1999). It possibly acts by improving the production of platelets from bone marrow due to its antioxidant (Eberly et al., 2007), immunomodulatory properties and cytotoxic effects. They have been reported to function by boosting phagocytosis activity of macrophages, production ROS in human neutrophil cells, enhancement in nitric oxide production by stimulation of macrophages (Namdeo, 2021). This beneficial role of *T. cordifolia* can be attributed to the presence of various phytochemical constituents like alkaloids (berberine, palmatine D, choline D, tinosporine, magnoflorine, tetrahydropalmatine), sesquiterpenoid (tinocordifolin) and glycosides (furanoidditerpene glycoside, tinocordiside, syringing and sysingine-apiosyglycoside) present in the stem (Kapil and Sharma, 1997). Phenylpropanoid glycosides such as cordifolioside A, cordifolioside B and syringin, have been reported to be main immunomodulatory active components. D-glucan, isolated from the stem of the plant has been shown to stimulate the immune system (Maurya et al., 1996).

Withania somnifera and/ or Ashwagandha is used as a general tonic known as Medharasayana. It exhibits antimicrobial, anti-inflammatory, immunomodulation, anti-oxidative, hepatoprotective and life prolonging properties. Ashwagandha have profound effects on the hematopoietic system acting as an immunoregulator and a chemoprotective agent (Namdeo, 2021; Uddin et al., 2012). Several recent reports have demonstrated immunomodulator and antitumor effects of ashwagandha (Agarwal et al., 1999). Ashwagandha acts as an anti-inflammatory agent through inhibition of complement, lymphocyte proliferation and delayed-type hypersensitivity (Rasool and Varalakshmi, 2006). Additionally, as the plant is rich in iron, it contributes to red blood cell count as well (Pal et al., 2015). *W. somnifera* show an immuno-potentiating and myeloprotective effects know roots by enhancing the levels of interferon (IFN)-γ, interleukin (IL)-2 and granulocyte macrophage colony stimulating factor in normal and cyclophosphamide-treated mice (Davis and Kuttan, 1999; Ziauddin et al., 1996). The active compound (withanolide A) in the roots of *W. somnifera* significantly increases the expression levels of T-helper 1 (Th1) cytokines as well as CD4 and CD8 counts. It also augments natural killer (NK) cell activity in a dose dependent manner with a faster recovery of CD4+ T cells in immune suppressed animals (Malik et al., 2006; Davis and Kuttan, 2002).

Asparagus racemosus/Satavar, shatavari, or shatamull is an important medicinal herb belongs to family Asparagaceae (Choudhary and Sharma., 2014).The word Asparagus is derived from Greek and it denotes ‘stalk’ or ‘shoot’ (Goyal et al., 2003). The phytochemical analysis of *Asparagus racemosus* possess anti-anemic and anti-thrombocytopenic properties and these could be associated to the phytoconstituents such as alkaloids, saponins, phytosterols, triterpenoids, polyphenols, carbohydrate, flavonoids, glycosides, phenolic compounds and tannins present in the extract (Chaudhary et al., 2016). Alkaloids have been proven effective against anti-platelet macrophages in patients suffering from Idiopathic Thrombocytopenic Purpura (ITP) Ahn et al. (1978). Saponins are known to inhibit platelet adhesion and aggregation, prevent thrombosis and also improve microcirculation. Saponins have been successfully used in the management of liver inflammation as tonic sedative formulas to promote and vitalize blood circulation (Wang et al., 2004; Shi et al., 2004). Flavonoids have anti-anaemic potential and veinotonic properties which protects the blood capillaries (Yenon et al., 2015). The

major active constituents of *Asparagus racemosus* are steroidal saponins. Isoflavones, asparagamine, racemosol, polysaccharides, mucilage, vitamins A, B1, B2, C, E, Mg, P, Ca, Fe, and folic acid present in roots (Joshi, 2016). The alcoholic extract has been found to enhance both, humoral and cell mediated immunity of albino mice injected with sheep red blood cells as particulate antigen. Alcoholic extract of root of *A. racemosus* has been shown to significantly reduce the enhanced levels of alanine transaminase, aspartate transaminase and alkaline phosphatase in $CC1_4$-induced hepatic damage in rats indicating antihepatotoxic potential of *A. racemosus* (Muruganadan et al., 2000).

Summary and Conclusions

CHAPTER- 6

SUMMARY AND CONCLUSIONS

Present investigation entitled "Ameliorative Potential of Polyherbal Formulation on Thrombocytopenia in Dogs with Ehrlichiosis'' has recorded ehrlichiosis associated clinical, haematological, biochemical alterations, canine specific platelet migration inhibition factor, coagulation profile (Prothrombin time and Activated partial thromboplastin time) and platelet indices associated with the disease. In present investigation total 62 dogs were screened for ehrlichiosis which was based on the clinical diagnosis criteria as stated earlier. All the screened dogs underwent for blood smear, buffy coat smear and PCR examination to confirm the dogs suffering with ehrlichiosis. Blood smear examination results showed 3 dogs positive for ehrlichiosis; buffy coat smear results show 6 dogs positive for ehrlichiosis; primary PCR revealed 18 positive dogs, however nested PCR confirmed 42 dogs positive for ehrlichiosis of all screened dogs.

Canine ehrlichiosis are a group of tick-borne illnesses which was described for the first time in Algeria in 1935 by Donatien and Lestoquard. Later, in 1970 it gained extensive attention when a large no. of dogs mainly GSD died from disease during Vietnam war. It is caused by lipopolysaccharide deficient, obligatory intracellular gram-negative bacteria of the genus *Ehrlichia* and family Anaplasmataceae. *Ehrlichia canis* (*E. canis*) (CME) causes a possibly fatal disease in dogs. CME requires a rapid and accurate diagnosis in order to initiate appropriate therapy leading to a favourable prognosis. CME also called tropical canine pancytopenia and has got worldwide prevalence and is transmitted by the bite of infected brown dog tick *Rhipicephalus sanguineus* and maintained in wild life hosts (red fox, coyote and golden jackal) as well as in domestic dogs.

In the present study important clinical signs exhibited by the ehrlichial positive dogs with highest frequency were fever (92.85%) and mucosal pallor (88.02%) followed by lymph adenomegaly (88.02%), melena (83.33%) depression (78.57%), tick infestation (42.85%) and weight loss (42.85%). Clinical symptoms with moderate frequency were epistaxis (21.42%), ascites (16.66%), ecchymotic and petechial hemorrhages (14.58%), ocular abnormalities (11.90%), vomiting (9.52%), hind limb/scrotal oedema / facial oedema (7.14 %), icterus (4.76%). Symptoms with

least frequency were hematuria (2.38%), hematemesis (2.38%) and CNS signs (2.38%).

Average value (Mean ± SE) of rectal temperature (°F), heart rate (per min), respiration rate (per min) and CRT (seconds) in healthy dogs was 101.85 ± 0.54 (°F), 82.97 ± 0.24 (per min), 19.13 ± 0.14 (per min) and 1.73 ± 0.23 sec, respectively while in Ehrlichia positive dogs having average value of rectal temperature (°F), heart rate (per min), respiration rate (per min) and CRT (seconds) was 104.57 ± 0.44 (°F), 99.74 ± 0.92 (per min), 33.98 ± 1.74 (per min) and 2.30 ± 0.32 sec, respectively.

Nested PCR was considered as standard test to compare other diagnostics in present investigation. Diagnostic relevance of various diagnostics was compared on sixty-two samples suspected for ehrlichiosis based on clinical examination. Leishman-stained thin blood smear examination of all 62 samples revealed the presence of *E. canis* in 3 samples, showing a percentage positivity of 4.8%. Leishman-stained buffy coat smear examination of all 62 samples revealed the presence of *E. canis* in 6 sample showing a percentage positivity of 14.28 %. Simple PCR of 62 blood samples, showed positivity in 18 samples for which the 42.85% positivity was calculated. All the 62 samples were finally undergoing for confirmation of disease by nested PCR, which yield 42 samples positive for the ehrlichiosis with a percentage positivity of 66.74 %.

In the present study percentage positivity of ehrlichiosis was recorded on the basis of age, breed, sex and season. Age group with highest positivity was category 3 (> 1 year) with a positivity of (61.90 %) followed by category 2 (> 6 months-1year), with a positivity of (23.80 %) and lowest positivity was recorded in category 1 (0-6 months) with a positivity of (14.28 %). On the basis of breed, Breed with highest positivity was German shepherd with a positivity of (38.09 %) followed by Rottweiler (14.28%), Labrador (14.28 %), non-descript (11.90 %), Pomeranian (9.52 %), Bull mastiff (4.76 %), Spitz (4.76%) and Pug (2.38 %). On the basis of sex, dogs were categorized in 2 categories male and female and positivity of male and female was (54.76 %) and (45.23 %), respectively. On the basis of season, dogs were categorized in 2 categories; summer and winter, for which the positivity was recorded as (78.57%) and (21.42 %), respectively.

In present study there was significant reduction in total leucocyte count, total erythrocyte count, haemoglobin concentration, packed cell volume and platelet count in all the treatment groups of dogs in comparison with control on the day of presentation. There was significant increase in the total leucocyte count, total erythrocyte count, haemoglobin concentration, packed cell volume and platelet count on day 7^{th} and on day 14^{th} after the treatment in all the treatment groups with highest recovery is seen in group 3. Therefore, improvement among all the treatment groups of dogs, best recovery was seen in group which was treated with conventional treatment combined with self-made polyherbal platelet booster containing extract of *Carica papaya*, extract of *Tinospora cordiofolia*, extract of *Withinia somnifera* and extract of *Asparagus racemosus*.

Present investigation shows that there was significant increase in neutrophil in all the treatment groups of dogs in comparison with control on the day of presentation. There was significant decrease in the neutrophil on day 7^{th} and on day 14^{th} after the treatment in all treatment groups. In present investigation it was found that there was significant decrease in lymphocyte in all the treatment groups of dogs in comparison with control on the day of presentation. There was significant increase in the lymphocyte on day 7^{th} and on day 14^{th} after the treatment in all treatment groups.

Present investigation revealed that there was significant increase in ALT, AST, ALP and BUN in all the treatment groups of dogs in comparison with control. There was significant decrease in the ALT, AST, ALP and BUN at day 7^{th} and day 14^{th} after the treatment in all treatment groups with highest recovery is seen in group 3.

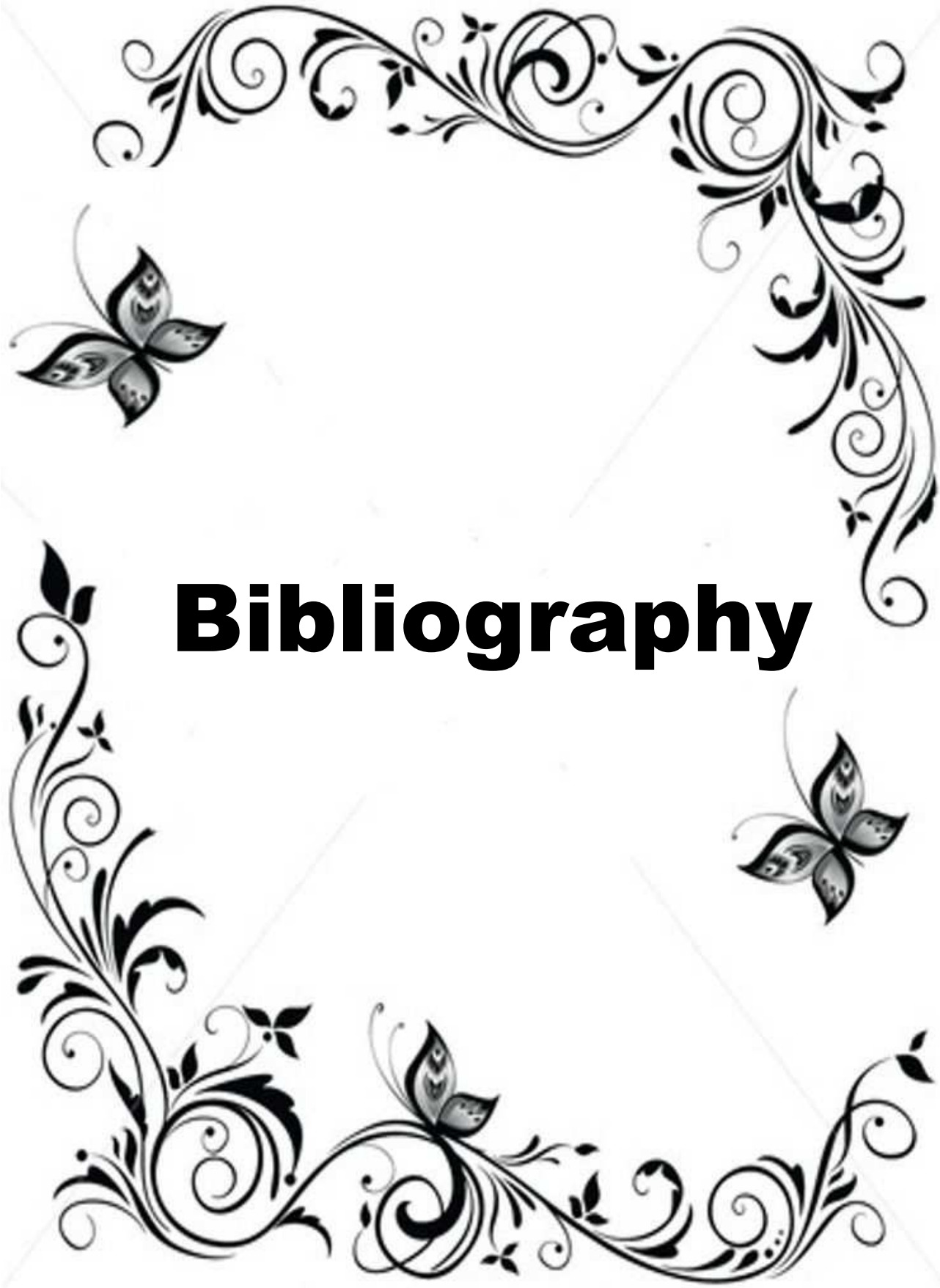
In present study there was significant decrease in total protein and albumin in all the treatment groups of dogs in comparison with control. There was significant increase in the total protein, albumin on day 7^{th} day and day14^{th} after the treatment in all treatment groups with highest recovery is seen in group 3.

Present investigation recorded that there was significant increase in the Activated partial thromboplastin time (aPTT) in all treatment group in comparison with control on day of presentation. There was significant decrease in (aPTT) on day 7^{th} day and on 14^{th} after the treatment in all treatment groups.

In present investigation it was found that there was non-significant increase in the C-PMIF in all treatment group in comparison with control on day 7th day and on day 14thafter the treatment in all treatment groups.

CONCLUSIONS:

1) High fever (pyrexia), pale mucus membrane, lymph adenomegaly, epistaxis melena, tick infestation and weight loss may be considered as classical clinical signs of canine ehrlichiosis.
2) Anaemia, thrombocytopenia and plateletcrit (PCT) were recorded as reliable haematological findings in CME and therefore can be employed for screening procedure for the disease.
3) Among different diagnostics blood smear and buffy coat smear examination having lowest diagnostic relevance in comparison to other diagnostics such as simple PCR and nested PCR.
4) Among the platelet index parameters mean platelet volume and platelet distribution width are not helpful for laboratory diagnosis of ehrlichiosis in dogs.
5) Activated thromboplastin time is found to be helpful for the detection of coagulopathies related to ehrlichiosis. Prothrombin time was not proven helpful for this purpose.
6) Supplementation of Polyherbal platelet booster (*Carica papaya* extract + *Tinospora cordifolia* extract+ *Withinia somnifera extract* +*Asparagus racemosus extract*) hasten the hemato-biochemical changes and clinical regaining towards normalcy in CME positive dogs in a faster way and has better therapeutic efficacy than another group when used with conventional treatment.
7) More studies are warranted to establish the role and exact mechanism of serum platelet migration inhibition factor in canine Ehrlichia induced thrombocytopenia.



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DEPARTMENT OF BOTANY
Babu Shivnath Agrawal College
Mathura -281004
(Estd. & Governed by Agrawal Shiksha Mandal, Mathura)

Ref. No. B.S.A/BOT-39/2020

Dated 18.12.2020

This is regarding the plant specimen provided by the student for identification and authentication certificate. On the basis of visual observation the given specimen identified as below.

S. No.	Botanical Name	Local Name	Family	Locality of Collection	Date of Collection
1.	Withania somnifera	Ashwagandha	Solanaceae	Mathura (U.P.)	18.11.2020
2.	Tinospora cordifolia	Giloy	Menispermaceae	Mathura (U.P.)	18.11.2020
3.	Asparagus racemosus	Shatavari	Solanaceae	Mathura (U.P.)	18.11.2020
4.	Carica papaya	Papaya	Caricaceae	Mathura (U.P.)	18.11.2020

Collector Name : Dr. Suraj Gulmire
Nature of sample : Plant specimen with flowers, fruits and roots
Purpose/intended use of material : For the study of antithrombocytopenic activity.

Kataria 18/12/2020

Dr. Suraj Gulmire
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High School	Maharashta State Board, Pune	2012	89.45%	Hindi, English, Math's, Science, Social science, Marathi

Number of seminar/ Conference / workshop / Training attended: 1

Medals / Honours / Fellowships received:

List of Publications (related to thesis work only):

Number of research article published:

Number of case report published:

Date: 07/10/2021

Place: Mathura

Signature

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Signature of Student