Studies on Add-on Effects of a Polyherbal Formulation on Clinical Recovery and Immuno-Competence of Dogs with Demodicosis

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BY

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Enrollment No. V- 1477/15

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

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(2017)

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This is to certify that the thesis entitled **"Studies on Add on Effects of a Polyherbal Formulation on Clinical Recovery and Immuno-Competence of Dogs with Demodicosis"** submitted by **Dr. Alok Singh**, Enrollment No. V-1477/15 in partial fulfilment of **Master of Veterinary Science in Veterinary Medicine** at U.P. Pandit Deen Dayal Upadhyaya Pashu chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura, 281001 (UP), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated...12/06/2017

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%	:	Per cent
<	:	Less-Than
>	:	Greater- Than
±	:	Plus-Minus
&	:	And
@	:	At the rate of
ALB	:	Albumin
ALKP	:	Alkaline Phosphatase
ALP	:	Alkaline Phosphatase
ALT	:	Alanine aminotransferase
AST	:	Aspartate aminotransferase
b.wt.	:	Body weight
CHOL	:	Cholesterol
e.g.	:	Exempli gratia
EDTA	:	Ethylene diamine tetra acetate
ELISA	:	Enzyme-linked immunosorbent assay
et al	:	Et alli/alia
etc.	:	And so forth
Fig.	:	Figure
G	:	Gram
GLU	:	Glucose
gm/dl	:	Grams per 100 millilitre
Hb	:	Haemoglobin
IFN- γ	:	Interferon- γ
IL-10	:	Interleukin-10
TNF-α	:	Tumor Necrosis Factor Alpha
IU	:	International unit
IU/L	:	International Unit per litre
kg	:	Killogram
L	:	Litre

mEq	:	milli equivalent
mg	:	Milligram
ml	:	Mililitre
mm	:	Millimeter
n	:	Number
PCV	:	Packed cell volume
pg	:	Picogram
RBC	:	Red blood cell
S.N.	:	Serial number
TEC	:	Total Erythrocytic count
TG	:	Triglyceride
TLC	:	Total Leukocyte count
TLR	:	Toll like receptor
TP	:	Total Protein
TVCC	:	Teaching veterinary clinical complex
WBC	:	White blood cells
X100	:	Magnification hundred times
µ	:	Micron
µg	:	Microgram
µl	:	Microlitre
ng	:	Nanogram
DSLS	:	Demodex induced skin lesions score
S.E.M	:	Standard error Mean
MCH	:	Mean corpuscular hemoglobin
MCHC	:	Mean corpuscular hemoglobin concentration
MCV	:	Mean corpuscular volume
TLC	:	Total Leucocytes Count
r.p.m.	:	Round Per Minute
HCT	:	Hematocrit

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CHAPTER-1

INTRODUCTION

Dermatological ailment accounts for sizeable percentage of the small animal sufferer (Khoshnegah *et al.,* 2013). Canine demodicosis is a common but exigent noncontagious parasitic dermatosis caused by overpopulation of the host-specific follicular mites of various *Demodex* species (Singh and Dimri, 2014). Recently it has been validated, that *Demodex* mite are the normal cutaneous microfauna in most of the healthy dogs (Ravera *et al.,* 2013) and pups acquire the parasite from the bitch during the first days of life (Greve and Gaffar, 1966). In canine demodicosis, cutaneous inflammation is associated with excessive numbers of proliferating mites, including immature form (eggs, larvae and nymphs), and the clinical cure is clearly associated with parasiticidal treatment and reduction in the number of *Demodex* mites (Forton, 2012; Miller *et al*., 2013). Therefore, *Demodex* mites are better considered as parasites that normally do not cause adverse effects on their host but that can act as opportunistic pathogens in certain circumstances (Ferrer *et al*., 2014). Canine demodicosis is differentiated into a localized versus a generalized form. Localized demodicosis has a good prognosis, with the overwhelming majority of cases spontaneously resolving without miticidal treatment (Scott *et al*., 2001). Generalized demodicosis may be a severe and potentially life-threatening dermatological condition in dogs (Mueller *et al*., 2012). It is may complicated with secondary bacterial and/or fungal folliculitis and/or furunculosis (Kuznetsova *et al*., 2012). The dogs with generalized demodicosis showing spontaneous cure is unknown presently. A breech in the immune status of the young dogs results into the occurrence of generalized demodicosis and the chance of occurrence in pure breed dog can be minimised by checking the breeding between healthy and diseased one (Beugnet *et al*., 2016). Castration of those dogs which are going to face the acaricidal therapy could be one of the strategies to curb the disease occurrence in dog population (Guaguere and Beugnet, 2008; Mueller *et al*., 2012).

The management of canine demodicosis remains one of the main challenges in veterinary dermatology. The disease is highly prevalent in certain dog breeds and can be very severe, even leading to euthanasia of affected dogs (Duclos *et al*., 1994; Plant *et al*., 2010). Moreover, many aspects of the disease remain unknown, making prevention and management of some cases difficult. Receptivity of dogs to demodicosis is influenced by numerous factors including; hereditary predisposition, alteration of skin's structure and biochemistry, immunological disorders, hormonal status, breed, age, nutritional status, oxidative stress, length of hair coat, stage of oestrus cycle, parturition, endoparasitism and debilitating diseases

(Ghubash, 2006; Dimri *et al*., 2008; Camkerten *et al*., 2009; Mederle *et al*., 2010; Plant *et al*., 2011; Singh and Dimri, 2014). The exact pathogenesis of generalized canine demodicosis is unknown but an aberration in immune status is thought to be the most significant (De Bosschere *et al*., 2007). Appearance of canine generalised demodicosis is greatly influenced by breech in T-cell immunity (Caswell *et al*., 1997), rather than any breech in hummoral immunity (Tizard, 2002). However, immunological abnormalities have not been noted in dogs with localized demodicosis (Scott *et al*., 2001). Literatures repeatedly make a statement that a genetically preprogrammed immunological defect is responsible for the exaggerated replication of mites in demodicosis (Scott *et al*., 2001). Alternatively, the decreased immune response could also be due to the *Demodex* mites themselves (Barriga *et al*., 1992; Paulik *et al*., 1996; Singh *et al*., 2010; Singh *et al*., 2011).

Cytokines, a large group of soluble extracellular proteins or glycoproteins, are key intercellular regulators and mobilizers. Cytokines secretion, by virtually every nucleated cell type, is usually an inducible response to injurious stimuli (Oppenheim, 2001). The mechanism of cytokine secretion from T lymphocytes has played important roles in the immune response of the dogs against mites induced skin dermatological conditions including the demodicosis (Felix *et al*., 2013; Singh and Dimri, 2014). Increased TGF-β mRNA expression might be a key factor for revealing differences in the mechanism of onset between localized and generalized demodicosis (Tani *et al*., 2002). Recently, Felix *et al*. (2013) revealed that elevated serum levels of IL-10 are strongly associated with recurrent demodicosis in dogs. They also demonstrated elevated levels of IL-10 in dogs encountered with demodicosis for the first time. IL-10 cytokine is an essential molecule in the mechanism underlying suppression mediated by T regulatory cells (Moore *et al*., 1990). IL-10 is also called the cytokine synthesis inhibitor factor, since it has the ability to inhibit the synthesis of Th1 cytokines (IL-1, IFN-γ, and TNF-α) as well as inhibiting the function of NK cells (Howard and O'garra, 1992). Its anti-inflammatory and suppressive effects on most hematopoietic cells, and it indirectly suppresses cytokine production and proliferation of antigen-specific CD4+ T effector cells, by inhibiting the antigen-presenting capacity of different types of professional antigen-presenting cells (Roncarolo *et al*., 2006). IL-10 mainly targets on Th1 cells, B cells, macrophages, NK cells, mast cells, and thymocytes (Tizard, 2002).

The clinical cure is clearly associated with parasiticidal treatment and reduction in the number of *Demodex* mites (Forton, 2012; Miller *et al*., 2013). Generalized demodicosis may be a severe and potentially life threatening disease and is commonly complicated with a secondary bacterial folliculitis and/or furunculosis (Kuznetsova *et al*., 2012). The management of canine demodicosis remains one of the main challenges in veterinary dermatology. Owing to wide prevalence of skin diseases increasing incidence of drug

resistance and detrimental side effects with the most of allopathic drugs there is quest for the safe alternative medicines. Traditional medicines hold a great promise as source of easily available effective therapy for skin diseases to the people particularly in tropical developing countries including India. An array of herbal medicines have been reported which are having immunomodulatory effects. It can be achieved by modulation of cytokine secretion, histamine release, immunoglobulin secretion, class switching, cellular co-receptor expression lymphocyte expression and phagocytosis (Mahima *et al*., 2012). The need of an immunorestorative therapy has been urged in generalized demodicosis for that immunorestorative drugs namely Immuplus a herbal drug and T11TS a sheep erythrocyte surface glycoprotein has been used in a study and get positive results (Sarkar *et al*., 2004). Amitraz plus inactivated *Parapoxvirus ovis* in the treatment of canine generalised demodicosis has recently been reported (Pekmezci *et al*., 2014). Generalised demodicosis requires a very long period for complete clinical and parasitological cure. Thus, frequent repeated administrations of miticides are needed. Repeated administrations of miticides are predisposing the parasites to develop the resistance against the allopathic drugs. Additionally, repeated administrations of allopathic medicine are also hazardous for the treated animals. Albeit, isoxazoline compounds with promising miticidal potential has recently been introduced for therapeutic management of canine demodicosis and opened novel hope for treatment of canine generalised demodicosis (Beugnet *et al.,* 2016; Six *et al.,* 2016). However, the immumo-pathology of generalised demodicosis evidently suggests the need of exploration of immunomodulatory therapeutics adjuct to the miticides for holistic and faster therapeutic management of generalised demodicosis in dogs. Owing to wide prevalence of canine demodicosis, increasing incidence of drug resistance and detrimental side effects with the most of allopathic drugs, there is quest for the safe alternative adjunctive medicines. Cost-effective and affordable medicines to an Indian animal owner are call for the commendable researchers. Traditional medicines hold a great promise as source of easily available effective therapy for skin diseases to the people, particularly in tropical developing counties, including India. Therefore, the present study was projected with following objective:

- **To evaluate the effects of a polyherbal formulation on clinical recovery of dogs with generalised demodicosis.**
- **To evaluate the effects of a polyherbal formulation on Immuno-competence of dogs with generalised demodicosis.**

CHAPTER-2

REVIEW OF LITERATURES

Canine Demodicosis and Pathobiology

The demodectic mites infest the hair follicles, sebaceous glands, melbomian glands, ceruminous glands, and internal organs of 11 orders of mammals, including dog, sheep, cat, and pig. For a long time, classification of *Demodex* mites has been based mainly on their hosts and phenotypic characteristics. Since Gustav Simon first identified *Demodex* in 1842, 140 species or subspecies have been identified. *Demodex* cannot be maintained or cultured *in vitro* so as to parasitize and infect other healthy animal hosts (Zhao *et al*., 2009; 2011). Sako (1964) describes the thermotactic zone of *D. canis* to be between 16^{o}C and 41^{o}C and the movement of the mites ceased below 15^{o}C of the environmental temperature.

Demodicosis is a disease caused by an excessive proliferation of the *Demodex* mites. Canine generalised demodicosis (GD) is frequently seen in practice (Plant *et al*., 2011). Until the introduction of amitraz it was not unusual that dogs with severe GD were euthanized. Since the introduction of amitraz and later the macrocyclic lactones to the small animal practitioners, the prognosis for dogs with this disease has dramatically improved (Mueller, 2004). The disruption of the cutaneous barrier due to the physical and chemical effects of the proliferating mites is likely to be present in all forms. The rupture of the hair follicles is facilitated by the inflammatory reaction, which is histologically characterized in the dog by a mural folliculitis accompanied by injury to follicular keratinocytes (Caswell *et al*., 1995; Caswell *et al*., 1997; Day, 1997). In human rosacea, the T-helper lymphocytes (CD4+, T-helper-17) have been identified as the main inflammatory cells present in the perifollicular and dermal inflammatory infiltrates (Rufli and Buchner, 1984). In the dog, the cells have been reported to be CD3+ and CD8 + T lymphocytes, and they are considered to be cytotoxic T cells, which may mediate the injury in the follicular epithelium (Caswell *et al*., 1997). Once in the dermis, the released mites, together with hair fragments and keratin, induce a granulomatous reaction, with a variable degree of lymphocytic infiltrates (Caswell *et al*., 1995; Caswell *et al*., 1997; Day, 1997; Forton, 2012). The granulomas have been associated with the resolution phase of the disease, when the clinical lesions regress (Caswell *et al*., 1997). The presence of a strong hypersensitivity reaction against the mites has been documented in papulopustular rosacea (Georgala *et al*., 2001). A similar hypersensitivity reaction to the mites has not been documented in the dog, but the presence of CD8+ lymphocytes in the inflammatory infiltrate could be an aberrant or exaggerated immune

response to the mites or against keratinocytes and Langerhans cells presenting *Demodex* antigens (Caswell *et al*., 1997).

It has been demonstrated that *Demodex* mites can contain, transport and interact with bacteria of the cutaneous microbiome (Forton, 2012; Jarmuda *et al*., 2012). In consequence, some of the lesions observed in demodicosis have been attributed to the interaction between *Demodex* and bacteria. In human rosacea, it has been demonstrated that *Demodex* overgrowth induces a proliferation of *Staphylococcus epidermidis*, which can lead to a skin infection, and a hypersensitivity reaction against bacterial antigens (*Bacillus oleronius*) (Lacey *et al*., 2007; Forton, 2012; O'Reilly *et al*., 2012). According to these authors, bacterial proteins and antigens would be responsible for the inflammatory and vascular changes characteristic of rosacea. In the dog, the association of *Demodicosis* with pyoderma is well established and is probably the most severe consequence of demodicosis (Miller *et al*., 2013). Treatment with antibiotics is prescribed if the clinical signs or cytological examination of exudates are suggestive of pyoderma. However, it is not known whether, as in humans, *Demodex* mites induce a proliferation of *Staphylococcus pseudintermedius*, or rather if this bacteria simply takes advantage of the epidermal barrier rupture that occurs in canine demodicosis. Although some recent papers have questioned the importance of the bacterial infection in canine demodicosis (Kuznetsova *et al*., 2012), it would probably be helpful to investigate the changes in the skin microbiome associated with canine generalized demodicosis.

In some dogs the pyoderma can progress to cause systemic signs such as fever, lethargy, anorexia and the potential of sepsis and death. It is known, that the prognosis is better in young dogs compared to dogs with adult onset demodicosis (Duclos *et al*., 1994). Juvenile GD is considered a disease with genetic background (Plant *et al*., 2011). There are many breeds, which have been reported to be predisposed (Plant *et al*., 2011; Scott *et al*., 2001). However, the genetic base of the dog population can vary significantly between countries and studies so far have focussed on dog populations in the United States, Australia and selected European countries. Woldemeskel *et al*., (2017) recently demonstrated the spreading of demodectic mites to the visceral lymph nodes via vascular channels which may considered as a part of pathogenesis of chronic, uncured, adult- onset of generalised canine demodicosis.

Cytokines and Demodicosis

TH1 cells release interleukin (IL)-1, interferon, and tumor necrosis factor (TNF) after antigen stimulation. This cascade of effects leads to cell-mediated immune responses, which are responsible for macrophage activation, delayed-type hypersensitivity reactions, and

defense against intracellular organisms. TH2 responses lead to release of IL-4, IL-5, IL-10, and IL-13, which stimulate B-cell proliferation and antibody secretion. Pathology associated with autoimmune, infectious and allergic diseases may be due to primary TH1- or TH2-like responses, and much recent research has focused on characterizing and modulating their responses (Duarte *et al.,* 1999; Singh *et al*., 1999). The evaluation of cytokine messenger RNA expression in mononuclear cells from the peripheral blood of dogs with demodicosis was performed using RT-PCR and semi-quantitative PCR (Tani *et al*., 2002). Results of PCR analysis suggest that increased TGF-mRNA expression might be a key factor for revealing the difference in the mechanism of onset between localized and generalized demodicosis (Tani *et al*., 2002). TGF-β is the prototypical member of a superfamily of pleiotropic cytokines, which regulate a multitude of biological processes including tissue homoeostasis, angiogenesis, migration and differentiation. TGF-β acts as a potent immunosuppressor by regulating the proliferation and survival of many cells of the immune system. In the same study authors demonstrated down regulation of TNF-α in dog with demodicosis (Tani *et al*., 2002). TNF-α is a pleiotropic pro-inflammatory cytokine that exerts multiple biological effects. At low level expression of TNF-α participates in beneficial tissue remodeling and host defense response. The expression of TNF-α is tightly controlled, because systemic over production of TNF-α activates inflammatory response to infection and injury, and mediates hypotension, diffused coagulation and widespread tissue damage. Additionally, Tani *et al*. (2002) suggest that IL-5 may be a key factor in monitoring the disease. Recently, Felix *et al*. (2013) revealed that elevated serum levels of IL-10 are strongly associated with recurrent demodicosis in dogs. They also demonstrated elevated levels of IL-10 in dogs encountered with demodicosis for the first time. IL-10 cytokine is an essential molecule in the mechanism underlying suppression mediated by T regulatory cells (Moore *et al*., 1990). Recently, Kumari *et al*. (2017) also evidently demonstrated the potential association of immunosuppressive circulatory cytokines with the localised as well as generalised demodicosis. They hypothesized the potential association of vagal immunosuppressive along with marked elevation in circulatory IL-10 level with the clinical pathology and progression of demodicosis in dogs.

IL-10 is also called the cytokine synthesis inhibitor factor, since it has the ability to inhibit the synthesis of Th1 cytokines (IL-1, IFN-γ, and TNF-α) as well as inhibiting the function of NK cells (Howard and O'garra, 1992). Its anti-inflammatory and suppressive effects on most hematopoietic cells, and it indirectly suppresses cytokine production and proliferation of antigen-specific CD4+ T effector cells, by inhibiting the antigen-presenting capacity of different types of professional antigen-presenting cells (Roncarolo *et al*., 2006). IL-10 mainly targets on Th1 cells, B cells, macrophages, NK cells, mast cells, and thymocytes

(Tizard, 2002). Recently, Singh *et al*. (2014) demonstrated remarkably higher level of mRNA expression for interleukin-4, interleukin-5 and transforming growth factor beta (TGF-β) mRNA expression in dogs with sarcoptic mange in comparison with the healthy dogs. Moreover, the same study revealed remarkably lower level of tumor necrosis factor alpha (TNF-α) dogs with scabies. Therefore, the possibility of *Demodex* induced overproduction of IL-10 and TGF-β as well as down regulation of TNF-α in conferring immune escape of proliferating mites is reasonable. The observations of the various researchers evidently suggest that dogs with generalized demodicosis show an innate and adaptative immunodeficiency (Singh and Dimri, 2014). Barboza *et al.* (2000) reported recuperation of the lymphocytic response by the effect of Thymostimulin can solve the clinical appearance of generalized demodicosis.

Demodicosis and miticide

Amitraz has been approved for the treatment of canine generalized demodicosis in many countries for decades and has been shown to be an effective treatment option in many studies (Mueller, 2004). It is the member of formamidine family. The mechanisms of action of the amitraz include the inhibition of prostaglandin synthesis, interaction with the octopamine receptors of the central nervous system and inhibition monoamine oxidases. The treatment protocol of the dog suffering with generalised demodicosis along with secondary deep pyoderma must not contain systemic antibiotics as a necessary component of treatment because intensive miticidal and topical antibacterial therapy may be sufficient to resolve the clinical situations (Kuznetsova *et al.,* 2012). Josephus *et al.* (2013) recently proved that Certifect® a combination of (fipronil 6.26% w/v, amitraz 7.48% w/v, and (S)-methoprene 5.63% w/v) produces effective result in treating dogs with generalized demodicosis when used both in monthly and bi-weekly manner over a 3-month period. Fipronil shows acaricidal action and there action is significantly potentiated when it is used in combination with Amitraz (Pfister, 2011; Prullage *et al.,* 2011). Keeping the view of providing safer and appropriate approach to cure this disease, findings of recent studies stablished topical products as a registered treatments protocol for curing demodicosis because these help in improving owner compliance, which results into increase in success rate. These topical products contain either amitraz (combined with the insecticide metaflumizone) as agile component or moxidectin (combined with the insecticide imidacloprid) which may applied as a spot-on at monthly or bi-weekly intervals (Fourie *et al.,* 2006; Fourie *et al.,* 2007; Heine *et al.,* 2005). Anecdotally, Amitraz caused headaches and asthma in owners, thus it is commonly recommended that dogs should be washed in a well-ventilated area (Mueller, 2004; Scott *et al*., 2001). Yohimbine (Yobine, Ben Venue Laboratories) is the antidote for amitraz, and can be used if side effects are excessive.

Atipamesole (50µg/kg intramuscularly) can reverse the signs of toxicosis within 10 minutes .The recommended concentration of Amitraz varies from 0.025% to 0.06%, with a frequency of once weekly to every 2 weeks (Mueller, 2004). Certain studies revealed that therapeutic management of those cases of canine generalised demodicosis which was not responding to normal conventional therapies can be achieved by using Amitraz intensively with daily rinsing of alternating body halves at a concentration of 0.125% (Medleau and Willemse, 1995) or weekly treatment with an amitraz concentration of 1.25% (Hugnet *et al.,* 2001). For the treatment of juvenile and adult onset demodicosis in dogs recently efficacy of metaflumizone plus amitraz was also studied (Rosenkrantz, 2009). Amitraz causing sudden death in Chihuahuas, so it should be avoided in this breed. Use of Amitraz should also be avoided in pregnant and nursing bitches and in puppies whose age is less than 12 weeks (Craig, 2003). Dog must be clipped properly before dipping, beside this dog should also not be bathed between applications of the miticidal drug so that efficacy of the medication increases (Scott *et al*., 2001). Newer parasiticides such as members of isoxazoline class of compounds have recently opened novel window in therapeutic management of canine demodicosis (Beugnet *et al.,* 2016; Six *et al,* 2016). Afoxolaner is an isoxazoline compound having insecticidal property along with good safety range and extended effectiveness against fleas and ticks on dogs following a single oral administration (Shoop *et al.*,2014). The novel insecticidal and acaricidal activities contaning isoxazolinen compounds shows there mode of action via acting on specific GABA/glutamatereceptor inhibiting the chloride ion channels of arthropods (Ozoe *et al*., 2010; Garcia-Reynaga *et al*., 2013; Gassel *et al*., 2014).

Demodicosis and alternative therapy

In recent scenario due to increasing incidence of drug resistance and detrimental side effects with the most of allopathic drugs there is quest for the safe alternative medicines. Natural remedies such as nutritious foods, herbs and other supplements can be used to treat the skin problem topically. The use of alternative medical therapy including herbs is increasing dramatically in the United States. Many of these herbal therapies have been used for centuries and show good anecdotal result (Bedi and Shenefelt,2002). Recent work has uncovered potential biochemical mechanisms involved in the immunomodulatory pathway of many supplemental vitamins (A, D, and E) that appear to affect the differentiation of $CD4^+$ cell TH-1 and TH-2 subsets (Mainardi *et al.,* 2009). Plant sterols and sterolins (phytosterols) are sterol molecules synthesized by plants and ingested by humans and animals in the form of fruits and vegetables. These compounds have been shown in animals to have antiinflammatory, antineoplastic, antipyretic, and immunomodulating activity. Phytosterols improve T-lymphocyte and natural killer cell activities. Overactive antibody responses are

also dampened to help control immune-mediated and autoimmune disease processes, and the dihydroepiandrsterone (DHEA): cortisol ratio is normalized (Dodds, 2000).

An antibacterial drug which is a constituent of two active components i.e. silver nanoparticles (AgNPs) having concentration 10–50 ppm and benzyldimethyl{3-[miristoylamino]-propyl}ammonium chloride having concentration 100 ppm, has been approved as an antiseptic during demodicosis in a more concentrated dosage form (up to 50 ppm of nanosilver) for the human patients. Significant reduction in the treatment period along with prevention of complications by the use of this antibacterial drug has been reported by Krutyakov *et al.* (2016). Successful management of refractory cases of canine demodicosis with homeopathy medicine Graphitis is also reported recently (Ranjan *et al*., 2013). Fish oil provides omega-3 fatty acids and can be very effective in easing an itch. Other sources of omega-3 fatty acids include flaxseed oil and pumpkin seed. *Jatropha curcas* oil along with *Withania somnifera root* extract revealed higher efficacy against demodectic mange (Singh and Dimri, 2010). The findings of study on demodicosis and alternative therapy show that Gliricidia (*Gliricidia sepium*) can be used as an alternative therapy for generalized canine demodicosis (David *et al*., 2000). Neem oil, together with lavender oil, can make an effective skin rinse against mange. Other herbs that are effective against mange include yellow dock, Echinacea, Calendula, and Aloe vera. Homeopathic treatment is also effective in treating canine mange. Commonly used homeopathic remedies are Sulphur, Psorinum, Silicea, Graphit. A treatment protocol for mange consists of the combined usage of neem oil (externally) and homeopathic (orally).

The uses of herbal plants as health promoters are gaining increasing attention in both consumer and scientific circle (Upadhayay *et al*., 2011; Hashemi and Davoodi, 2012). Hrebal drugs/Indeginous drugs exhibited multiple immunomodulatory actions including modulation of cytokine secretion, histamine release, immunoglobulin production, immunoglobulin class switching, cellular co-receptor expression, lymphocyte proliferation and phagocytosis promotion (Spelman *et al*., 2006). Clove (*Syzygium aromaticum*) shows its anti-inflammatory action by eugenol, eugenol in noncytotoxic concentrations exert immunomodulatory/anti-inflammatory action on cytokine production by murine macrophages (Bachiega *et al*., 2012). In recent time, compliance and switching over to herbal therapies from conventional medicine is advocated globally as herbal therapies can often improve or even reverse a condition by identifying and treating the root cause. Herbal medicines are also effective in chronic conditions, such as asthma, allergies, recurring skin conditions and cancers (Mahima *et al.*, 2012). The various herbal drugs like *Ascophyllum nodosum, Cucumis melo, Carica papaya, Aloe vera, Haematococcus pluvialis, Curcuma longa*, *Camellia sinensis*, *Punica granatum*, *Piper nigrum*, *Polygonum cuspidatum, Echinacea purpurea*, *Grifola frondosa*,

and *Glycine max* in the diet correlated with a significant decrease in TH-1 response, in terms of INF-γ production. Such evidence highlighted the immunomodulatory potentials of these specific botanicals (Guidetti *et al.*, 2016). *Withania somnifera* (WSE) can be used to augment the activities of antioxidant enzymes and level of reduced glutathione and decrease lipid peroxidation and oxidative stress in dogs with demodicosis (Singh *et al.,* 2010). Singh *et al*. (2011) also demonstrated the ameliorative potential of *Withania somnifera* extract against the altered $CD4^+/CD8^+$ balance of dogs with demodicosis. The cyanobacterium *Spirulina* is an aquatic microorganism that contains a huge amount of essential nutrients and bioavailable to the body, widely used in the world because of its nutritional richness and character immunostimulant. According to recent studies it reveals that *Spirulina* aids in the early recovery of dogs with demodicosis and does not lead to undesirable effects resulting from its chemical composition, revealing its benefits as adjuvant therapy for the treatment of canine demodicosis (Bezerra *et al*., 2013). The recent trends of immuno-pathology of canine generalised demodicosis (Kumari *et al*., 2017) evidently suggest the need of supplementations of safe alternative immunomodulators along with the conventional miticidal therapies for holistic management of canine demodicosis.

CHAPTER-3

MATERIALS AND METHODS

3.1. Selection criteria of animals and design of the study

Client-owned dogs presented with dermatological ailments at Teaching Veterinary Clinical Complex (Kothari Hospital) of the University for clinical and dermatological examination were examined and diagnosis of demodicosis was made by detection of mature and immature *Demodex canis* mites in scrapings and/or hair pluck samples from lesional skin. The dogs were diagnosed with generalized demodicosis when they have either minimum of five affected areas (>10 cm^2 each) or have a single-affected body region (>100 cm^2) or have at least one affected paw (pododemodicosis) (Paterson *et al*. 2009). The dogs must be older than 7 weeks of age and weighing >4kg.

3.2. Animal inclusion criteria

The dogs generalised demodicosis that have not been treated with ectoparasiticides or steroidal anti-inflammatory drugs in the last 30 days prior to clinical examination were included in this study. Diseased dogs have history of regular routine deworming and were free of any other concurrent diseases. The participated demodicosed dogs were also free of other ecto-parasites infestations, except for *D. canis* mites. Dogs were also found negative for haemoprotozoa on thin blood smears examination and have physiological parameters like body temperature, respiratory-rate and heart-rate within the normal reference range. When skin cytology of impression smears revealed presence of neutrophils and intracellular Cocci (Fig.1.3), a diagnosis of concurrent secondary pyoderma was made.

3.3. Treatment plan

The dogs diagnosed with generalised demodicosis were allocated into two groups (Group 1 and 2). Demodicosed dogs of Group 1 (n= 09) and Group 2 (n=10). The demodicosed dogs of Group 1 were treated with 0.0375% solution v/v of amitraz rinse at weekly intervals for 8 weeks. Whereas, demodicosed dogs of Group 2 were treated with 0.0375% solution v/v of amitraz rinse at weekly intervals and oral with one capsule (250 mg) twice in a day of a polyherbal formulation (Pyodermacare-G capsules) for 8 weeks. Pyodermacare-G is a polyherbal preparation developed under ICAR outreach programme on Ethnoveterinary Medicine (*Grant No.1-72/(EVM-Outreach Programme)/2009/Med dated 05.02.2010)* by Department of Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, DUVASU, Mathura. A patent has been filed for the polyherbal (Pyodermacare-G) preparation and the product is still under the process of patent. The

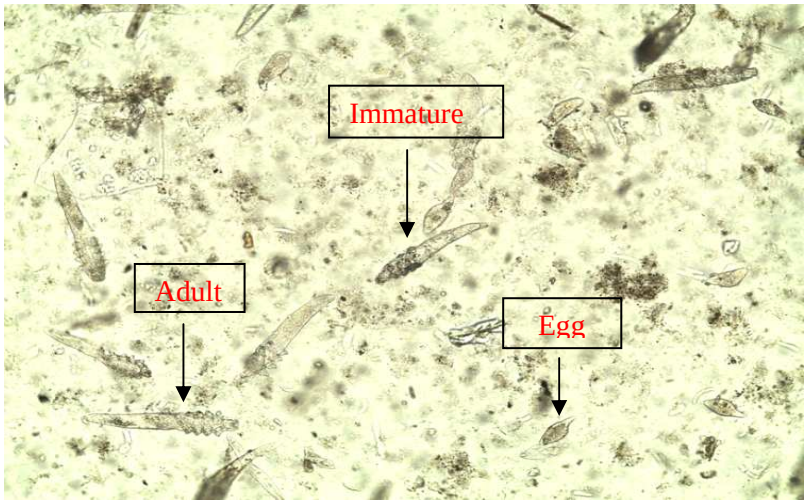

Fig. 1.1: Micrograph of skin scrapping showing various stages of *Demodex canis* mites

Fig.1.2: Micrograph of an adult *Demodex* mite

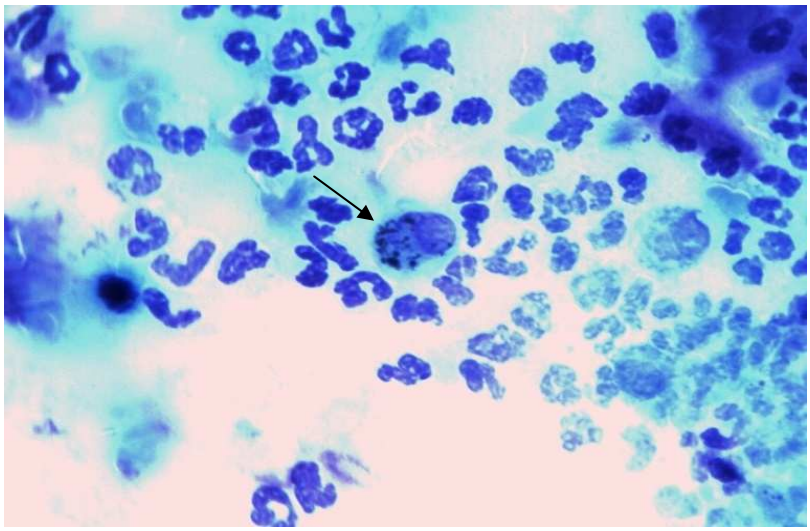

Fig.1.3: Micrograph of impression smear showing engulfed bacterial Cocci by WBCs

preparation Demodicosed dogs of both of the groups were bathed thoroughly with 2.5% benzoyl peroxide shampoo and towel dried before each application of amitraz rinse. Demodicosed dogs diagnosed with concurrent pyoderma were also treated with injection lincomycin at a dose rate of 20 mg per kg of body weight once a day, intramuscularly for a period of minimum five days and first amitraz rinse was applied on day fifth in these dogs. The diseased dogs were clinically examined at the intervals of 30 ± 03 days post-therapy for clinical and parasitological recovery assay.

3.4. Faecal sample examination

By using sterilized swabs anal swab samples were obtained from each of the participated dogs before start of any treatment. Swab obtained were subjected for routine examination methods for detection of endo-parasites infestations.

3.5. Thin blood smears examination

For preparation of blood smear, a drop of blood sample was obtained by aseptic pricking of ear tips of diseased dogs and spread over a clean slide to prepare the blood smear. Blood smear examination was conducted by routine methods for detection of any haemoprotozoa infection and differential leukocyte counts.

3.6. Dermatological investigation

3.6.1. Mite counts

Mite counts were performed in skin scraping materials obtained from two different skin-scraping sites of one squire centimetre. The same sites were sampled on each subsequent examination (at 4 wks intervals). For taking skin scrap the selected 1 cm^2 area was squeezed by holding the area of skin between thumb and fore finger. Further, with the help of blunted knife a deep skin scrap was taken until the blood ooze out. The scraped sample was transferred into a glass test tube. Further, 2-3 ml of 10% KOH solution was added into the scraped samples and subjected for mild heating over a sprit lamp until 1or 2 bumps arises in the KOH solution. The tube was allowed to stand for few min at room temperature to make it cool down and further subjected for centrifugation at 2500 rpm for 5 min. The supernatant was discarded and the aliquot was used for counting the mites. Skin scrape samples were systematically read using the 10X objective lens. Absolute counts for each life stage (adult, larvae and egg) Figs. (1.2 and 1.3) were recorded as per method suggested by Paterson *et al*. (2009)

3.6.1. Skin impression smears cytology

Pressure impression smear was prepared from the skin lesions by using the clean grease free glass slides. The air dried impression smear should be fixed in methanol for 1

minute and dry it in air. Further, the fixed smear was stained with Field Stain B (Red Stain) {approximately 1ml} for 30 seconds. After washing under tap water and the slide was flooded over with Field Stain A (Blue Stain) {approximately 1ml} for 10 to 20 seconds. Further subjected for washing under running tap water and dried in air. Microscopic examination was performed under 100 x oil immersion objective.

3.6.2. Clinical recovery score analysis

The presence and severity of *Demodex*-induced skin lesions were recorded. Four clinical symptoms e.g., the extent of Erythema, Scales⁄Crusts, Comedones⁄Papules⁄Pustules and Alopecia were assessed and rated on a scale from 0 (absent) to 6 (extremely severe) with a maximum total score of 24 at one sits. All the four scores were summed up for each affected area of the body and expressed as *Demodex*-induced skin lesions score (DSLS) for each affected site. The mean of DSLS at different sites of affected areas was calculated and used for assessment of clinical recovery for each of the diseased dog. Per cent clinical recovery was calculated by using formula e.g., % clinical recovery = [(Day 0 DSLS – DSLS at day post-therapy) / Day 0 DSLS] X 100.

3.7. Blood sample collection

With the informed verbal consent of the pet owners, approximately 3 mL of blood samples were obtained from each dog in clot activators containing tubes on every examination during the period of the therapy and used for harvesting serum. The tubes containing blood samples were kept in a slanted position for 15-20 min at normal room temperature, followed by subjected to centrifuge at 1500 rpm for 5 min. Supernatant serum was aspirated using micropipette and transferred into cryovials and stored at -20 °*C* until estimation various panels. Additionally, 2 mL blood samples were also obtained into vials containing EDTA from each of the diseased dog on every examination during the period of the therapy and were subjected for routine haematology. Blood samples were collected before on day 0 (start of the therapy); Day 30 post-therapy and day 60 post-therapy. In similar manner blood samples were also obtained from the healthy dogs and were used as reference value (Group 3).

3.7.1. Haematology

Haematology of all blood samples were carried out by using fully automated haematology analyzer (BS-2800 Vet Haematology analyzer, Mindray Electronic Co. Ltd) on each sampling time. Total erythrocyte counts, haemoglobin, haematocrit (HCT), MCH, MCHC, differential leukocyte counts, granulocytes, monocytes, lymphocytes, eosinophils counts were measured.

3.7.2. Serum biochemistry

Serum biochemical panels were estimated by routine methods on each sampling time with the help of automated biochemistry analyzer (BS-120 Chemistry Analyzer; 2007- 2010 Shenzhen Mindray Biochemical Electronics Co. Ltd.) by using the kits from Span Diagnostics Ltd. Sero-biochemical panels, namely glucose, total cholesterol, triglycerides, albumin, total protein, urea and creatinine levels and activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALKP) were estimated. Globulins level was estimated by deducting the albumin content from total proteins and the content of albumin was divided with globulin content to derive the albumin/globulin (A/G) ratio.

3.7.3. Quantification of circulatory cytokines by ELISA

Circulatory levels of interleukin-10 (IL-10), tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were estimated at day 0 and day 60 post-therapy by using canine specific ELISA kits (RAB0524-IL-10; RAB0526-TNF-α; RAB0523-IFN-γ, Sigma-Aldrich, USA) following the procedure as described by the manufacturer. Levels of IL-10 and IFN-γ were expressed as ng/mL, where TNF-α level was expressed as pg/mL. The intra-assay and inter-assay reproducibility coefficients of variation of the all assayed cytokines were <10% and <12%, respectively.

3.7.3.1. Quantification of interleukin-10

Reagents Required:

Assay/ Sample Diluent Buffer dilution

Assay/Sample dilution buffers B and D (Provided in kit) were diluted 5 fold with deionized or distilled water before use. 1x sample diluents buffer was further used for serum sample dilution.

Preparation of standard

Item C (provided in kit) was briefly spinned and 80ng/ml standard solution was prepared by adding 500µl of 1x diluents buffer D. pipette up 400 µl of 1x diluents buffer D in to 7 tubes. Then 200 µl of prepared standard solution was used to produce a dilution series. Each tube was properly mixed before the next transfer. 1X diluent buffer is used as blank.

Preparation of concentrate Biotinylated Antibody

Detection antibody vial (provided in kit) was briefly spinned and 100µl of 1X diluents buffer B is added to prepare a detection antibody concentrate. Prepared mixture is gently mixed and stored at 4ºC for further use.

Preparation of working Biotinylated Antibody

Prepared concentrate biotinylated antibody was further diluted 80 fold with 1x diluent buffer B to get a working solution.

Dilution of HRP- streptavidin Concentrate

HRP- streptavidin concentrate vial (provided in kit) was mixed properly and diluted 350 fold with 1x diluent buffer B.

Washing solution- already provided in the kit.

TMB reagent- provided in the kit.

Procedures

Before starting the protocol all reagents and samples were allowed to reach at room temperature. 100 µl of each standard and samples were added into appropriate wells of coated ELISA plate (provided in kit). Wells were covered and incubated for 2.5 hours at room temperature with gentle shaking. After incubation, the solution was discarded and 300 µl of wash solution was added in to each well for washing. Washing solution was completely removed from wells by gentle tapping. This step was performed for four consecutive times. After washing, 100 µl 1X prepared biotinylated antibody was added into each well and incubated for 1 hour at room temp temperature with gentle shaking followed by discarding of the solution and repeated the washing was given. Further, 100 µl of prepared HRP-Streptavidin solution was added into each well and incubated for 45 min at room temperature with gentle shaking. Washing step was repeated and the washing solution was completely removed from each well. 100 µl of ELISA Colorimetric TMB Reagent was added to each well and incubated for 30 min at room temperature in the dark with gentle shaking. At the completion of the incubation period, 50 µl of stop solution was added to each well and reading was taken immediate at 450nm with help microplate ELISA reader.

Calculation

Absorbance for each set of standard, control and samples were calculated and subtracted the average zero optical density. Standard curve was plotted using sigma plot software with standard concentration on the X-axis and absorbance on the Y-axis. Circulatory levels of IL-10 were expressed as ng/mL.

Quantification of TNF-α

Reagents Required:

Assay/ Sample Diluents Buffer dilution

Assay/Sample dilution buffers B (Provided in kit) was diluted 5 fold with deionized or distilled water before use.

Sample diluents buffer

1x sample diluents buffer (provided in kit) was used for serum sample dilution.

Preparation of standard

Item C (provided in kit) was briefly spinned and 100 ng/ml standard solution was prepared by adding 400µl of 1x diluents buffer A. Powder was thoroughly dissolved by gentle mixing. 7 µl of TNF-α standard was added into a tube having 993µl of assay diluents B to prepare a 700 pg/ml standard solution. 300µl of diluents buffer A was pipette in to 8 tubes. Then 200 µl of prepared standard solution (700 pg/ml) was used to produce a dilution series. Each tube was properly mixed before the next transfer. 1X diluent buffer B was used as blank.

Preparation of concentrate Biotinylated Antibody

Detection antibody vial (provided in kit) was briefly spinned and 100µl of 1X diluent buffer B was added to prepare a detection antibody concentrate. Prepared mixture was gently mixed and stored at 4ºC for further use.

Preparation of working Biotinylated Antibody

Prepared concentrate biotinylated antibody was further diluted 80 fold with 1x diluent buffer B to get a working solution.

Dilution of HRP- streptavidin Concentrate

HRP- streptavidin concentrate vial (provided in kit) was mixed properly and diluted 350 fold with 1x diluent buffer B.

Washing solution- already provided in the kit.

TMB reagent- provided in the kit.

Procedure

Before starting the protocol all reagents and samples were allowed to reach at room temperature. 100 µl of each standard and samples were added into appropriate wells of coated ELISA plate (provided in kit). Wells were covered and incubated for 2.5 hours at room

temperature with gentle shaking. After completion of incubation period, the solution was discarded and 300 µl of wash solution was added into each well for washing. Washing solution was completely removed from wells by gentle tapping. This step was performed for four times. After washing, 100 µl 1X prepared biotinylated antibody was added into each well and incubated for 1 hour at room temperature with gentle shaking. Further, the solution was discarded and washing procedure was repeated. 100 µl of prepared HRP-Streptavidin solution was added into each well and incubated for 45 min at room temperature with gentle shaking. Further, washing step was repeated and the washing solution was completely removed from each well. 100 µl of ELISA colorimetric TMB reagent was added to each well and incubated for 30 min at room temperature in the dark with gentle shaking. After completion of the incubation period, 150 µl of stop solution was added to each well and reading was taken immediately at 450 nm by microplate ELISA reader.

Calculation

Absorbance for each set of standard, control and samples were calculated and subtracted the average zero optical density. Standard curve has been plotted using sigma plot software with standard concentration on the X-axis and absorbance on the Y-axis and circulatory TNF-α level was expressed as pg/mL.

Quantification of IFN-γ

Reagents Required:

Assay Diluents Buffer dilution

Assay/Sample dilution buffers B (Provided in kit) was diluted 5 fold with deionized or distilled water before use.

Sample Diluent Buffer

Sample diluent buffer A (provided in kit) was used for serum dilution.

Preparation of standard

Item C (provided in kit) was briefly spinned and 100 ng/ml standard solution was prepared by adding 400 µl of 1x diluents buffer A.7 µl of standard solution from vial C was added into another tube having 993 µl of assay diluents buffer A to prepare a 700 ng/ml standard solution. Pipette up 300 µl of 1x diluents buffer A in to 8 tubes. Then 200 µl of prepared standard solution is used to produce a dilution series. Each tube was properly mixed before the next transfer. 1X diluent buffer A was used as blank.

Preparation of concentrate Biotinylated Antibody

Detection antibody vial (provided in kit) is briefly spinned and 100µl of 1X diluents buffer B was added to prepare a detection antibody concentrate. Prepared mixture was gently mixed and stored at 4ºC for further use.

Preparation of working Biotinylated Antibody

Prepared concentrate biotinylated antibody was further diluted 80 fold with 1x diluent buffer B to get a working solution.

Dilution of HRP- streptavidin Concentrate

HRP- streptavidin concentrate vial (provided in kit) was mixed properly and diluted 350 fold with 1x diluent buffer B.

Washing solution- already provided in the kit.

TMB reagent- provided in the kit.

Procedure

Before starting the protocol all reagents and samples are allowed to reach at room temperature (18-25 ºC). 100 µl of each standard and samples were added into appropriate wells of coated ELISA plate (provided in kit). Wells were covered and incubated for 2.5 hours at room temperature with gentle shaking. At the completion of incubation, the solution was discarded and 300 µl of wash solution was added into each well for washing. Washing solution was completely removed from each well by gentle tapping. This step was performed for four times. Further, 100 µl 1X prepared biotinylated antibody was added into each well and incubated for 1 hour at room temperature with gentle shaking. Again, the solution was discarded and washing procedure was repeated. 100 µl of prepared HRP-Streptavidin solution was added into each well and incubated for 45min at room temperature with gentle shaking. Further, washing step was repeated and the washing solution was completely removed from each well. Further, 100 µl of ELISA colorimetric TMB reagent was added into each well and incubated for 30 min at room temperature in the dark with gentle shaking. At the completion of incubation period, 50 µl of stop solution was added to each well and the absorbance was read immediately at 450nm by using micropalte ELISA reader.

Calculation

Absorbance for each set of standard, control and samples were calculated and subtracted the average zero optical density. Standard curve was plotted using sigma plot software with standard concentration on the X- axis and absorbance on the Y-axis. Circulatory levels of IFN-γ were expressed as ng/mL.

Statistical analysis

All data were expressed as mean ± S.E.M. Statistical analysis were conducted to determine the difference between the groups by using one-way ANOVA, post-hoc Tukey's test with general linear models in SPSS 16. While, the comparison among the values within the same group at different time intervals were analyzed by the Repeated Measures approach using ANOVA with mixed linear models in SPSS 16. The level of statistical significance for all the comparisons made was established at $P<0.05$.

CHAPTER-4

RESULTS

Effects on Haematology

The haematology of diseased dogs at the studied sampling days is depicted in Table 1. The ameliorative potential of Pyodermacare-G on haematology of dogs with generalised demodicosis was evaluated in terms of alterations in the both leukograms and haemograms. Remarkable alterations in the both leukograms and haemograms were not recorded on day 0 (before start of the therapy). Whereas, remarkable alterations in the both leukograms were recorded in both of the groups on day 30 and day 60 post-therapy when compared with their own day 0 values (Table 1). Moreover, remarkable alterations in the both leukograms and haemograms were also recorded in both of the groups on day 60 when compared with their own day 30 values (Table 1).

The demodicosed dogs of control group (Group 1), that were not supplemented with Pyodermacare-G, found have no significant reduction in total leukocyte counts (TLC) at day 30 and day 60 post-therapy as compared with their own day 0 values. Moreover, significant amelioration in other panels of the leukograms for instance lymphocytes, granulocytes and monocytes counts as well as lymphocytes, neutrophils, monocytes and eosinophils percentage was also not recorded in this groups at day 30 and day 60 post-therapy as compared with their own day 0 values. Likewise, significant amelioration in all the panels of leukograms were not revealed by the dogs of control group at day 60 post-therapy as compared with their own day 30 values. Whereas, the demodicosed dogs supplemented with Pyodermacare-G (Group 2) found have significant ($P<0.001$) reduction in TLC at day 30 and day 60 post-therapy as compared with their own day 0 values (Fig.10.11). Moreover, significant ($P<0.001$) reduction in granulocytes counts was recorded in this groups at day 30 and day 60 post-therapy as compared with their own day 0 values (Fig.10.5). At day 30 post-therapy, significant ($P<0.001$) reduction lymphocytes counts was revealed by Pyodermacare-G supplemented dogs as compared with their own day 0 values. Moreover at day 60 post-therapy these dogs also revealed significant ($P<0.001$) reduction in the both lymphocytes and monocytes counts as compared with their own day 0 values. The dogs of this group also found to have significantly ($P<0.05$) lower percentage of neutrophils at day 60 post-therapy as compared with their own day 0 and day 30 values (Fig.10.10). Whereas, significantly higher percentage of lymphocytes ($P<0.01$) (Fig.10.9) and monocytes ($P<0.05$) at was recorded in this group at day 60 post-therapy as compared with own day 0 values. Remarkable reduction in eosinophis percentage was not recorded in dogs of both of the groups at day 30 and day 60 post-therapy as compared with their own day 0 values.

Table 1: Effects of Pyodermacare-G supplementation on leukogram of dogs with generalised demodicosis.

Panels	Controls (Group 1; n=09)			Pyodermacare-G supplemented (Group 2; n=10)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
TLC ($10^3/\mu L$)	27.26±2.64	24.10±2.9^{A}	20.21±1.85^{A,B}	24.40±1.9$^{¥}$	11.57±0.64^{a,†}	7.67±0.37^{a,B,†}
Lymphocytes ($10^3/\mu L$)	5.26±0.41	4.45±0.32^{A}	4.54±0.40^{A,B}	5.01±0.52$^{¥}$	2.48±0.21^{a,†}	2.03±0.10^{a,B,†}
Monocytes ($10^3/\mu L$)	0.97±0.14	0.68±0.07^{A}	0.63±0.08^{A,B}	1.06±0.16$^{¥}$	0.68±0.14^{A}	0.45±0.03^{a,B,‡}
Granulocytes ($10^3/\mu L$)	20.77±2.40	18.33±3.01^{A}	14.66±1.52^{A,B}	18.34±1.70$^{¥}$	7.96±0.47^{a,†}	5.04±0.26^{a,B,†}
Lymphocytes (%)	20.07±2.02	20.36±1.60^{A}	22.83±1.42^{A,B}	20.71±1.71$^{¥}$	23.08±1.41^{A}	26.64±0.65^{a,B,‡}
Monocytes (%)	3.71±0.45	2.98±0.26^{A}	3.36±021^{A,B}	4.37±0.61$^{¥}$	5.09±0.32^{A,†}	5.79±0.19^{b,B,†}
Neutrophils (%)	72.60±2.08	73.68±1.82^{A}	70.81±1.57^{A,B}	71.77±2.53$^{¥}$	68.86±1.17^{A,‡}	65.73±0.51^{b,c,†}
Eosinophils (%)	3.36±0.57	3.18±0.52^{A}	3.87±0.71^{A,B}	3.05±0.68$^{¥}$	2.05±0.46^{A}	1.89±0.24^{A,B,†}

^{A}Non-significant difference, when compared with day 0 values of the same group; ^{B}Non-significant difference, when compared with day 30 values of the same group; ^{a}Significant (P<0.01) difference, when compared with day 0 values of the same group; ^{b}Significant (P<0.05) difference, when compared with day 0 values of the same group; ^{c}Significant (P<0.05) difference, when compared with day 30 values of the same group; †Significant (P<0.01) difference, when compared with same day post-treatment values of Group 1; ‡Significant (P<0.05) difference, when compared with same day post-treatment values of Group 1; $^{¥}$ Non-significant difference, when compared with day 0 values between the groups.

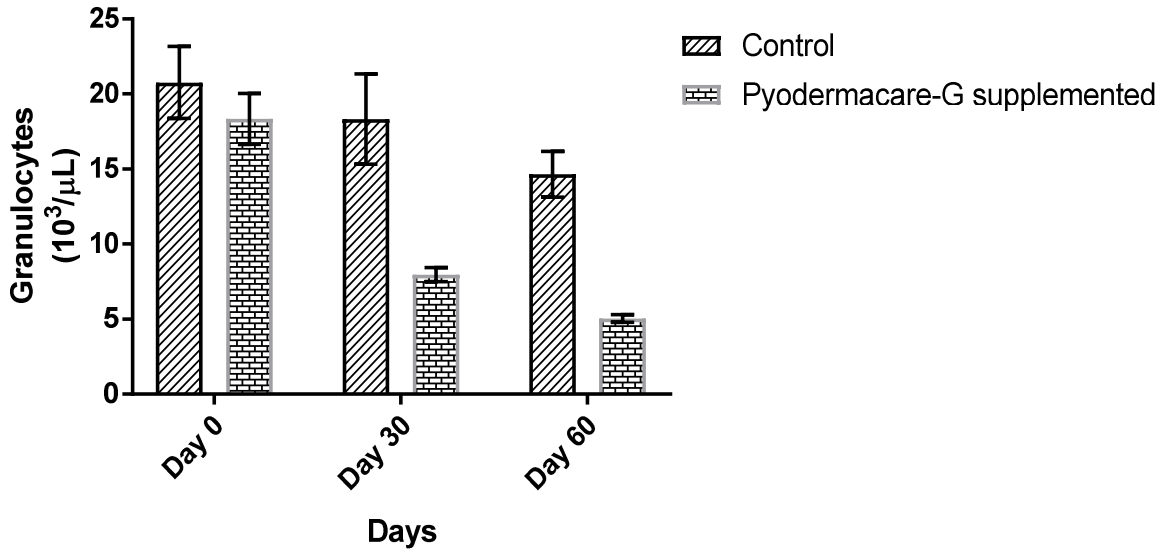

Fig.10.5: Effect of Pyodermacare-G capsule supplementation on absolute numbers of Granulocytes (Mean+SE)

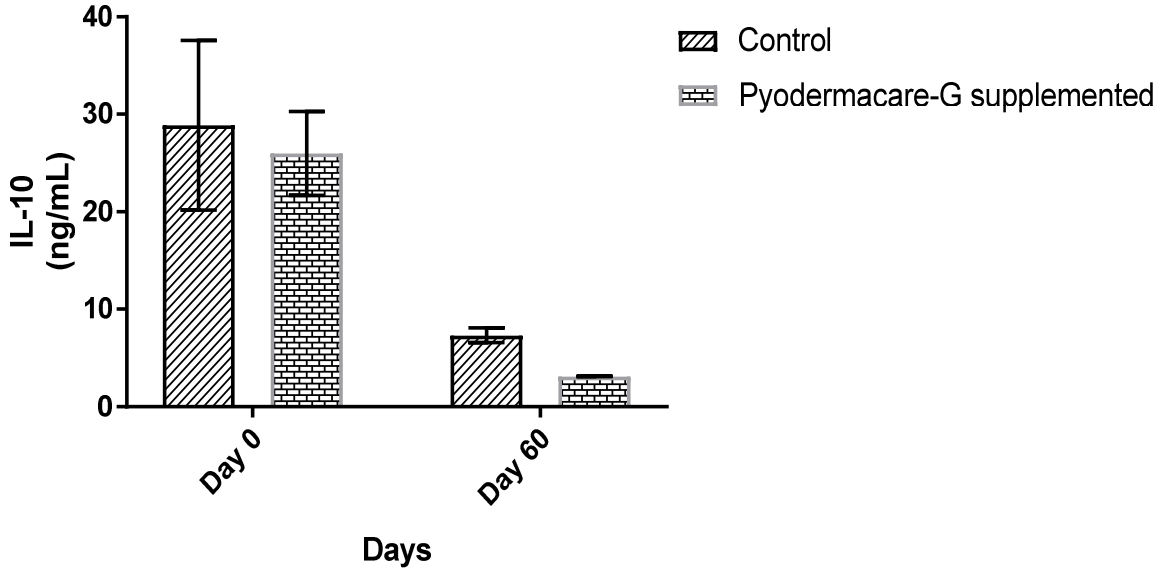

Fig. 10.6: Effect of Pyodermacare-G capsule supplementation on circulatory IL-10 level (Mean+SE)

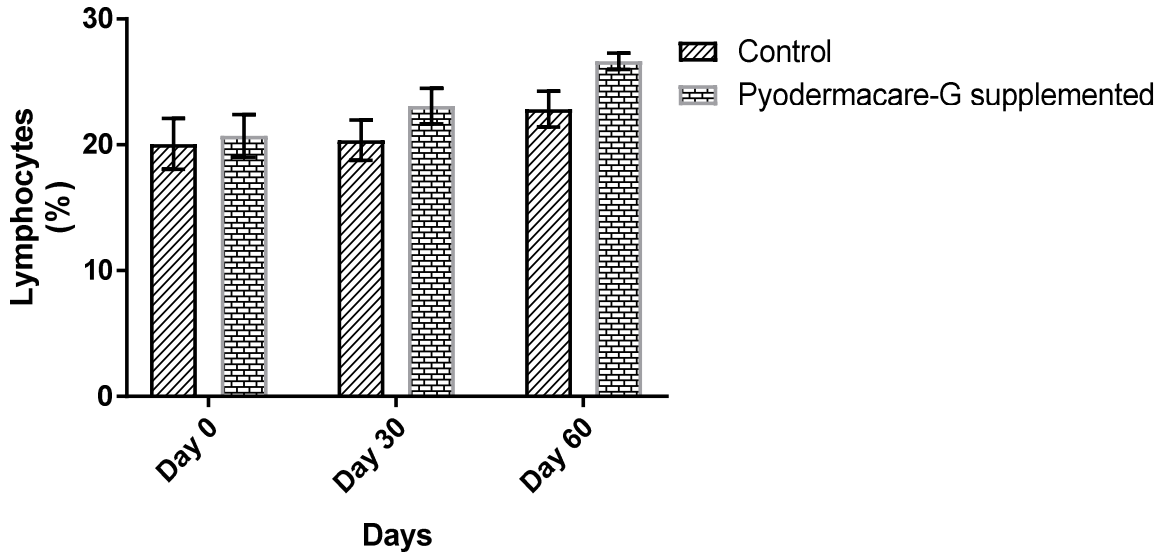

Fig.10.9: Effect of Pyodermacare-G capsule supplementation on Lymphocytes percentage (Mean+SE)

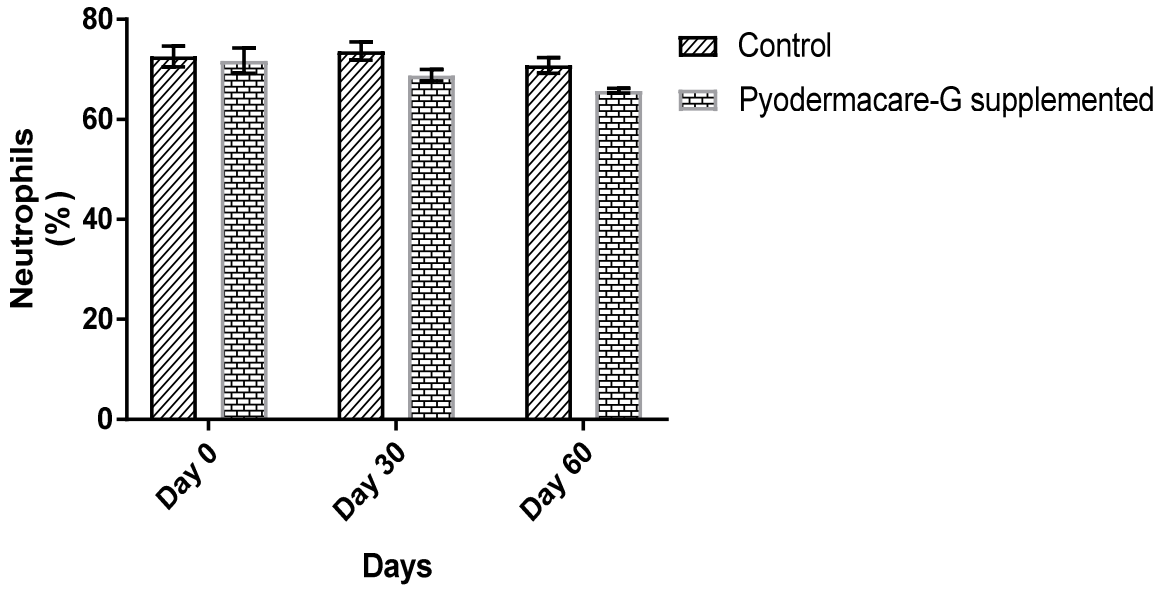

Fig. 10.10: Effect of Pyodermacare-G capsule supplementation on Neutrophils percentage (Mean+SE)

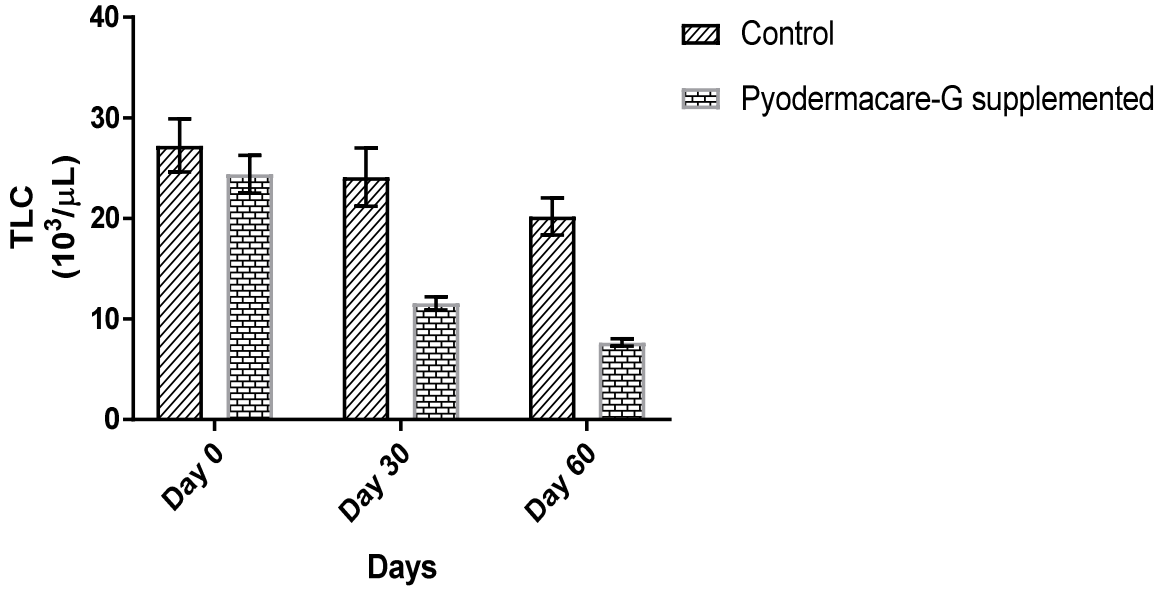

Fig. 10.11: Effect of Pyodermacare-G capsule supplementation on TLC (Mean+SE)

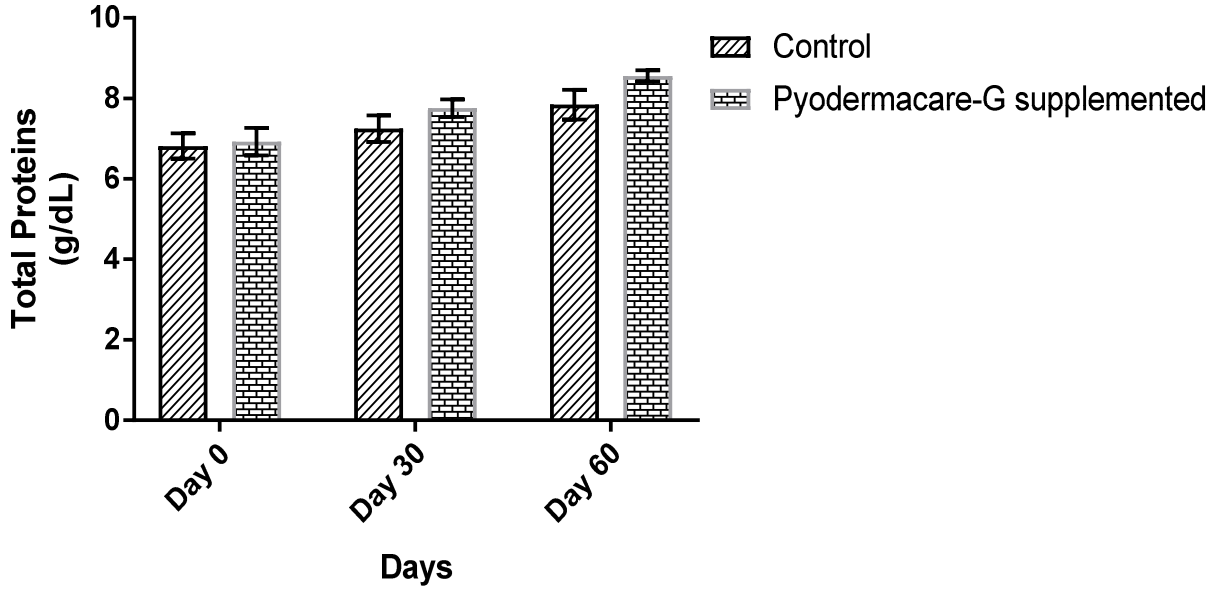

Fig. 10.12: Effect of Pyodermacare-G capsule supplementation on Total Protein level (Mean+SE)

At day 30 post-therapy, the dogs supplemented with Pyodemacare-G found to have significantly (P<0.01) lower values of TLC, granulocytes and lymphocytes as compared with day 30 post-therapy values of non-supplemented dogs (Group 1). Additionally at day 30 post-therapy, percentage of neutrophils was significantly (P<0.05) lower in the dogs supplemented with Pyodemacare-G as compared with the same day values of Group 1. Whereas, Pyodemacare-G supplemented dogs found to have significantly (P<0.001) elevated percentage of monocytes at day 30 post-therapy as compared with same day's values of non-supplemented group. Moreover at day 60 post-therapy, the dogs supplemented with Pyodemacare-G found to have significantly lower values of TLC (P<0.001), granulocytes (P<0.001), lymphocytes (P<0.001) and monocytes (P<0.05) as compared with day 60 post-therapy values of non-supplemented dogs (Group 1). Additionally at day 60 post-therapy, significantly lower percentage of neutrophils (P<0.005) and eosinophils (P<0.01) was recorded in the dogs supplemented with Pyodemacare-G as compared with the same day values of Group 1. Whereas, Pyodemacare-G supplemented dogs found to have significantly elevated percentage of lymphocytes (P<0.02) and monocytes (P<0.001) at day 60 post-therapy as compared with same days values of non-supplemented group.

Remarkable alterations in haemograms were recorded in the both within and between the studied groups (Table 2).The demodicosed dogs of control group (Group 1), that were not supplemented with Pyodermacare-G, found have no significant amelioration haemograms panels for instance TEC, Hb, HCT, MCV, MCH and MCHC at day 30 and day 60 post-therapy as compared with their own day 0 values. Whereas, significant improvement in haemograms panels for instance TEC (P<0.001), Hb (P<0.001) (Fig.10.13), HCT (P<0.001), MCH (P<0.01) and MCHC (P<0.04) at day 30 post-therapy was revealed by the dogs of Pyodermacare-G supplemented group as compared with their own day 0 values. Additionally, the dogs of this group also found to have significant improvements in TEC (P<0.001), Hb (P<0.001), HCT (P<0.001), MCH (P<0.04) and MCHC (P<0.004) at day 60 post-therapy as compared with their own day 0 values. Likewise at day 60 post-therapy the dogs of this group found to have further significant improvements in TEC (P<0.03) and Hb (P<0.01) as compared with their own day 30 values.

At day 30 post-therapy, the dogs supplemented with Pyodemacare-G found to have significantly higher Hb (P<0.01), HCT (P<0.004) and MCH (P<0.01) as compared with day 30 post-therapy values of non-supplemented dogs (Group 1). In tandem, at day 60 post-therapy the dogs supplemented with Pyodemacare-G found to have significantly higher TEC (P<0.003), Hb (P<0.001), and MCH (P<0.004) as compared with day 30 post-therapy values of non-supplemented dogs (Group 1).

Table 2: Effects of Pyodermacare-G supplementation on haemogram of dogs with generalised demodicosis.

Panels	Controls (Group 1; n=09)			Pyodermacare-G supplemented (Group 2; n=10)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
RBC (10^6/µL)	5.12±0.5	5.65±0.36^{A,}	6.20±0.29^{A,B}	4.88±0.37$^{¥}$	6.37±0.13^{a}	7.33±0.16^{a,c,†}
HB (gm/dL)	9.25±0.96	9.94±0.82^{A}	11.02±0.48^{A,B}	8.7±0.60$^{¥}$	12.14±0.15^{a,†}	13.79±0.24^{a,d,†}
HCT (%)	38.58±3.4	39.30±2.39^{A}	42.90±2.20^{A,B}	35.99±2.5$^{¥}$	47.34±0.84^{a,†}	52.78±1.93^{a}
MCV (fL)	71.93±1.5	70.26±1.3^{A}	69.15±1.4^{A,B}	75.58±1.92$^{¥}$	75.51±2.34^{A}	71.99±1.32^{A,B}
MCH (pg)	19.6±1.18	16.74±0.75^{A}	17.43±0.34^{A,B}	17.37±0.5$^{¥}$	19.08±0.41^{a,†}	18.79±0.23^{b,†}
MCHC (g/dL)	24.85±0.86	24.35±1.0^{A}	25.76±0.46^{A,B}	24.01±0.29$^{¥}$	25.62±0.27^{b}	26.26±0.66^{a}
Platelets (10^3/µL)	442±91	276±22^{A}	316±27^{A,B}	317±40$^{¥}$	269±17^{A}	288±21^{A,B}

^{A}Non-significant difference, when compared with day 0 values of the same group; ^{B}Non-significant difference, when compared with day 30 values of the same group; ^{a}Significant (P<0.01) difference, when compared with day 0 values of the same group; ^{b}Significant (P<0.05) difference, when compared with day 0 values of the same group; ^{c}Significant (P<0.05) difference, when compared with day 30 values of the same group; ^{d}Significant (P<0.01) difference, when compared with day 30 values of the same group; †Significant (P<0.01) difference, when compared with same day post-treatment values of Group 1; $^{¥}$ Non-significant difference, when compared with day 0 values between the groups.

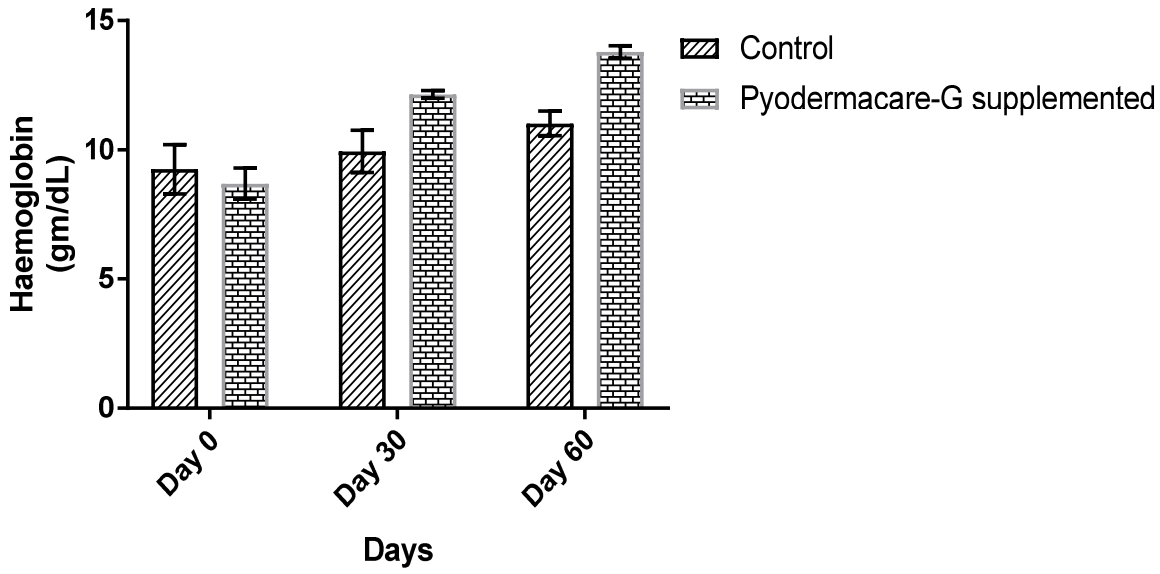

Fig. 10.13: Effect of Pyodermacare-G capsule supplementation on Haemoglobin content (Mean+SE)

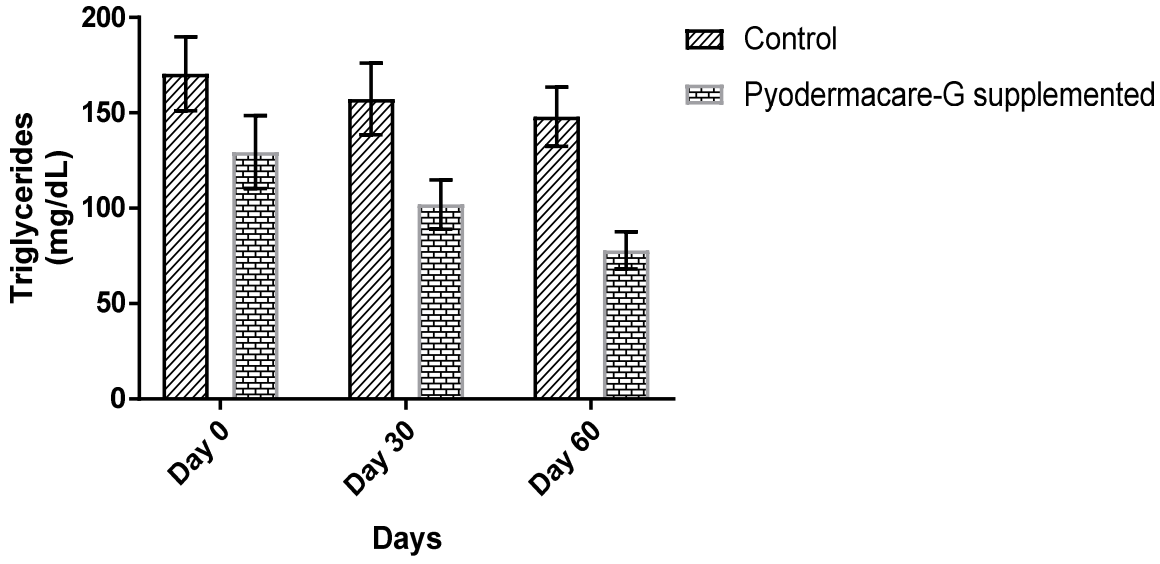

Fig. 10.14: Effect of Pyodermacare-G capsule supplementation on Triglycerides level (Mean+SE)

Effects on serum biochemistry

The ameliorative potentials of Pyodermacare-G capsules on serum biochemical panels of dogs with generalised demodicosis are depicted in Table 3. Demodicosed dogs of the control group (Group 1) have not revealed remarkable alteration in most of the altered serum biochemical panels including cholesterol, triglycerides, and albumin contents at day 30 and day 60 post-therapy as compared with their own day 0 values. However, significant ($P<0.05$) amelioration in ALKP activity was recorded in this group at day 60 post-therapy as compared with their own day 0 values. But remarkable reduction in serum ALKP activity was not estimated in this group at day 30 post-therapy as compared with their own day 0 values.

Whereas, the demodicosed dogs supplemented with Pyodermacare-G (Group 2) found have significant reduction in total cholesterol level ($P<0.05$) at day 60 post-therapy as compared with their own day 0 values (Fig.10.3). But at day 30 post-therapy, significant amelioration in serum total cholesterol level was not revealed by the dogs of this group as compared with their own day 0 values. Moreover, significant increment in total protein ($P<0.001$) (Fig.10.12), albumin ($P<0.001$) (Fig.10.2) and globulins ($P<0.05$) was revealed by the dogs of this group at day 60 pot-therapy as compared with their own day 0 values. Significant increment in total protein ($P<0.05$) and albumin ($P<0.01$) was also recorded at day 30 post-therapy as compared with their own day 0 values. Additionally at day 60 post-therapy, significant ($P<0.05$) increment in albumin content was also revealed by the dogs of this group as compared with their own day 30 post-therapy values.

At day 30 post-therapy, the dogs supplemented with Pyodermacare-G found to have significantly ($P<0.02$) lower levels of total cholesterol and triglycerides as compared with day 30 post-therapy values of the non-supplemented dogs (Group 1). Moreover at day 60 post-therapy, the dogs supplemented with Pyodemacare-G found to have significantly lower levels of total cholesterol ($P<0.02$) and triglycerides ($P<0.001$) (Fig.10.14) as compared with the same day post-therapy values of non supplemented group (Group 1). Contrarily, the dogs supplemented with Pyodemacare-G found to have significantly ($P<0.03$) higher albumin level as compared with the day 60 post-therapy values of non-supplemented dogs (Group 1).

Effects on Circulatory Cytokines

The effects of supplementation with Pyodermacare-G on circulatory cytokines levels of dogs with generalised demodicosis are depicted in Table 4. The demodicosed dogs of control group, that were not supplemented with Pyodermacare-G, found have significant ($P<0.02$) reduction in IL-10 level at day 60 post-therapy as compared with their own day 0 values. Whereas remarkable amelioration in circulatory levels of TNF-α and IFN-γ was not

Table 3: Effects of Pyodermacare-G supplementation on serum biochemistry of dogs with generalised demodicosis.

Panels	Controls (Group 1; n=09)			Pyodermacare-G supplemented (Group 2; n=10)		
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
Glucose (mg/dL)	88.67±12.54	76.88±6.76^{A}	72.22±6.63^{A,B}	65.90±3.42^{a}	62.80±2.97^{a,A}	73.10±3.54^{a,A,B}
Cholesterol (mg/dL)	188.4±21.3	179.77±16.6 ^{A}	172.77±20.7^{A,B}	151.4±7.2^{a}	136.2±8.1^{b,A}	119.6±9.2^{b,C,B}
Triglycerides (mg/dL)	170.5±19.4	157.33±18.8^{A}	148.0±15.6^{A,B}	129.4±19.1^{a}	102±12.9^{b,A}	77.90±9.6^{c,A,B}
Total Proteins (g/dL)	6.82±0.32	7.25±0.33^{A}	7.85±0.37^{A,B}	6.93±0.34^{a}	7.76±0.22^{a,C}	8.56±0.14^{a,D,B}
Albumin (g/dL)	2.11±0.07	2.22±0.10^{A}	2.43±0.11^{A,B}	2.04±0.05^{a}	2.44±0.10^{a,D}	2.80±0.11^{b,D,C}
Globulins (g/dL)	4.7±0.31	5.02±0.32^{A}	5.42±037^{A,B}	4.9±0.29^{a}	5.31±0.19^{a,A}	5.76±0.21^{a,C,B}
Creatinine (mg/dL)	0.52±0.08	0.54±0.05^{A}	0.59±0.06^{A,B}	0.55±0.04^{a}	0.49±0.05^{a,A}	0.55±0.04^{a,A,B}
Urea (mg/dL)	12.47±0.80	13.02±0.62^{A}	12.93±1.02^{A,B}	11.98±0.4^{a}	13.10±0.86^{a,A}	13.91±1.35^{a,A,B}
Aspartate Aminotransferase (AST) (u/L)	31.3±4.7	28.02±4.6^{A}	29.41±3.9 ^{A,B}	26.34±3.18^{a}	28.82±3.2^{a,A}	24.83±2.2^{a,A,B}
Alanine Aminotransferase (ALT) (u/L)	31.2±5.1	24.41±3.2^{A}	23.50±2.42^{A,B}	25.11±3.3^{a}	20.19±1.3^{a,A}	18.75±1.5^{a,A,B}
Alkaline Phosphatase (ALKP) (u/L)	64.7±4.6	57.33±4.35^{A}	48.0±4.4^{C,B}	58.9±8.5^{a}	47.1±4.4^{a,A,B}	44.8±4.1^{a,A,B}

^{A}Non-significant difference, when compared with day 0 values of the same group; ^{B}Non-significant difference, when compared with day 30 values of the same group; ^{C}Significant (P<0.05) difference, when compared with day 0 values of the same group; ^{D}Significant (P<0.01) difference, when compared with day 0 values of the same group; ^{a} Non-significant difference, when compared with values of same days of treatment of Group 1; ^{b}Significant (P<0.05) difference, when compared with values of same days of treatment of Group 1; ^{c}Significant (P<0.01) difference, when compared with values of same days of treatment of Group 1.

Table 4: Effects of Pyodermacare-G supplementation on circulatory cytokines of dogs with generalised demodicosis.

Estimated Cytokines	Controls (Group 1; n=09)		Pyodermacare-G supplemented (Group 2; n=10)	
	Day 0	Day 60	Day 0	Day 60
Interleukin-10 (ng/mL)	28.87±8.7	7.33±0.75^{A}	25.97±4.3$^{¥}$	3.13±0.02^{C,†}
Tumour necrosis factor-α (pg/mL)	153.05±27.5	107.55±9.8^{B}	154.48±27.9$^{¥}$	145.15±11.3^{B,‡}
Interferon-γ (ng/mL)	3.83±0.08	3.84±0.09^{B}	3.81±0.09$^{¥}$	6.43±0.65^{C,†}

^{A}Significant (P<0.05) difference, when compared with day 0 values of the same group; ^{B}Non-significant difference, when compared with day 0 values of the same group; ^{C} Significant (P<0.01) difference, when compared with day 0 values of the same group; $^{¥}$ Non-significant difference, when compared with day 0 values between the groups. †Significant (P<0.01) difference, when compared with day 60 values between the groups; ‡Significant (P<0.05) difference, when compared with day 60 values between the groups.

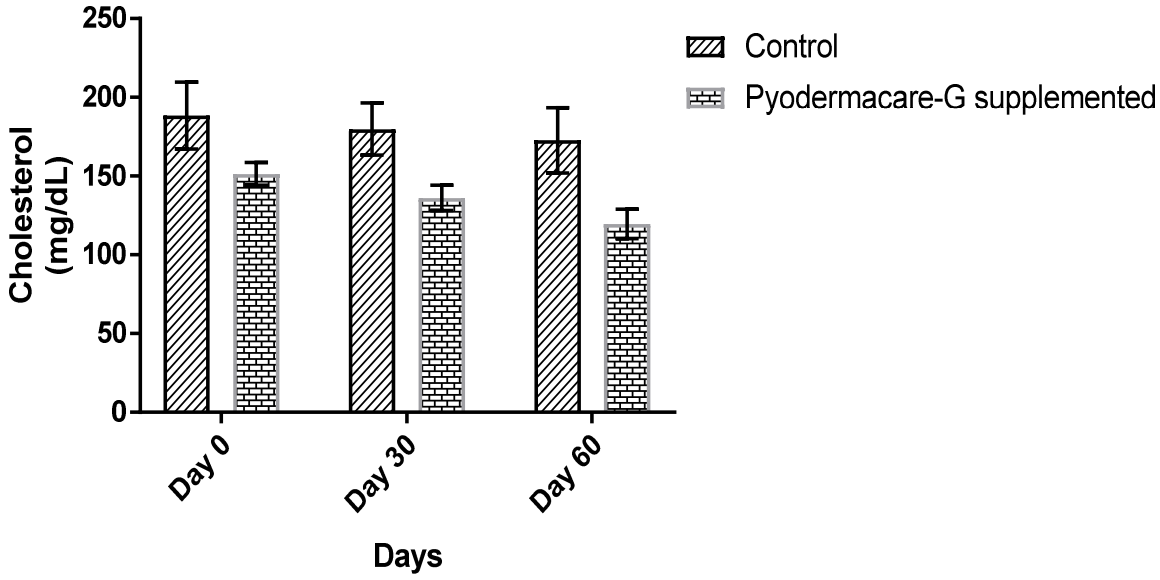

Fig. 10.3: Effect of Pyodermacare-G capsule supplementation on Cholesterol level (Mean+SE)

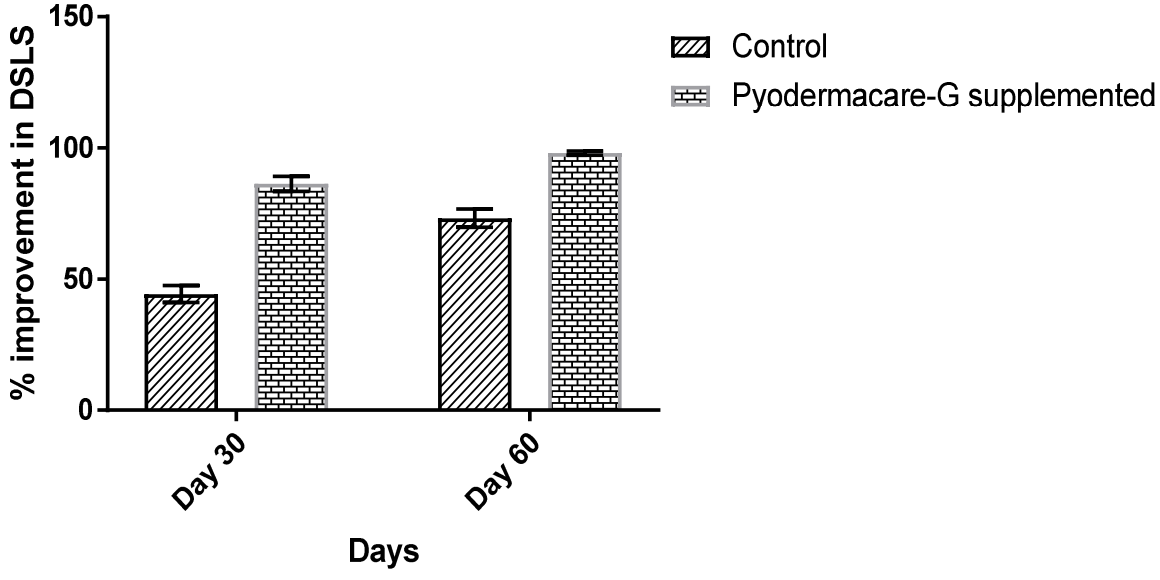

Fig. 10.4: Effect of Pyodermacare-G capsule supplementation on per cent Improvement in DSLS (Mean+SE)

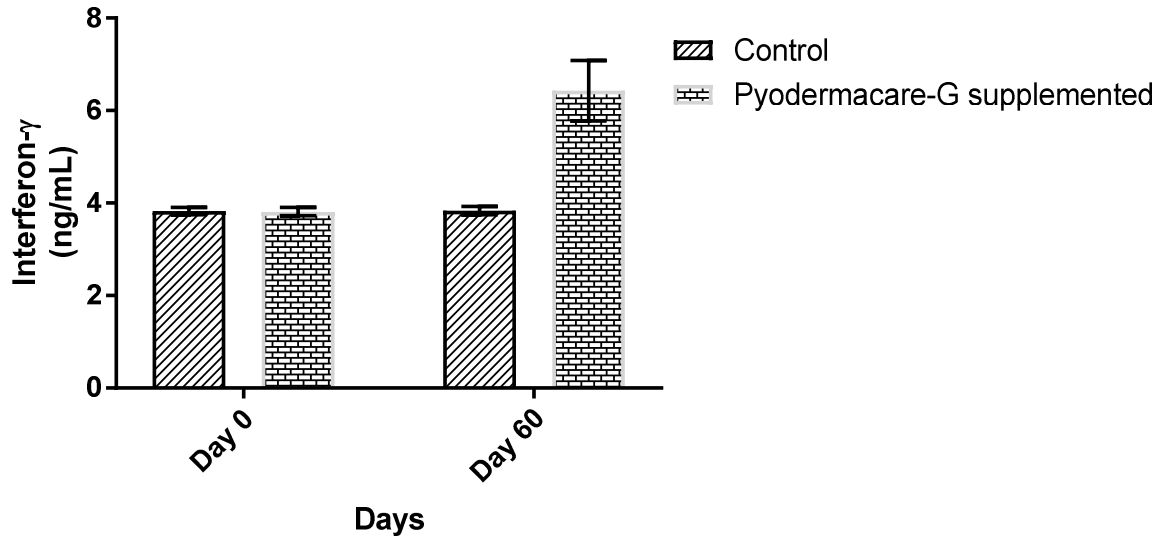

Fig.10.7: Effect of Pyodermacare-G capsule supplementation on circulatory Interferon-γ level (Mean+SE)

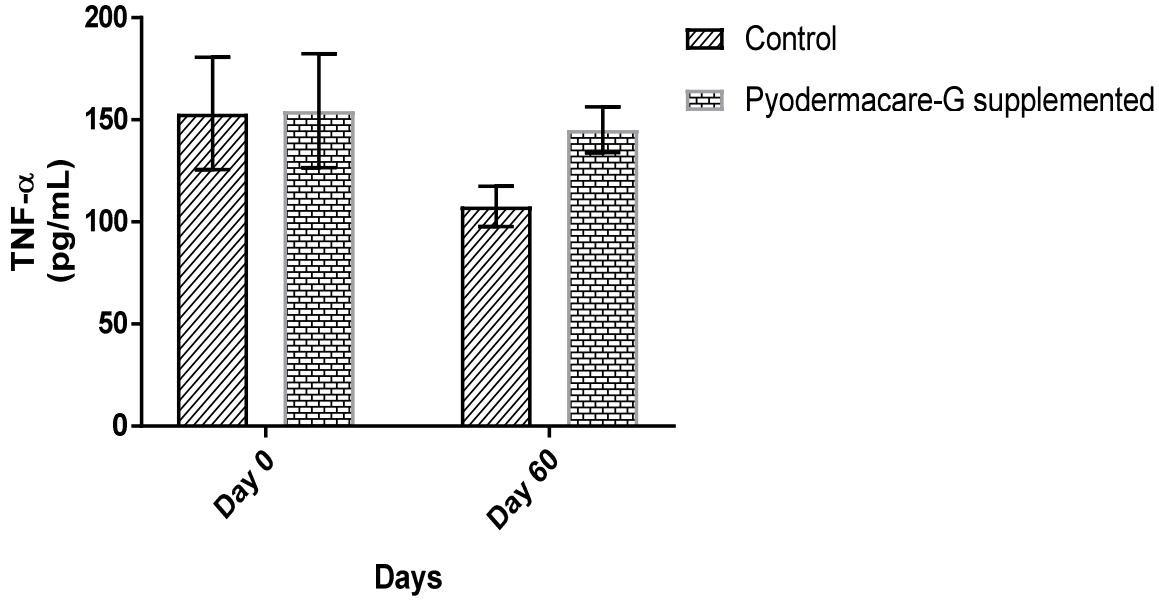

Fig. 10.8: Effect of Pyodermacare-G capsule supplementation on circulatory TNF-α level (Mean+SE)

revealed by the dogs of this group at day 60 post-therapy as compared with their own day 0 values.

The demodicosed dogs supplemented with Pyodermacare-G (Group 2) found have significant ($P<0.001$) reduction in circulatory IL-10 (Fig.10.6) and significant ($P<0.001$) increment in IFN-γ levels (Fig.10.7) at day 60 post-therapy as compared with their own day 0 values. In tandem, remarkable reduction in circulatory level of TNF-α was not revealed by this group at day 60 post-therapy (Fig.10.8) as compared with their own day 0 values.

Noticeably at day 60 post-therapy, the demodicosed dogs supplemented with Pyodermacare-G found to have significantly ($P<0.001$) lower circulatory IL-10 level as compared with day 60 post-therapy values of control group (Group 1). Agreeably, Pyodermacare-G supplemented dogs also revealed significant higher circulatory TNF-α ($P<0.02$) and IFN-γ ($P<0.001$) levels as compared with the same day values of control dogs (Group 1).

Effects on parasitological recovery

The parasitological recovery e.g. reduction in number of total mites counts (developing and adult stages of *Demodex* mites) and percentage reduction in total mite counts of both the studied groups of demodicosed is depicted in Table 5. At day 30 post-therapy, the per cent reduction in total mites counts in Pyodermacare-G supplemented dogs was significantly ($P<0.0001$) higher as compared with the same days values of non-supplemented group (Group 1). Similarly at day 60 post-therapy, the per cent reduction in total mites counts in Pyodermacare-G supplemented dogs was also significantly ($P<0.0001$) higher as compared with that of the same day values of non-supplemented group. A total of 52.42±3.83 % and 92.87±2.3 % reduction in mite counts was revealed by the dogs of control group at day 30 and day 60 post-therapy, respectively. Whereas, per cent reduction in mite counts of Pyodermacare-G supplemented dogs was 82.27±1.27 and 99.5±0.28 at day 30 and 60 post-therapy, respectively (Fig.10.1). None of the dogs of control group were found negative for the presence of mites and their developmental stages at day 60 post-therapy. Whereas, out of 10 demodicosed dogs supplemented with Pyodermacare-G, nine dogs were found negative for the presence of mites and their developmental stages at day 60 post-therapy.

Effects on clinical recovery

The clinical recovery in skin lesions at various days of therapy of control group has been shown in Figs (2.1, 2.2, 2.3 and 3.1, 3.2, 3.3). The clinical recovery in skin lesions of Pyodermacare-G supplemented demodicosed dogs at various days of therapy has been shown in Figs (4.1, 4.2, 4.3, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.2, 8.3, and 9.1, 9.2, 9.3). The clinical recovery e.g. improvement in Demodex-induced skin lesions score (DSLS) of

Table 5: Effects of Pyodermacare-G supplementation on parasitological recovery of dogs with generalised demodicosis.

Days post-therapy	Controls (Group 1; n=09) (% reduction in mites count)	Pyodermacare-G supplemented (Group 2; n=10) (% reduction in mites count)	*P values*
Day 30	52.42±3.83	92.87±2.3[¥]	0.0001
Day 60	82.27±1.27	99.5±0.28[¥]	0.0001

[¥]Significant (*P<0.0001*) difference, when compared with same day post-treatment values of Group 1.

Table 6: Effects of Pyodermacare-G supplementation on clinical recovery of dogs with generalised demodicosis.

Days post-therapy	Controls (Group 1; n=09) (% improvement in DSLS)	Pyodermacare-G supplemented (Group 2; n=10) (% improvement in DSLS)	*P values*
Day 30	44.31±3.23	86.29±2.88[¥]	0.0001
Day 60	73.19±3.44	98.01±0.85[¥]	0.0001

[¥]Significant (*P<0.0001*) difference, when compared with same day post-treatment values of Group 1.

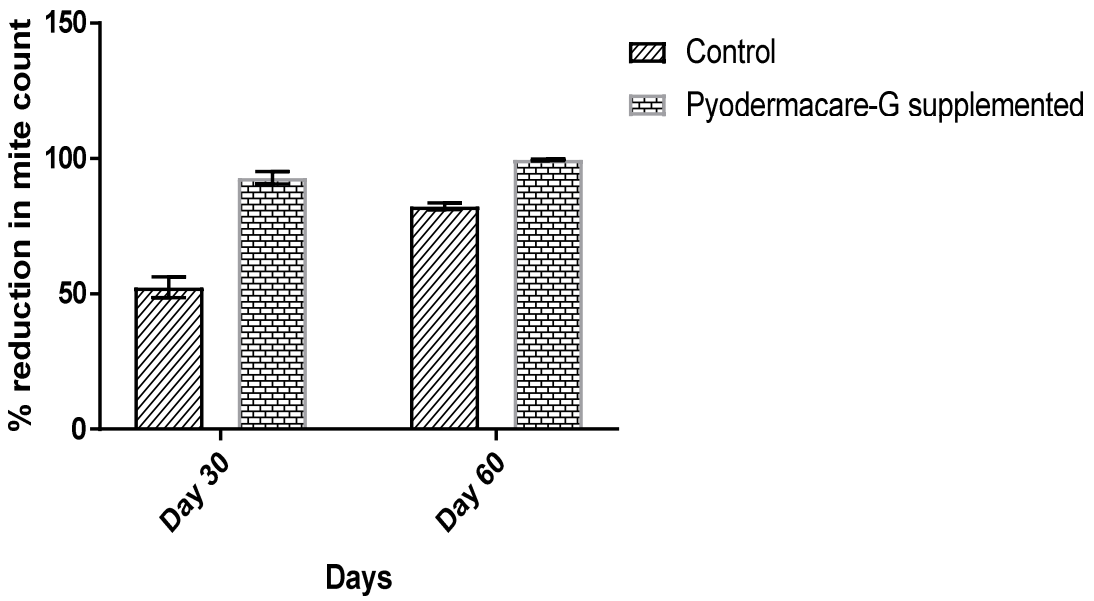

Fig 10.1: Effect of Pyodermacare-G capsule supplementation on per cent reduction in mite counts (Mean+SE)

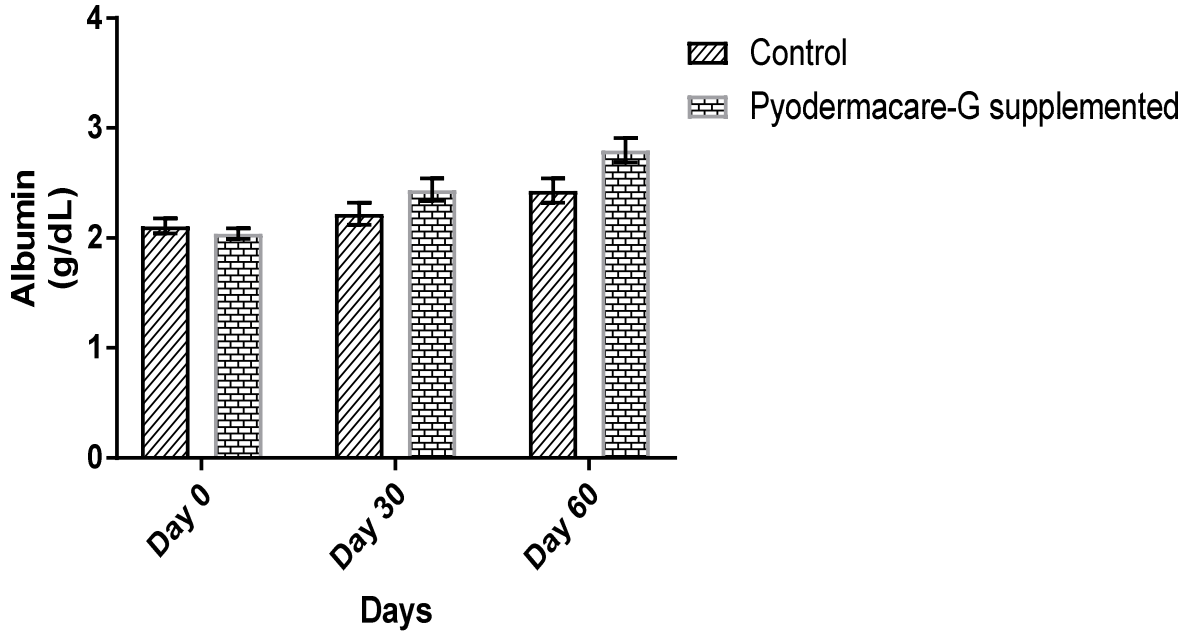

Fig.10.2: Effect of Pyodermacare-G capsule supplementation on Albumin level (Mean+SE)

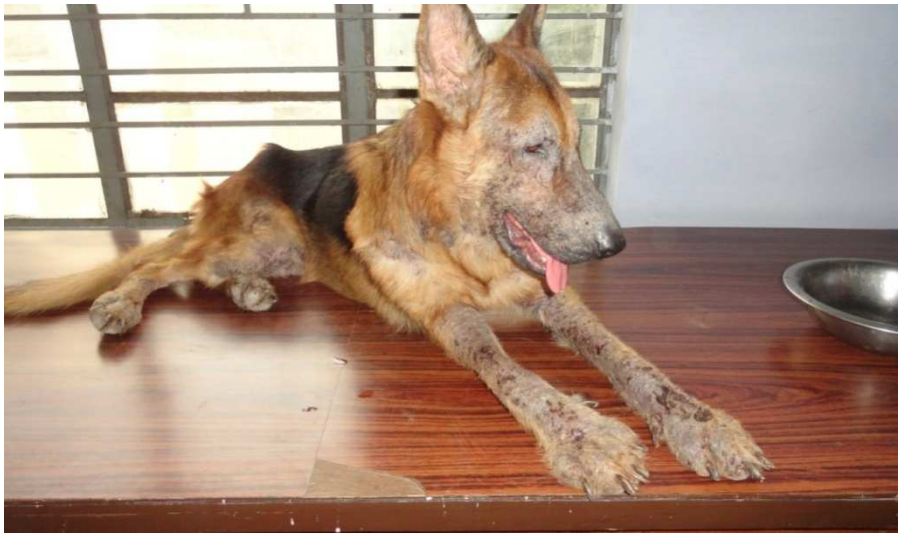

Fig. 2.1: A 18-month-old male demodicosed German Shepherd dog of control group before therapy

Fig. 2.2: Same dog of control group at day 30 post-therapy

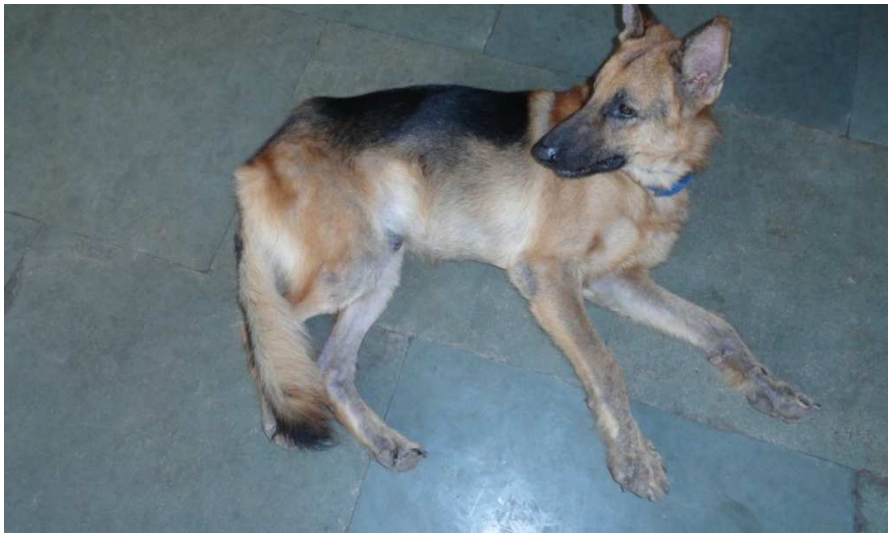

Fig. 2.3: Same dog of control group at day 60 post-therapy

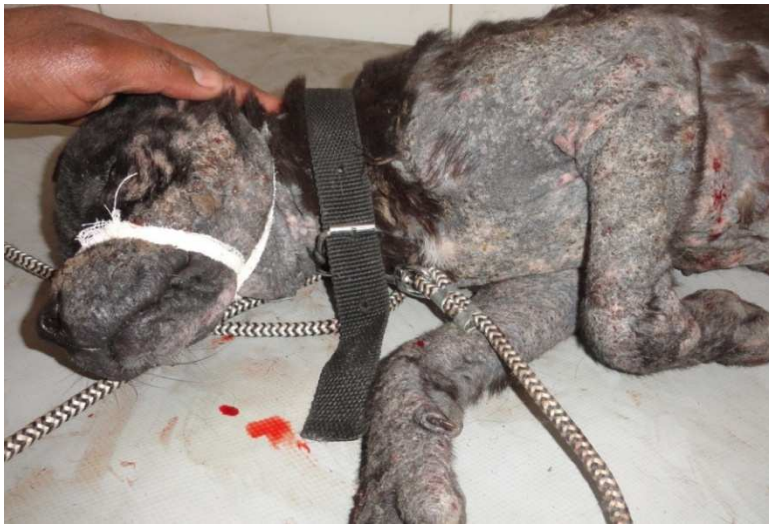

Fig. 3.1: A 12-month-old female demodicosed Mongrel dog of control group before therapy

Fig. 3.2: Same dog of control group at day 30 post-therapy

Fig. 3.3: Same dog of control group at day 60 post-therapy

diseased of both the studied groups of demodicosed is depicted in Table 6. At day 30 post-therapy, the per cent improvement DSLS in Pyodermacare-G supplemented dogs was significantly ($P<0.0001$) higher as compared with the same days values of non-supplemented group (Group 1). Similarly at day 60 post-therapy, the per cent improvement DSLS in Pyodermacare-G supplemented dogs was also significantly ($P<0.0001$) higher as compared with the same days values of non-supplemented group (Group 1)**.** Albeit, 44.31±3.23 % and 86.29±2.88 % improvements in DSLS were revealed by the dogs of control group at day 30 and day 60 post-therapy, respectively. However, per cent improvements in DSLS of Pyodermacare-G supplemented dogs were 73.19±3.44 and 98.01±0.85 at day 30 and 60 post-therapy, respectively (Fig.10.4). Moreover, an appreciable improvement in food intake and body coat lustre was revealed by the dogs of Pyodermaccare-G supplemented group as compared with the control ones. None of the diseased dogs of Pyodermacare-G supplemented group revealed recurrence of the clinical diseases during the 3-6 months of follow-up period. Whereas out of nine enrolled dogs control group, seven dogs revealed recurrence of the clinical diseases within 3 months of the withdrawal of miticidal therapy. Additionally, the demodicosed dogs of control group required 120-150 days to become negative for the presence of the mites on skin scraping examination during the recommended regimen of miticidal application. The demodicosed dogs of control group with concurrent pyoderma require antibiotic therapy for prolong period (10-20 days), whereas Pyodermacare-G supplemented demodicosed dogs with concurrent pyoderma required antibiotic therapy on for five days. Marker improvement in appetite and body coat lustre was also noticed in demodicosed dogs supplemented with Pyodermacare-G.

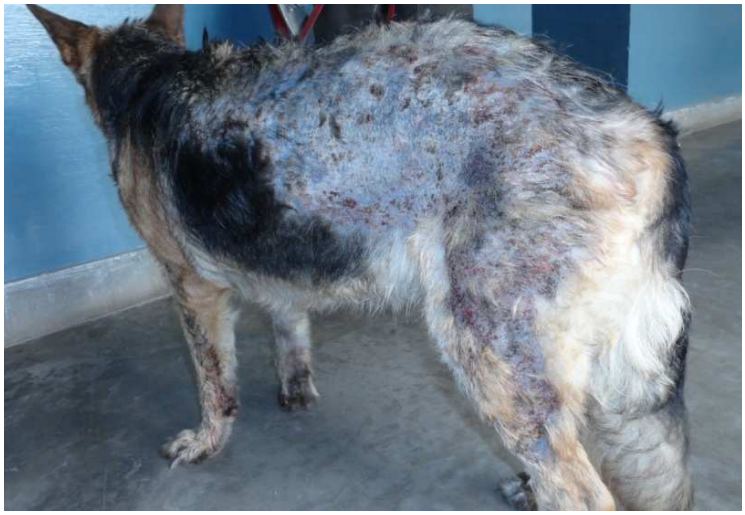

Fig. 4.1: A 24-month-old female demodicosed German Shepherd dog of Pyodermacare-G supplemented group before therapy

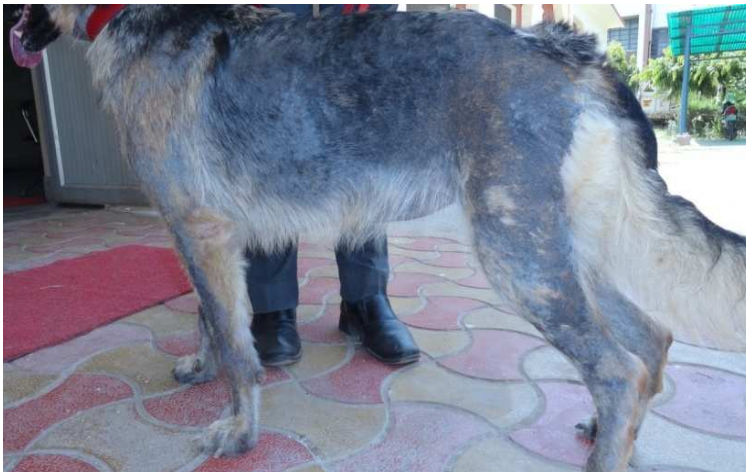

Fig. 4.2: Same dog of Pyodermacare-G supplemented group at day 30 post-therapy

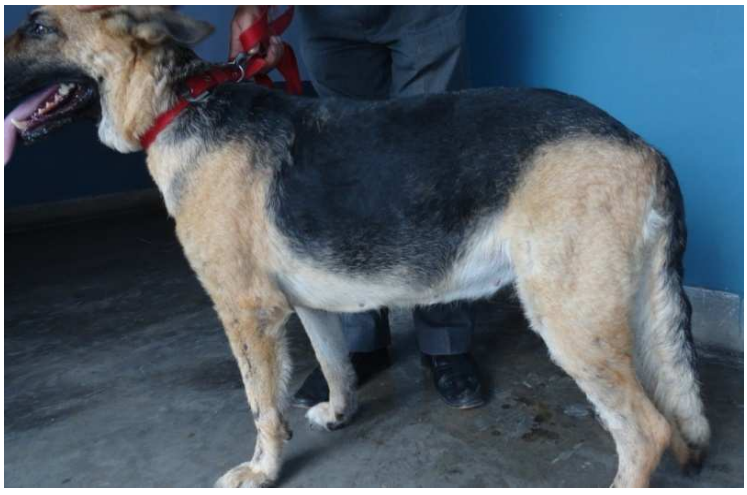

Fig. 4.3: Same dog of Pyodermacare-G supplemented group at day 60 post-therapy

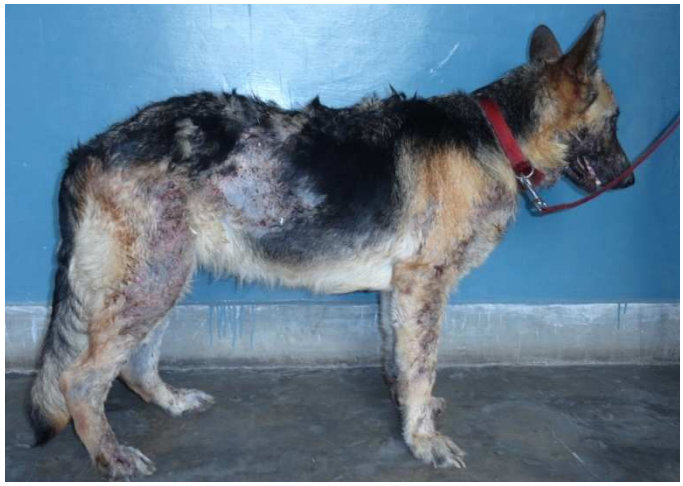

Fig. 5.1: Same 24-month-old female demodicosed German Shepherd dog of Pyodermacare-G supplemented group before therapy in another view

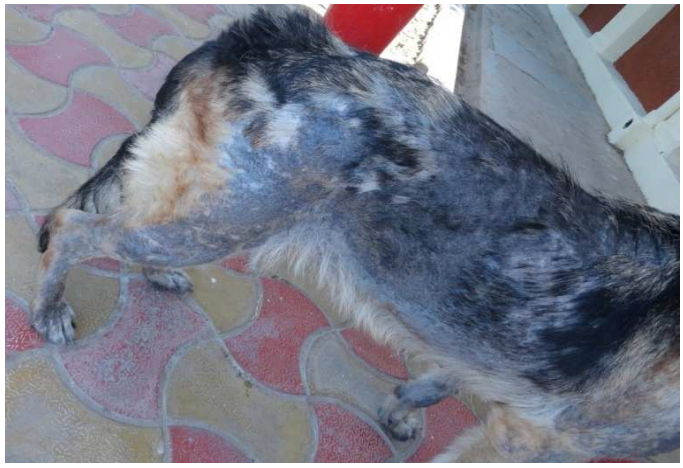

Fig. 5.2: Same dog of Pyodermacare-G supplemented group at day 30 post-therapy

Fig. 5.3: Same dog of Pyodermacare-G supplemented group at day 60 post-therapy

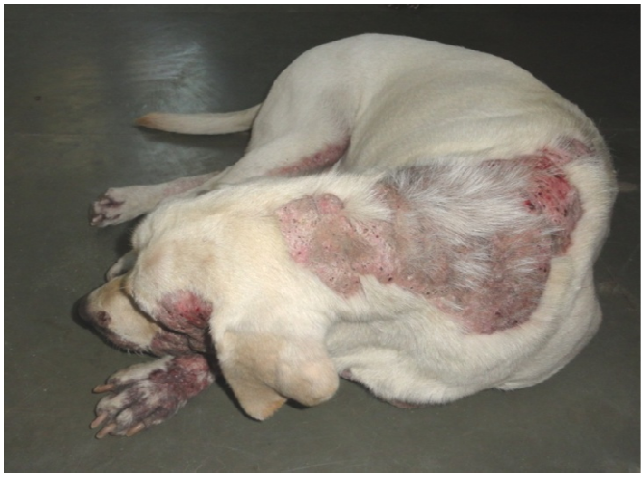

Fig. 6.1: A 30-month-old female demodicosed Labrador Retriever dog of Pyodermacare-G supplemented group before therapy

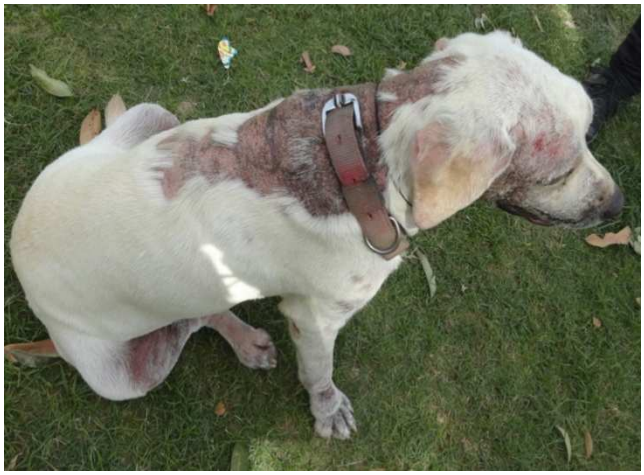

Fig. 6.2: Same dog of Pyodermacare-G supplemented group at day 30 post-therapy

Fig. 6.3: Same dog of Pyodermacare-G supplemented group at day 60 post-therapy

Fig.7.1: Same 30-month-old male demodicosed Labrador Retriever dog of Pyodermacare-G supplemented group before therapy in another view

Fig.7.2: Same dog of Pyodermacare-G supplemented group at day 30 post-therapy

Fig. 7.3: Same dog of Pyodermacare-G supplemented group at day 60 post-therapy

Fig. 8.1: A 15-month-old female demodicosed German Shepherd dog of Pyodermacare-G supplemented group before therapy

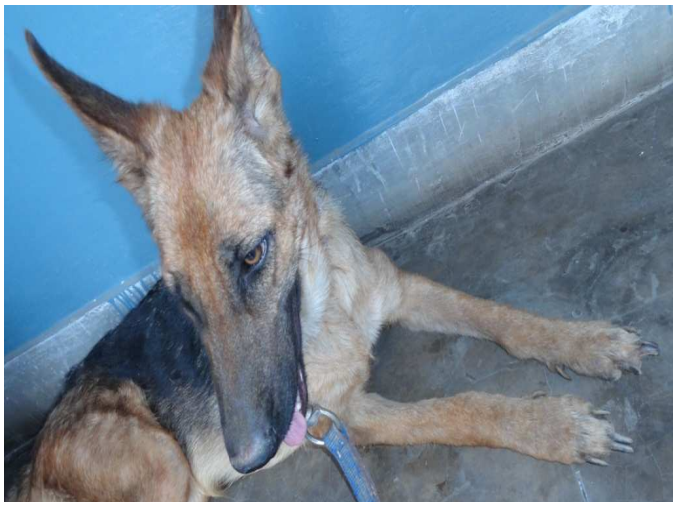

Fig. 8.2: Same dog of Pyodermacare-G supplemented group at day 30 post-therapy

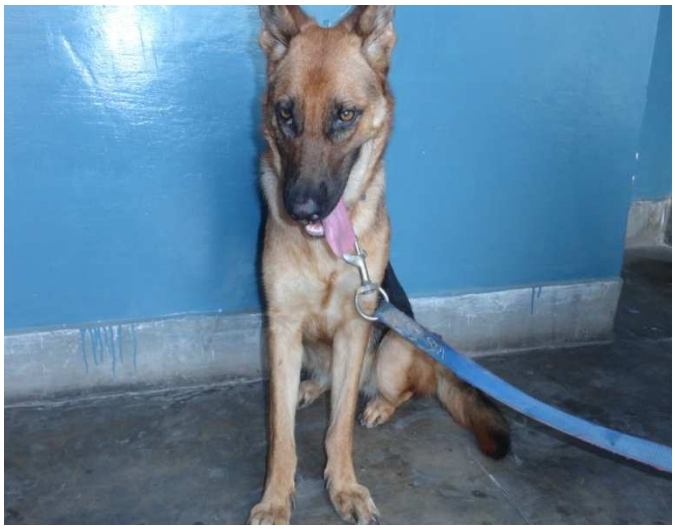

Fig. 8.3: Same dog of Pyodermacare-G supplemented group at day 60 post-therapy

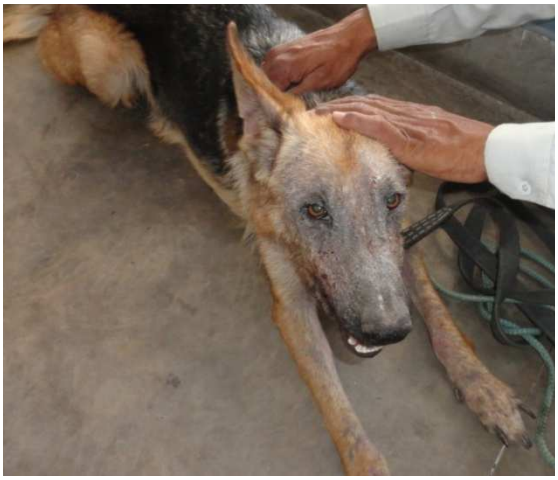

Fig. 9.1: A 8-month-old female demodicosed German Shepherd dog of Pyodermacare-G supplemented group before therapy

Fig. 9.2: Same dog of Pyodermacare-G supplemented group at day 30 post-therapy

Fig. 9.3: Same dog of Pyodermacare-G supplemented group at day 60 post-therapy

CHAPTER- 5

DISCUSSION

Effects on Haematology

The results of the present study evidently indicate the marked alterations in haematological panels of dogs with generalised demodicosis and need of amelioration for holistic management of diseased animal health. The demodicosed dogs of control group that were only treated with the miticide have not revealed remarkable amelioration in the leukograms at days 30 and 60 post-therapy. Whereas, the demodicosed dogs adjunctly supplemented with Pyodermacare-G found to have remarkably ameliorated leukograms, mainly TLC and granulocytes at days 30 and 60 post-therapy. Moreover at days 30 and 60 post-therapy, the dogs of this group also found to have remarkably lower values of TLC, granulocytes and lymphocytes as compared with the same days post-therapy values of non-supplemented dogs (Group 1). Therefore, the results of our study evidently suggest that the bungling inflammatory process is still operation at day 60 post-therapy in the demodicosed dogs underwent for miticidal therapy only.

Forton (2012) reported that cutaneous inflammation is associated with excessive proliferation of mites and clinical recovery of the diseased dogs often entails parasiticidal treatment and reduction in the mite's population. Neutrophils play a critical role in the innate immune system by phagocytosing, killing and digesting microbial cells (Bellocchio et al., 2004) and have been implicated in the pathogenesis of rosacea of human patient where *Demodex* has the key role in pathogenesis (O'Reilly *et al.*, 2012). Recently, a bacterium (*Bacillus oleronius*) isolated from a *D. folliculorum* mite from a patient with rosacea produced proteins that induced an inflammatory immune response in 72% of patients with papulopustular rosacea (O'Reilly *et al*., 2012). At day 60 post-therapy, most of the demodicosed dogs of control group were found to have presence of mites and complete parasitological cure was not revealed by them. Therefore, remarkably elevated TLC and granulocytes at day 60 post-therapy in control group could be the resulted from the dog's immune response to ameliorate the mite's proliferation. In agreement to our observation, Reddy *et al*. (2015) demonstrated an elevated total TLC and absolute number of granulocytes in dogs with demodicosis. The diseased dogs supplemented with Pyodermacare-G revealed remarkable amelioration in the leukograms e.g., reduction in absolute granulocytes counts and increment in lymphocytes percentage at days 30 and 60 post-therapy. As remarkable reduction in mite counts was revealed by the dogs of Pyodermacare-G supplementation group and most of the dogs were found negative for mites at day 60 in this group, the mitcidal effects amitraz might be augmented its supplementation via mitigation of immunological

dents of demodicosed dogs. The absence of mites and/or remarkably reduced mites at days 30 and 60 post-therapy in the dogs of this group might responsible for amelioration of leukograms. These finding validates the potential immuno-restorative activities of the polyherabal formulation.

Moreover at days 30 and 60 post-therapy, remarkable improvements in haemograms including TEC, Hb and HCT were not revealed by the demodicosed dogs of control group. Whereas, at the same days of therapy; the demodicosed dogs supplemented with Pyodermacare-G revealed remarkable amelioration in the haemograms. These dogs also revealed remarkably higher levels of TEC, Hb and HCT as compared with the same day's values of control group. Reddy *et al*. (2015) demonstrated an elevated remarkably lower haemograms including TEC, Hb and HCT in dogs with generalised demodicosis. Remarkably shoddier haemograms of dogs with generalised demodicosis is attributed to the loss of skin protein (Deb *et al.*, 2000). Moreover, the current literature does clearly support the general concept that oxidative stress causes extensive cellular damage throughout the body and can result in compromised immune and inflammatory reactions (Victor *et al*., 2004; Nicolls *et al*., 2007). An overproduction of free radicals, shifting the antioxidative status toward oxidative side has been demonstrated in dogs with demodicosis (Dimri *et al*., 2008). It is known that inflammatory cells are increased as a result of inflammation in animals with mange; recruited neutrophils and macrophages produce reactive oxidants such as hydrogen peroxide (H2O2), hypochlorite and oxygen radicals. These reactive oxygen substances produced by cells of immune system shows potent cytotoxic effects on parasites and as well as on other pathogenic organisms (Gurgoze *et al.*, 2003). Free radicals can also induce or contribute adverse effects on the host (Bickers and Athar, 2006). Therefore, free radical mediated damage and/or production of erythrocytes may be attributed to alterations in the haemograms of dogs with generalised demodicosis. This hypothesis is also supported by the findings of the present study that the dogs of control group revealed remarkable clinical recovery at days 30 and 60 post-therapy but still they found to have markedly high levels of inflammatory cell mainly granulocytes. Whereas, the dogs supplemented with the polyherbal formulation revealed marked reduction in inflammatory cell as well as improvement in haemograms. Herbal drugs/Indigenous drugs exhibited multiple immunomodulatory actions including modulation of cytokine secretion, histamine release, immunoglobulin production, immunoglobulin class switching, cellular co-receptor expression, lymphocyte proliferation and phagocytosis promotion (Spelman *et al*., 2006). The antioxidant potentials of various herbal medicines have been well validated in the both clinical as well as in laboratory animal studies. Singh *et al*. (2010) demonstrated the antioxidant augmenting potential *Withania somnifera* extract (WSE) in dogs with demodicosis (Singh *et al.*, 2010). Singh *et al*. (2011) also demonstrated

the ameliorative potential of *Withania somnifera* extract against the altered $CD4^+/CD8^+$ balance of dogs with demodicosis. Nutritional status, immunological aberrations and misbalance of oxidant/antioxidant status of the demodicosed dogs may be associated in the progression of clinical disease condition (Dimri *et al*., 2008; Singh *et al*., 2010, 2011). Therefore, it is quite possible that Pyodermacare-G supplementation might has lucratively ameliorated the altered leukograms and oxidant/antioxidant balance of dogs with generalised demodicosis to augment the haemograms at days 30 and 60 post-therapy.

Effects on serum biochemistry

Demodicosed dogs of the control group (Group 1) did not revealed remarkable alteration in most of the altered serum biochemical panels including cholesterol, triglycerides, and albumin contents at day 30 and day 60 post-therapy as compared with their own day 0 values. Whereas, the demodicosed dogs supplemented with Pyodermacare-G (Group 2) found have remarkably lower level of total cholesterol and higher levels total protein, albumin and globulins at day 60 post-therapy as compared with their own day 0 values. Additionally the dogs of this group found to have remarkably lower levels of total cholesterol and triglycerides and higher level of albumin compared with the day same days post-therapy values of non-supplemented dogs. Recently, hypo-albunemia and hyper-lipidemia has been reported in dogs with generalised demodicosis (Pradhan *et al*., 2012; Reddy *et al*., 2015). Lower albumin levels in dogs with generalised demodicosis could be attributed to continuous loss of plasma protein through oozing from the *Demodex* induced severe skin lesions in adjunct to the reduced appetite. Hyper-lipidemia could be resulted from the stress of infection and body fat mobilisation due reduced appetite of the severely infected dogs. Remarkable amelioration in lowered albumin levels in Pyodermacare-G supplemented dogs could be attributed to faster clinical recovery in skin lesions as well restoration of the appetite of affected dogs. Liver is the primary organ for synthesis of most of the plasma proteins except immunoglobulins. Especially albumin levels indicate the synthetic function of liver (Sunanda *et al*., 2012). Most of the herbs have augmenting properties to the hepatic functions. Our polyherbal formulation (Pyodermacare-G) may have hepatio-stimulatory activity helping to restore the appetite of demodicosed dogs and augmenting the protein synthesis resulting into faster amelioration of albumin level. Moreover, a faster reduction in circulatory lipids in Pyodermacare-G supplemented dogs could be attributed to shifting of catabolic status of demodicosed dogs towards the anabolic side via restoration of appetite, faster healing of skin lesions, amelioration of elevated leukograms mediated stress and hepato-stimulatory activities.

Alkaline phosphatase (ALKP) is a group of enzymes that remove phosphate groups from many types of molecules in the body. Alkaline phosphatases are present in many tissues, including bone, intestine, kidney, liver, placenta and white blood cells (Marshall, 1972). The

dogs of control group found to have remarkably elevated leukocytes counts at days 30 and 60 post-therapy as compared with the Pyodermacare-G supplemented groups. An elevated level of ALKP in control group dogs with generalised demodicosis at days 30 and 60 post-therapy could be of leukocytes origin. As the demodicosed dogs supplemented Pyodermacare-G have successfully ameliorated the leukograms at days 30 and 60 post-therapy, amelioration of leukograms could be the possible rationale for the mitigation of elevated ALKP in this group.

Effects on Circulatory Cytokines

Albeit, demodicosed dogs of control group, that were not supplemented with Pyodermacare-G, found have significant ($P<0.02$) reduction in IL-10 level at day 60 post-therapy as compared with their own day 0 values. Circulatory IL-10 level in this group was still significantly ($P<0.001$) higher than the Pyodermacare-G supplemented group at day 60 post-therapy. Contrarily, the dogs of Pyodermacare-G supplemented group revealed significantly higher circulatory TNF-α ($P<0.02$) and IFN-γ ($P<0.001$) levels as compared with the dogs of control group at day 60 post-therapy. Additionally, Pyodermacare-G supplemented dogs found have significant ($P<0.001$) reduction in circulatory IL-10 and significant ($P<0.001$) increment in IFN-γ levels at day 60 post-therapy as compared with their own day 0 values. Whereas, remarkable amelioration in circulatory TNF-α and IFN-γ levels was not revealed by the dogs of control group at day 60 post-therapy as compared to their own day 0 values.

The T helper cells (Th) play an important role in the immuno-regulation and immuno-stimulation and can be classified in Th1, Th2 and Th17 cells, which produce different patterns of cytokines (Saito *et al*., 2010). Th1 and Th2 produce cytokines with opposite effects, pro-inflammatory and anti-inflammatory respectively, but their production is suppressed by immunoregulatory cytokines, such as transforming growth factor (TGF)-β and IL10 or by cell-to-cell interaction (Saito *et al*., 2010). A balance between Th-1 and Th-2 cell subpopulation and production of cytokines coordinates immune response to ensure pathogen elimination without causing the disease (Carter and Dutton, 1996). The exact pathogenesis of generalized canine demodicosis is obscure, however, an aberration in immune status is thought to be most significant (De Bosschere *et al.* 2007; Singh *et al.,* 2010; Ferrer *et al.,* 2014). Host's immune system appears to detect and tolerate the presence of these mites, and also has an inhibitory effect on mites proliferation and keeps the mites number low without inducing an inflammatory response (Akilov *et al.*, 2004; Forton, 2012). Obstinately, it is quite possible that *D. canis* mites regulate the host immune response and induce immunosuppressive pathways to evade the host's defence system and perpetuate in the microenvironment (Akilov *et al.,* 2005; Singh *et al.,* 2011; Singh and Dimri, 2014). Scientific studies have demonstrated the potential association of anti-inflammatory and/or

immunosuppressive cytokines including transforming growth factor beta (TGF-β) and IL-10 with immunosuppressive pathogenesis of canine demodicosis (Tani *et al.* 2002, Felix *et al.* 2013; Yarim *et al*. 2013). Felix *et al*. (2013) reported that the elevated serum levels of IL-10 are strongly associated with recurrent demodicosis in dogs. They also demonstrated elevated levels of IL-10 in dogs encountered with demodicosis for the first time. IL-10 cytokine is an essential molecule in the mechanism underlying suppression mediated by T regulatory cells (Moore *et al*., 1990). IL-10 is also called the cytokine synthesis inhibitor factor, since it has the ability to inhibit the synthesis of Th1 cytokines (IL-1, IFN-γ, and TNF-α) as well as inhibiting the function of NK cells (Howard and O'garra, 1992). It has anti-inflammatory suppressive effects on the most of hematopoietic cells. It indirectly suppresses cytokine production and proliferation of antigen-specific CD4+ T effecter cells, by inhibiting the antigen-presenting capacity of various types of professional antigen-presenting cells (Roncarolo *et al*., 2006). IL-10 mainly targets on Th1 cells, B cells, macrophages, NK cells, mast cells, and thymocytes (Tizard, 2002). Tani *et al.* (2002) also demonstrated down regulation of TNF-α in dog with demodicosis. TNF- α is a pleiotropic pro-inflammatory cytokine that exerts multiple biological effects. At low level expression of TNF-α participates in beneficial tissue remodelling and host defense response. The expression of TNF-α is tightly controlled, because systemic over production of TNF-α activates inflammatory response to infection and injury, and mediates hypotension, diffused coagulation and widespread tissue damage. Recently, Kumari *et al.* (2017) demonstrated the potential association of circulatory cytokines with progression of the clinical demodicosis in dogs. They reported remarkably elevated circulatory levels of IL-10 and lowered level of TNF-α in dogs with generalised demodicosis in comparison to dogs with localised demodicosis and healthy ones.

Recent studies on the function of T lymphocytes and their involvement in the immune response of dogs with clinical disease provide an explanation to the cellular reactions which occur in demodicosis (Fukata *et al*., 2005). Previously, we have demonstrated the specific downregulating activity of *D. canis* mites on CD4+ T cells and hypothesized the possibility of an increased rate of apoptosis or immunological exhaustion of CD4+ T cells in demodicosed dogs (Singh *et al*., 2010). Detrimental effects caused by apoptosis can be triggered by parasitic infection, depending upon the specific host–parasite situations (Bienvenu *et al*., 2010). Moreover, we have also endorsed an increased apoptosis of peripheral leukocytes of dogs with demodicosis conferring the progression of the clinical manifestations (Singh *et al*., 2011). Therefore, Demodex mites could also induce immuno-incompetence in the demodicosed dogs and thus can lead to the flare-up of clinical disease (Singh and Dimri, 2014). At day 60 post-therapy remarkably lower circulatory TNF-α and higher IL-10 levels in the dogs of control group clearly indicates that immunosuppressive pathways are still

operational at day 60 pot-therapy in control group. This also signifies the paramount requisite of supplementations having immunomodulatory potentials along with the conventional miticidal therapies to manage generalised demodicosis in dogs. The results of the present study evidently demonstrated the promising ameliorative potential of our polyherbal formulation (Pyodermacare-G) over circulatory cytokines contents in dogs with generalised demodicosis. Marked elevations in circulatory IFN-γ and TNF-α levels in Pyodermacare-G post-therapy; signifies the potential Th-1 cells up-regulatory activities of this polyhedral formulation. Additionally, marked mitigation of circulatory IL-10 level was revealed by the demodicosed dogs supplemented with Pyodermacare-G, signifies the holistic immuno-restorative potential of this novel polyherbal formulation in dogs with generalised demodicosis. Thus, it can be evidently said that the novel polyherbal formulation (Pydermacare-G) could holistically regulate Th1/Th2 cytokines balance in dogs with generalised demodicosis and can shift the balance towards the Th-1 side to enhance the clinical and parasitological recovery form demodicosis.

Effects on parasitological and clinical recovery

Remarkable reduction in total mites counts was recorded dogs supplemented with Pyodermacare-G at days 30 and 60 post-therapy as compared with the same days values of non-supplemented control group. Additionally, none of the dogs of control group were found negative for the presence of mites and their developmental stages at day 60 post-therapy. Whereas, out of 10 demodicosed dogs supplemented with Pyodermacare-G, nine dogs were found negative for the presence of mites and their developmental stages at day 60 post-therapy. Moreover at days 30 and 60 post-therapy, improvement in Demodex-induced skin lesions score (DSLS) was also remarkably higher in Pyodermacare-G supplemented dogs as compared with the same days values of non-supplemented control dogs. None of the diseased dogs of Pyodermacare-G supplemented group revealed recurrence of the clinical diseases during follow-up period. Whereas, most of the dogs of control group revealed recurrence of the clinical diseases within 3 months of the withdrawal of miticidal therapy. The body coat lustre and increment in appetite of the Pyodermacare-G supplemented dogs was also appreciable.

The *Demodex* mite, moving with four pairs of legs, each of them bearing two claws, is responsible for the erosion of the epithelium (Forton, 2012). While eating cells the mite can penetrate into the dermis (Hsu *et al*., 2009) as the cutaneous barrier becomes disrupted, the TLR are, as a rule, stimulated (Schauber *et al*., 2007) and the *Demodex* antigens are exposed to the host immune system. Stimulated TLRs could be attributed to the migration and infiltration of leukocytes at the site of infestation and further induction of the skin lesions of demodicosis. It is striking that the majority of the cytokines, which disrupt the barrier

function of the epidermis in model systems, are also deregulated in skin diseases (Hänel *et al.*, 2013). Thus, it is quite obvious that the management of cytokine expression and activity is important to control and ameliorate disease development in the skin (Hänel *et al.,* 2013). Faster clinical recovery in Pyoderma-G supplemented groups could also be attributed to the healing effects of the novel formulation by repairing the breach in skin barrier functions as well as immunological regulation. Therefore, the rates of clinical and parasitological recoveries as well as non-recurrence of the clinical condition after the withdrawal of miticidal therapy in Pyoderma-G supplemented groups designates the holistic mitigating potentials of this novel polyherbal formulation over the *Demodex*-induced immuno-clinico-pathology in dogs. In tandem, the immuno-clinico-pathological status of the demodicosed dogs of control group at day 60 post-therapy evidently suggest the outmost need of adjunctive supplements along with the miticidal therapy to defeat the indomitable canine generalised demodicosis.

CHAPTER-6

SUMMARY AND CONCLUSIONS

Canine demodicosis is a common but exigent noncontagious parasitic dermatosis caused by overpopulation of the host-specific follicular mites of various *Demodex* species. In canine demodicosis, cutaneous inflammation is associated with excessive numbers of proliferating mites, including immature form (eggs, larvae and nymphs), and the clinical cure is clearly associated with parasiticidal treatment and reduction in the number of *Demodex* mites. Generalized demodicosis may be a severe and potentially life-threatening dermatological condition in dogs. The dogs with generalized demodicosis showing spontaneous cure is unknown presently. The management of canine demodicosis remains one of the main challenges in veterinary dermatology. Appearance of canine generalised demodicosis is greatly influenced by breech in T-cell immunity, rather than any breech in hummoral immunity. Literatures repeatedly make a statement that a genetically preprogrammed immunological defect is responsible for the exaggerated replication of mites in demodicosis. Alternatively, the decreased immune response could also be due to the *Demodex* mites themselves. A breach in the immune status of the young dogs results into the occurrence of generalized demodicosis**.** The mechanism of cytokine secretion from T lymphocytes has played important roles in the immune response of the dogs against Demodex-induced dermatological conditions. Increased TGF-β mRNA expression might be a key factor for revealing differences in the mechanism of onset between localized and generalized demodicosis**.** Recently studies revealed that elevated serum levels of IL-10 are strongly associated with recurrent as well as progression of demodicosis in dogs. IL-10 cytokine is an essential molecule in the mechanism underlying suppression mediated by T regulatory cells. Owing to wide prevalence of canine demodicosis, increasing incidence of drug resistance and detrimental side effects with the most of allopathic drugs, there is quest for the safe alternative adjunctive medicines. Cost-effective and affordable medicines to an Indian animal owner are call for the commendable researchers. Traditional medicines hold a great promise as source of easily available effective therapy for skin diseases to the people, particularly in tropical developing counties, including India. Therefore, the present study aimed to evaluate the effects of a polyherbal formulation on clinical recovery and immuno-competence of dogs with generalised demodicosis.

Client-owned dogs presented with dermatological ailments at Teaching Veterinary Clinical Complex (Kothari Hospital) of the University for clinical and dermatological examination were examined and diagnosis of demodicosis was made by detection of mature

and immature *Demodex canis* mites in scrapings and/or hair pluck samples from lesional skin. Dogs with generalised demodicosis of juvenile onset have only been included in the present study. The dogs diagnosed with generalised demodicosis were allocated into two groups (Group 1 and 2). Demodicosed dogs of Group 1 (n= 09) and Group 2 (n=10). The demodicosed dogs of Group 1 were treated with 0.0375% solution v/v of amitraz rinse at weekly intervals for 8 weeks. Whereas, demodicosed dogs of Group 2 were treated with 0.0375% solution v/v of amitraz rinse at weekly intervals and oral with one capsule (250 mg) twice in a day of a polyherbal formulation (Pyodermacare-G capsules) for 8 weeks. Demodicosed dogs of both of the groups were bathed thoroughly with 2.5% benzoyl peroxide shampoo and towel dried before each application of amitraz rinse. Demodicosed dogs diagnosed with concurrent pyoderma were also treated with injection lincomycin at a dose rate of 20 mg per kg of body weight once a day, intramuscularly for a period of minimum five days and first amitraz rinse was applied on day fifth in these dogs. The diseased dogs were clinically examined at the intervals of 30 ± 03 days post-therapy for clinical and parasitological recovery assay. The effects of polyherbal formulation on parasitological and clinical recovery were evaluated. Amelioration in heamto-biochemical panels was also evaluated. To evaluates the immunomodulatory potential of the polyherbal formulation competence, circulatory levels of interleukin-10 (IL-10), tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were estimated at day 0 and day 60 post-therapy by using canine specific ELISA kits following the procedure as described by the manufacturer. Statistical analysis were conducted to determine the difference between the groups by using one-way ANOVA, post-hoc Tukey's test with general linear models in SPSS 16. While, the comparison among the values within the same group at different time intervals were analyzed by the Repeated Measures approach using ANOVA with mixed linear models in SPSS 16.

The ameliorative potential of Pydermacare-G on haematology of dogs with generalised demodicosis was evaluated in terms of alterations in the both leukograms and haemograms. Remarkable alterations in the both leukograms and haemograms were not recorded on day 0 (before start of the therapy). Whereas, remarkable alterations in the both leukograms was recorded in both of the groups on day 30 and day 60 post-therapy when compared with their own day 0 values. Moreover, remarkable alterations in the both leukograms were also recorded in both of the groups on day 60 when compared with their own day 30 values. Demodicosed dogs of the control group (Group 1) have not revealed remarkable alteration in most of the altered serum biochemical panels including cholesterol, triglycerides, and albumin contents at day 30 and day 60 post-therapy as compared with their own day 0 values. However, significant amelioration in ALKP activity was recorded in this group at day 60 post-therapy as compared with their own day 0 values. Whereas, the

demodicosed dogs supplemented with Pyodermacare-G found have significant reduction in total cholesterol level at day 60 post-therapy as compared with their own day 0 values. Moreover, significant increment in total protein, albumin and globulins was revealed by the dogs of this group at day 60 pot-therapy as compared with their own day 0 values. At day 30 post-therapy, the dogs supplemented with Pyodermacare-G found to have significantly lower levels of total cholesterol and triglycerides as compared with day 30 post-therapy values of the non-supplemented dogs. Moreover at day 60 post-therapy, the dogs supplemented with Pyodemacare-G found to have significantly lower levels of total cholesterol and triglycerides as compared with the same day post-therapy values of non supplemented group. Contrarily, the dogs supplemented with Pyodemacare-G found to have significantly higher albumin level as compared with the day 60 post-therapy values of non-supplemented dogs. The demodicosed dogs of control group, that were not supplemented with Pyodermacare-G, found have significant reduction in IL-10 level at day 60 post-therapy as compared with their own day 0 values. Whereas remarkable amelioration in circulatory levels of TNF-α and IFN-γ was not revealed by the dogs of this group at day 60 post-therapy as compared with their own day 0 values. The demodicosed dogs supplemented with Pyodermacare-G (Group 2) found have significant reduction in circulatory IL-10 and significant increment in IFN-γ levels at day 60 post-therapy as compared with their own day 0 values. In tandem, remarkable reduction in circulatory level of TNF-α was not revealed by this group at day 60 post-therapy as compared with their own day 0 values. Noticeably at day 60 post-therapy, the diseased dogs supplemented with Pyodermacare-G found to have significantly lower circulatory IL-10 level as compared with day 60 post-therapy values of control group. Agreeably, Pyodermacare-G supplemented dogs also revealed significant higher circulatory TNF-α and IFN-γ levels as compared with the same day values of control dogs.

At day 30 post-therapy, the per cent reduction in total mites counts in Pyodermacare-G supplemented dogs was significantly higher as compared with the same days values of non-supplemented group. Similarly at day 60 post-therapy, the per cent reduction in total mites counts in Pyodermacare-G supplemented dogs was also significantly higher as compared with that of the same day values of non-supplemented group. At day 30 post-therapy, the per cent improvement DSLS in Pyodermacare-G supplemented dogs was significantly higher as compared with the same days values of non-supplemented group. Similarly at day 60 post-therapy, the per cent improvement DSLS in Pyodermacare-G supplemented dogs was also significantly higher as compared with the same days values of non-supplemented group**.** None of the diseased dogs of Pyodermacare-G supplemented group revealed recurrence of the clinical diseases during the 3-6 months of follow-up period. Whereas out of nine enrolled dogs control group, seven dogs revealed recurrence of the clinical diseases within 3 months of

the withdrawal of miticidal therapy. Additionally, the demodicosed dogs of control group required 120-150 days to become negative for the presence of the mites on skin scraping examination during the recommended regimen of miticidal application. The demodicosed dogs of control group with concurrent pyoderma require antibiotic therapy for prolong period (10-20 days), whereas Pyodermacare-G supplemented demodicosed dogs with concurrent pyoderma required antibiotic therapy on for five days. Marker improvement in appetite and body coat lustre was also noticed in demodicosed dogs supplemented with Pyodermacare-G.

The results of the present study evidently indicate the marked alterations in haematological panels of dogs with generalised demodicosis and need of amelioration for holistic management of diseased animal health. The results of the present study also evidently suggest that bungling inflammatory process is still operation at day 60 post-therapy in the demodicosed dogs underwent for miticidal therapy only. The diseased dogs supplemented with Pyodermacare-G revealed remarkable amelioration in the leukograms e.g., reduction in absolute granulocytes counts and increment in lymphocytes percentage at days 30 and 60 post-therapy. The polyherbal formulation revealed marked reduction in inflammatory cell as well as improvement in haemograms. Therefore, it is quite possible that Pyodermacare-G supplementation might had lucratively ameliorated the altered leukograms and oxidant/antioxidant balance of dogs with generalised demodicosis to augment the haemograms at days 30 and 60 post-therapy. Remarkable amelioration in lowered albumin levels in Pyodermacare-G supplemented dogs could be attributed to faster clinical recovery in skin lesions as well restoration of the appetite of affected dogs. Moreover, a faster reduction in circulatory lipids in Pyodermacare-G supplemented dogs could be attributed to shifting of catabolic status of demodicosed dogs towards the anabolic side via restoration of appetite; faster healing of skin lesions, amelioration of elevated leukograms mediated stress and hepato-stimulatory activities.

The T helper cells (Th) play an important role in the immuno-regulation and immuno-stimulation and can be classified in Th1, Th2 and Th17 cells, which produce different patterns of cytokines. Th1 and Th2 produce cytokines with opposite effects, pro-inflammatory and anti-inflammatory respectively, but their production is suppressed by immunoregulatory cytokines, such as transforming growth factor (TGF)-β and IL10 or by cell-to-cell interaction. A balance between Th-1 and Th-2 cell subpopulation and production of cytokines coordinates immune response to ensure pathogen elimination without causing the disease**.** At day 60 post-therapy remarkably lower circulatory TNF-α and higher IL-10 levels in the dogs of control group clearly indicates that immunosuppressive pathways are still operational at day 60 pot-therapy in control group. The results of the present study evidently demonstrated the promising ameliorative potential of our polyherbal formulation (Pyodermacare-G) over

circulatory cytokines contents in dogs with generalised demodicosis. Marked elevations in circulatory IFN-γ and TNF-α levels in Pyodermacare-G at day 60 post-therapy; signifies the potential Th-1 cells up-regulatory activities of this polyherbal formulation. Additionally, marked mitigation of circulatory IL-10 level was revealed by the demodicosed dogs supplemented with Pyodermacare-G, signifies the holistic immuno-restorative potential of this novel polyherbal formulation in dogs with generalised demodicosis. Therefore, it can be evidently said that the novel polyherbal formulation (Pydermacare-G) could holistically regulate Th1/Th2 cytokines balance in dogs with generalised demodicosis and can shift the balance towards the Th-1 side to enhance the clinical and parasitological recovery form demodicosis. Faster clinical and parasitological recovery in Pyoderma-G supplemented groups could also be attributed to the healing effects of the novel formulation by repairing the breach in skin barrier functions as well as immunological regulation. Therefore, the rates of clinical and parasitological recoveries as well as non-recurrence of the clinical condition after the withdrawal of miticidal therapy in Pyodermacare-G supplemented groups designates the holistic mitigating potentials of this novel polyherbal formulation over the *Demodex*-induced immuno-clinico-pathology in dogs. In tandem, the immuno-clinico-pathological status of the demodicosed dogs of control group at day 60 post-therapy evidently suggest the outmost need of adjunctive supplements along with the miticidal therapy to defeat the indomitable canine generalised demodicosis.

Therefore, it can be concluded that miticidal therapeutic regimens of canine generalised demodicosis warrants supplementary medicines having immunomodulatory potential for the holistic management and to get rid of the wretched clinical condition. The polyherbal formulation (Pyodermacare-G) could be promising candidate for the holistic managements of immuno-clinico-pathological anarchies of canine generalised demodicosis. Further studies are required to validate the précised immuno-regulatory activity of Pyodermacare-G particularly in terms of orchestrated regulation innate immunity (Toll like Receptors) and healing potentials particularly in terms of maintenance and/or repair in the breach of skin barrier of dogs with generalised demodicosis.

ABSTRACT

A breach in the immune status of the young dogs may results into the occurrence of generalized demodicosis**.** Cytokines secretions from T lymphocytes have played important roles in the immune response of the dogs against generalised demodicosis. Therefore, the present study aimed to evaluate the effects of a polyherbal formulation on clinical recovery and immuno-competence of dogs with generalised demodicosis. Total 19 client-owned dogs with generalised demodicosis of juvenile onset were allocated into two groups. Control group (n=9) were treated with conventional miticide (Amitraz), whereas other 10 demodicosed dogs were supplemented with a polyherbal formulation (Pyodermacare-G) adjunct with Amitraz regime. Clinico-haemato-biochemical and circulatory cytokines (TNF-α, IFN-γ and IL-10) were estimated pre- and post-therapies. Demodicosed dogs of the control group have not revealed remarkable amelioration in most of the altered serum haemato-biochemical and immunological panels at day 60 post-therapy. Remarkable parasitological and clinical recovery could not be achieved by the dogs of control group at day 60 post-therapy. Whereas, the demodicosed dogs supplemented with Pyodermacare-G revealed remarkable amelioration in haemato-biochemical and immunological panels (TNF-α, IFN-γ and IL-10) at day 60 post-therapy. Remarkable improvements in clinical and parasitological recovery were also revealed by the demodicosed dogs of Pyodermacare-G supplemented group. Therefore, it can be concluded that miticidal therapeutic regimens of canine generalised demodicosis warrants supplementary medicines having immunomodulatory potential for the holistic management and to get rid of the wretched clinical condition. The polyherbal formulation (Pyodermacare-G) could be promising candidate for the holistic managements of immuno-clinico-pathological anarchies of canine generalised demodicosis.

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