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CONTENTS

CONTENTS

Review Article

PANCHGAVYA: PHARMACO-THERAPEUTIC PERSPECTIVES

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ABSTRACT

Panchgavya means therapy of human or animal ailments using five cow products i.e. cows urine, milk, curd, dung and ghee. In India, panchgavya chikitsa has a unique place in Ayurveda. It is an alternate prophylactic and therapeutic approach for sound human and livestock health, which is safe, cheaper and without any side effects. The ancient ayurvedic literature (Vir Charak Samhita, Sushrut, Gad Nigrah) suggests a number of pharmacological applications of the substances used in panchgavya. In Ayurveda, there is a long tradition of using cow products for positive health, pharmaceutical processes and in therapeutics. All five products either alone or with each other possess many pharmaco-therapeutic properties against wide numbers of diseases and disorders of animals and human. The cow derived products are proved to possess anticancer, antidiabetic, antimicrobial, antiseptic, antibacterial, antifungal, immunomodulatory, hepatoprotective, anticonvulsant, antistress, analgesic, antihemorroids and many other therapeutic uses which are still to be explored. This review includes many diversified pharmaco-therapeutic utility of the components of panchgavya for health of animals and humans.

Keywords: Cow milk, cow urine, cowpathy, panchgavya, pharmacology.

INTRODUCTION

Pancha means five and gavya means substance obtained from cow. That is the mixture of 5 products of cow such as cow's urine, milk, curd, cow dung, and ghee in a proper ratio that allows it to ferment and the end product is known as Panchagavya. Historically, Maharshi Dhanvantri coined the word "Panchgavya" and in sanskrit all these five products are individually called "Gavya" and collectively termed as Panchgavya. Panchgavya had reverence in the script of Vedas and Vrikshayurveda. Cowpathy means the therapy of human/ animal ailments through cow products. All five products possess different medicinal properties against many disorders and are used alone or in combination or with some other herbs (Chauhan, 2005). Present review highlights the salient points regarding applications, benefits, uses and importance of panchgavya in day to day life, well supported by the known scientific facts.

INDIAN COW

In veda, cow is considered the most valuable animal and is called mother of all. Cow is the backbone of Indian culture and known as "Kamdhenu" and "Gaumata" because of its nourishing nature like mother, the giver of all to huminity and is a store of medicines. The indigenous cattle scientifically called as *Bos indicus* or Zebu cattle, mainly inhabitant the Indian subcontinent. It is thought to be world's oldest domesticated cattle. Historically also it is now proved by the fact that humped cattle were found in Mohanjodaro site of Indus Valley indicating their presence in India even before the arrival of Aryans. Indigenous cattle or zebu cattle is considered as blessed in Indian literature because its products like milk, curd, ghee, urine and dung which has many great medicinal properties.

PHARMACOLOGY OF PANCHGAVYA AND ITS COMPONENTS

Cowpathy is an old system of medicine mentioned in ancient Indian literature (Ayurveda) as Panchgavya Chikitsa. Panchgavya Chikitsa has been proposed as an alternate prophylactic and therapeutic approach for sound livestock and poultry health along with safeguarding human health (Dhama *et al.,* 2005; Mathivanan *et al.,* 2008). Panchgavya products have been found to be beneficial in curing several human ailments and enhance the body's immunity and resistance to fight various infections. Cowpathy induces immunomodulation by enhancing both cellular and humoral immune responses, up-regulates the lymphocyte proliferation activity, secretion of cytokines and macrophage activity; reduces apoptosis in lymphocytes thus helping them to survive and fight infection; acts as anti-aging factor by preventing the free radicals formation and efficiently repairing the damaged DNA (Dhama *et al.,* 2005). Also, immunity is reducing drastically as a result of the environmental pollution, use of agrochemicals in agriculture and presence of pesticides, heavy metals and fungal toxins in the food chain, in this scenario cowpathy is a good alternative (Dhama *et al.,* 2013).

The hepatoprotective activity of Panchagavya Ghrita (PG) was studied against carbon tetrachloride

induced hepatotoxicity. The parameters like serum glutamate oxaloacetate transaminase (SGOT) serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP) and acid phosphatase (ACP) was determined. Silymarin was used as the standard drug for comparison. Panchgavya Ghrita (150-300 mg/kg, p.o.) markedly prevented CCl_4 induced elevation of levels of SGPT, SGOT, ACP and ALP. The results were comparable to that of standard drug Silymarin. Histopathological comparision of liver tissues exhibited almost normal architecture, as compared to control group (Achliya *et al.*, 2003)

Panchgavya Ghrita was evaluated for some neurological parameters in rats and it was found that PG protected rats from maximal electroshock (MES) induced convulsions, increased the spontaneous motor activity as measured by actophotometer and inhibited the pentobarbitone induced sleep time in rats without much influence on the general behavior of the rats except increase in the general activity. PG appears to possess anti-convulsant property but the degree of protection might not be sufficient to use it as single antiepileptic agent. It is concluded that PG can be used as adjuvant in treatment of epilepsy (Gosavi, 2012).

The effect of PG in maximal electroshock (MES) induced seizures model and its pharmacodynamic and pharmacokinetic interaction with phenytoin (PHT) and carbamazepine (CBZ) was evaluated in rats. It was concluded that Co-administration of PG with low doses of PHT and CBZ caused complete seizure protection. This suggests the potential of PG as an adjunct in epilepsy with improved efficacy and tolerability (Joshi *et al.*, 2015).

Neurotropic activity of Panchgavya Ghrita (PG) was studied using diazepam induced amnesia in mice and Morris Water Maze (MWM) test in rat model. Piracetam was used as standard drug. Panchgavya ghrita successfully reversed the amnesia induced by Diazepam (1mg/kg, i.p.). PG was administered in three dose levels as 2.5, 5 and 10 g/kg in mice and 1.75, 3.5 and 7 g/kg in rats for 21 days. Elevated plus maze (EPM) test showed significant effect of 5g/kg dose of Panchgavya ghrita. Also, Piracetam and Panchgavya ghrita at 3.5g/kg have significant memory enhancement action in MWM test in rats suggesting possible use of PG as adjuvant in mental disorder treatments (Pandey and Pawar, 2015).

Panchagavya was evaluated for its antioxidant potential by HPTLC-DPPH bioautography method as well as assays for Ferric reducing antioxidant power (FRAP), DPPH – free radical scavenging activity and Superoxide radical scavenging activity. In addition total phenolic content was also estimated which was in fairly good amount. HPTLC-DPPH bioautography study revealed the presence of several antioxidant compounds in Panchagavya (Athavale *et al.,* 2012*)*

Antifungal activity of panchgavya was evaluated for its use in microbiological medium. 10µl, 100µl, 500µl and 1000µl of panchagavya mixed with 1.5% water agar medium and after sterilization, incubated at room temperature. After 5 days of incubation, the initial lower dilution showed 100% fungal growth and middle dilutions showed moderate growth. Though higher dilution axes the significant fungal growth, but sticks the identical bacterial colonies and even no bacterial growth on 10 µl, 100 µl and 500 µl concentrations indicates less growth promotional and more antifungal source. According to these data, the higher dilutions of panchagavya are promising source for simple and naturally derived less expensive bacteriological media with antifungal effect with growth promotion (Joseph and Sankarganes, 2011)

Panchagavya in appropriate dilutions has been found as promising growth enhancer of micro-organism hence it adds to the soil fertility by avoiding the use of chemical fertilizers, and with marked antifungal properties it can be used as a successful microbiological growth medium as well. Panchagavya at higher dilution are found to be promising source for simple and naturally derived bacteriological media that are less expensive because of additional antifungal effect with growth promotion (Joseph and Sankarganesh, 2011). Panchgavya when used along with plant like *Andrographis paniculata* can act as an alternative to antibiotic growth promoter and enhances productivity of broiler industry (Mathivanan *et al.,* 2006). It also possesses ameliorative effect on certain viral diseases (e.g. New castle disease in layer chicken) (Sumithra *et al.,* 2013).

COW URINE/ COW URINE DISTILLATE

Cow urine therapy has a unique place in Ayurveda. It is the most effective secretion of animal origin with innumerable therapeutic values (Dhama *et al.*, 2005). In India, drinking of cow urine has been practiced since thousands of years. In the “Vedas”, the sacred Hindu scriptures, which is said to be the oldest books in Asia (approx. 1,500 BC), it is mentioned that “amrita” (beverage of immortality), the nectar of the god is urine. Some recognized cow urine as “water of life”. The contents of cow urine include: 95% water, 2.5% urea; minerals and salt; 2.5% hormones and enzymes. The biochemical estimation of cow urine has shown that it contains sodium, nitrogen, sulphur, Vitamin A, B, C, D, E, manganese, iron, copper, silicon, chlorine, magnesium, citrate, succinate, calcium salts, phosphate, lactose, carbolic acid, enzymes, creatinine and hormones (Jain *et al.,* 2010). Copper is capable of abolishing diseases and comprehensively act as an

antidote. Probable role in immune enhancement are played by cytokines and amino acids. Singularly, cow urine has such potentialities and capabilities that it able to removes all the ill effects and imbalances in the body (Chauhan *et al.,* 2001; Chauhan, 2004). According to the Chinese pharmaceuticals dictionary "Shang Han Lun", urine had been used as a medium for delivery of medicinal herbs to strengthen their effects as using herbs to get her economized the quantity of precious medicinal herbs (Chauhan and Garg, 2003).

In a study of cow urine and its distillate to assess the analgesic effect using rat tail immersion method it was observed that the animals received cow urine showed comparable analgesic effects to standard drug group received diclofenac sodium. Their analgesic activity is attributable to the steroidal moieties and some volatile fatty acids whose presence in cow urine is established through other parallel studies involving chemical and instrumental analysis (Wate *et al.*, 2012).

The effect of cow urine formulation (Gomutra ark, GoA) on experimental alloxan-induced diabetes in rats was studied. Wistar albino rats of either sex weighing 200-250 g were used. The biochemical parameters observed were blood sugar, vitamin C and malondialdehyde (MDA) release. GoA significantly lowers blood glucose in diabetic rats although the observed effect was found to be less than glibenclamide. It is suggested that GoA might have a significant protective effect against alloxan-induced type I Diabetes Mellitus. GoA contains volatile fatty acids like acetic acid 2 propenyl ester, acetic acid methyl ester, 2 2 3 trichloro propionic acid, Butanoic acid-3methyl, propyl ester, 1H indol-3-acetate, acetic acid phenyl ester, quinoline, which act as an antioxidant. The antioxidant potential might be contributing for the antihyperglycemic effect, by preventing formation of the free radicals which cause damage to the beta cells of pancreas. It significantly lowers the level of malondialdehyde and vitamin C in diabetic rats. No toxicity was observed even when cow urine was given 32 times of the study dose in acute toxicity and no significant change were observed when it was used chronically, suggesting that cow urine is having a very high therapeutic index. The findings of the study supported the traditional use of cow urine in diabetes and have a high therapeutic index and safety profile for chronic use (Sachdev *et al.*, 2012).

In a study of use of cow urine distillate at three different doses in streptozotocin (50 mg/kg bw., i.p) induced diabetes rats. Streptozotocin was dissolved in citrate buffer (0.1 M, pH 4.5). The anti diabetic effect of standard drug glibenclamide (0.25 mg/kg, p.o) was also studied in these diabetic rats. The parameters employed in the study included assessment of fasting blood glucose levels, serum lipid profiles, liver glycogen levels and initial and final changes in body weight. The cow urine distillate produced a significant ($P<0.05$) reduction of the elevated blood glucose, serum cholesterol and serum triglycerides level when compared with the diabetic control. The diabetic rats treated with cow urine distillate also showed a significant increase in HDL levels and gain in body weight when compared with the diabetic control (Gururaja *et al.*, 2011).

Cow urine was also evaluated for efficacy on various cancer patients through a questionnaire in a clinical study. The symptoms (pain, inflammation, burning sensation, difficulty in swallowing, irritation, etc.) of cancer patients were categorized into severe, moderate and mild categories, respectively. During survey 7.35% patients withdraw themselves from the treatment and 92.64% patients continued the therapy. There was a high proportion (30.87%) of throat cancer and the other prevalent cancer was breast cancer (14.70%) followed by cervix and uterine cancer (5.88%), buccal cavity cancer and sinus (4.41%) lung cancer, lymphoma and bone cancer (2.94%), both throat and buccal (5.88%) and other cancer (8.82%), respectively. The study revealed that the degree of severe, moderate and mild symptoms were 82.16%, 15.8% and 1.58% on the first day and 7.9%, 55.3% and 36.34% on the eighth day, respectively. It was observed that patients who were receiving cow urine therapy since 2-3 months were most benefited. Hence, this traditional therapy may really a boon to cancer patients (Jain *et al.*, 2010).

Study of wound healing activity of cow urine in wistar albino rats by excision wound model revealed significant healing activity of cow urine. The parameter studied was the rate of wound contraction. The studies on excision wound healing revealed that there was a decrease in wound area. External application of urine showed significant increase in wound healing in male and female rats after day four as compared to all other groups. However till the end of 14th day animals showed that only 0 % and 0.4 % of healing was left, which may be due to normal immunity of the animals whereas nitrofurazone, standard drug treated animals showed 0 % and 0.3 % healing. Study revealed that the cow urine on external application to the wound fastened the healing process (Sanganal *et al.*, 2011). Study demonstrated that cow urine ark significantly increases wound healing in diabetic wound patient. Thus, it helps in accelerating wound healing in diabetic patients because of its property of enhancing granulation tissue formation (Hirapara *et al.*, 2016). Cow urine with wheat grass and *Aloe vera* is found to be very effective antibacterial and comparatively cheaper than antibiotics (Sharma *et al.*, 2013). Cow urine distillate has concentration dependent inhibitory effect on Candida species and is effective on the isolates that are either resistant or sensitive to the routinely used antifungal

agents (Hoh and Dhanashree, 2017)

Anti-urolithiatic effect of cow urine ark (CUD) on ethylene glycol (EG) induced renal calculi was studied in 36 male Wistar rats. Group I animals served as vehicle control and received distilled water for 28 days. Group II to VI animals received 1% v/v EG in distilled water for 28 days. Group II served as EG control. Group III and IV (preventive groups) received cow urine ark orally for 28 days in doses of 1 ml/kg and 2 ml/kg, respectively. Group V and VI (treatment groups) received 1 ml/kg and 2 ml/kg cow urine ark orally, respectively from 15th to 28th days. 24-hour urine samples were collected on day 0 and 28. Urine volume and oxalate levels were measured. On day 28, blood was collected for biochemical parameters. Animals were sacrificed and kidneys were harvested, weighed and histopathologically evaluated for calcium oxalate (CaOx) crystals. EG significantly increased urine oxalate, serum creatinine, blood urea level, kidney weight and CaOx deposits. Provision of cow urine ark resulted in significantly lower levels of urine oxalate, serum creatinine, blood urea and CaOx depositions as compared to group II. It also significantly restored kidney weight. Cow urine ark inhibited 40% and 35% crystallization of CaOx and calcium phosphate, respectively (Shukla *et al.*, 2013).

Effect of cow urine and combination of antioxidants against lindane induced genotoxicity was evaluated in mice. In this study, lindane brought about a non-significant increase in structural chromosomal aberrations and the number of micronucleated polychromatic erythrocytes and a significant depression in mitotic index. Pretreatment with cow's urine and combination of antioxidants showed decrease in chromosomal aberrations and micronucleated polychromatic erythrocytes whereas an increase in mitotic index (Nagda and Bhatt, 2015).

Assessing the effect of Gir cow urine distillate on immunity and hemato-biochemical parameters was planned to evaluate immunostimulatory effects and hemato-biochemical alterations following 28 days repeated oral administration of CUD in normal healthy mice. The study was conducted on twenty four male swiss albino mice dividing them in four groups having six mice in each group. Group I served as normal control group given normal saline orally for 28 days. Group II, III and IV served as treatment group given cow urine distillate @ 2, 4 and 6 ml/kg, body weight orally for 28 days. At the end of experiment, all the mice were subjected to blood collection; blood and serum sample were analyzed for hematological and biochemical parameters, respectively. Humoral and cell mediated immune response were measured and histopathogical examination of spleen and thymus were carried out. The result obtained clearly indicated that the oral administration of cow urine distillate in normal mice showed immunostimulatory effect by increasing both the antibody titer and cell mediated immune response (Pancha *et al.,* 2015).

Study on immunomodulatory effect of Gir CUD was planned to evaluate its effect on humoral and cell mediated immunity in cyclophosphamide induced immunosuppressive mice. Swiss albino mice were randomly divided into 5 groups (numbered I to V), each containing 6 mice. Animals of groups I were administrated normal saline for 28 days. Group II were administered cyclophosphamide @ 60 mg/kg b.w. (body weight), daily once orally for 28 days. Group III, Group IV and Group V were administrated Cow urine distillate @ 2 ml/kg, 4 ml/kg and 6 ml/kg b.w., for 28 days, respectively along with cyclophosphamide @ 60 mg/kg b.w. for 28 days. On 29th day humoral and cell mediated immune responses, TLC (Total leukocyte counts), DLC (Differential leukocyte counts), serum total protein, globulin, albumin and histopathological examination of tissues were conducted. The result obtained clearly indicated that oral administration of Cow urine distillate in immunosuppressed mice having immunomodulatory effect in dose dependent manner by increasing both the cell mediated immune response and antibody titer. So the present study suggests that repeated oral administration of Cow urine distillate for 28 days in immunosuppressive mice showed immunomodulatory effect (Pancha *et al.,* 2017).

COW DUNG

Cow dung is considered as gold mine because it's wide applications in the field of agriculture and therapeutic. Thin coating of dung for cooking place, havan kund, mud house, floors and walls provides antiseptic surface (Dhama *et al.*, 2005). Cow dung can act as skin tonic. When mixed with crushed neem leaves and smeared on skin, it proves good for boils and heat rashes. Cow dung also used as tooth polish and relieves toothache. Cow dung also contains an antifungal substance which inhibits the growth of Corprophilous fungi (Lehr *et al.*, 2009). Since the presence of microbial source in cow dung. Patulodin-like compounds CK2108A and CK2801B produced by *Eupenicillium bovifimosum* present in cow dung has more antifungal activity (Dorothy *et al.,* 2002). Cow dung also posses antifungal components which inhibits growth of even coprophilous fungi and their activity is increased when used in combination with cow urine (Kulkarni, 2009). The fresh cow dung kills the germs of Malaria and T.B. Cow dung is antiseptic and posses prophylactic (disease preventive) properties (Dhama *et al.*, 2005 and Schnurer and Magnusson, 2005).

COW MILK

Cow milk is a healthy food because of low

calorie, low cholesterol and high micro-nutrients, protein, calcium, vitamins, and plays an important role in meeting requirements of various essential nutrients. It contains carotenes, Vitamin A, Vitamin B complex group and Vitamin C. Cow milk is healthy food because of low cholesterol and high micro-nutrient. A specific fatty acid (a cis-trans isomer of linoleic acid) present in cow milk has anticancer property (Belury *et al.*, 1996). It has bio-protective role in human health and is easily digestible (Dhama *et al.*, 2005 and Schnurer and Magnusson, 2005). It is found to be effective in curing fever, pain, tumors, diabetes and weaknesses and importantly act as a medium to administer medicine. It delay the processes involved in aging (Sowrirajan, 2006).

Cow milk contains about 85% water. The remaining 15% is the milk sugar lactose, protein, fat and minerals. Beta-casein is about 30% of the total protein content in milk. A2 milk contains only the A2 type of beta-casein protein whereas A1 milk contains only A1 beta casein or A1A2 type variant. A1 protein variant is commonly found in milk from crossbred and European breeds of cattle. A2 milk is found basically in indigenous cows and buffaloes of Asia and particularly India (Asia as a whole) (Boro *et al.,* 2016). Beta-casein comes in twelve genetic variations, namely A1, A2, A3, B, C, D, E, F, H1, H2, I and G. Out of these twelve variations, A1, A2 and B forms are the commonly found genetic variations. Initial studies on indigenous cow (Zebu type), buffalo and exotic cows (taurine type) have revealed that A1 allele is more frequent in exotic cattle while Indian native dairy cow and buffalo have only A2 allele (Mishra *et al.*, 2009). A1 and A2 beta-casein protein differs from each other in single amino acid sequencing at "67th" position. For A2 beta-casein protein, at 67th place "Proline" is present, whereas for A1 beta-casein protein in 67th place, "Histidine" is found. This polymorphism is responsible to cause changes in digestion patterns. A1 beta-casein digestion by digestive enzymes develops bioactive seven-amino-acid peptide called as "Beta-Casomorphin-7" (BCM-7) and on the other hand A2 beta casein digestion results in minimal development and least release of BCM-7. In hydrolysed milk with variant A1 of beta-casein, BCM-7 level is 4-fold higher than in A2 milk (Mishra *et al.*, 2009). Epidemiological evidences claim that A1 beta-casein cow milk consumption was related to health risks like heart disease, type-I diabetes, sudden infant death syndrome, autism and schizophrenia (Laugesen and Elliott, 2003; Tailford *et al.*, 2003 and Banerjee, 2018). Populations, which consume milk containing high levels of β-casein A2 variant, have a lower incidence of cardiovascular disease and type-1 diabetes (Sodhi *et al.*, 2012)

COW CURD

Cow curd is regard as digestive, nutritive and useful in gastrointestinal ailments. Cow milk curd is considered as vatanashak, blood purifier, tridoshnashak and found useful in pitha, blood related problems, piles and gastrointestinal disorders. Butter milk with sugar has tridoshnashak action and found useful in piles and gastrointestinal disorders (Singh and Chauhan, 2004). Curd is an efficient probiotic with potential to control infections in a nondrug manner. Lactic acid producing bacteria are present in curd and butter milk that produces antifungal metabolites viz. cyclic dipeptides, phenyl lactic acid as well as proteinaceous compounds and 3-hydroxylated fatty acid (Dhama *et al.*, 2005; Schnurer and Magnusson, 2005).

COW GHEE

Cow ghee (the butter fat obtained from the cow milk) traditionally believed to improve memory, voice, vision, intelligence and body's resistance to infections. Cow ghee has been claimed to have many medicinal

Table 1:
Pharmacological Properties/Activities of Cow Urine/ Cow Urine Distillate.

Sr. No.	Pharmacological Properties/ Activities	References
1)	Immunostimulant / Immunomodulator	Kumar *et al.*, 2004, Chauhan, 2004; Banga *et al.*, 2005; Singla and Garg, 2013.
2)	Antibaterial, antifungal, antiviral and anthelmintic activity	Panthi and Chaudhuri, 2006; Patil, 2007; Kekuda *et al.,* 2010; Dhama *et al.,* 2013; Sarsar *et al.,* 2013; Kumar, 2013
3)	Anticancer activity	Dhama *et al.*, 2005; Raja and Agrwal, 2010
4)	Antiurolithiatic activity	Shukla *et al.*, 2013
5)	Anti-leishmanial effect	Singh, 2005
6)	Antioxidant activity	Bhardawaj and Varshneya, 2011; Sachdev *et al.*, 2012
7)	Hepatoprotective activity	Achliya *et al.,* 2003; Gururaja *et al.*, 2009
8)	Hypoglycemic activity	Jarald *et al.,* 2008; Kadagi *et al.,* 2012
9)	Ameliorate reproductive toxicity	Khan and Srivastava, 2005
10)	Growth promoter (poultry)	Garg *et al.* 2005; Patel and Sharma, 2013

properties like it is rejuvenating, bestows luster and beauty, enhances memory and stamina, increases the intellect and promotes longevity. It is an aphrodisiac and protects the body from various diseases (Chunekar, 1960). Ayurvedic formulation containing *Emblica officinalis* and *Glycyrrhiza glabra* and cow *ghee* has sedative action (Achliya *et al.,* 2004). Cow ghee processed with herbals known as "ghrita" has immunomodulatory activity (Fulzele *et al.*, 2003), hepatoprotective effect (Achliya *et al.,* 2003), wound healing activity (Charde *et al.*, 2003), sedative and anticonvulsant activity (Achliya *et al.,* 2004) which has been reported on experimental animals. When we offer ghee in fire as part of ritualistic sacrifices, it strengthens the ozone layer and shields the earth from harmful radiations from sun (Khan *et al.*, 2015).

In a study of feeding cow ghee versus soyabean oil on 7, 12-dimethylbenz(a)-anthracene (DMBA) induced mammary carcinogenesis and expression of COX-2 and peroxisome proliferators activated receptors-γ(PPAR-γ) in mammary glands of rats revealed anticancer potential of cow ghee. In the DMBA treated groups, the animals given soybean oil had higher tumour incidence (65.4%), tumour weight (6.18 g) and tumour volume (6285 mm^3) compared to those fed with cow ghee (26.6%, 1.67g, 1925 mm^3, respectively). Tumour latency period was increased to 27 week on cow ghee treated rats as compared to 23 week on soyabean oil. Histological analysis of tumours showed more rapid progression of carcinogenesis on soybean oil than on cow ghee fed rats. The study proved that dietary cow ghee opposed whereas soybean oil attenuates mammary carcinogenesis induced by DMBA. Also, the effect is mediated by decreased expression of cyclooxygenase-2 and increased expression of PPAR-γ in the former group (Rani and Kansal, 2011)

Further study carried out to know the exact mechanism behind the anticancer potential of cow ghee versus soyabean oil by observing the effects on carcinogen metabolizing enzymes in the rats. It was observed that cow ghee relative to soyabean oil decreased the activities of Cytochrome P450 (CYP) enzymes- CYP1A, CYP1A2, CYP1B1, and CYP2B2 which are responsible for activation of carcinogens in liver. The activities of Uridinediphospho-glucuronosyl transferase (UDPGT) and quinone reductase (QR) in liver, and γ-glutamyltranspeptidase (GGTP) and QR in mammary tissue were significantly increased in cow ghee fed rats than soyabean oil fed animals. The hepatic GGTP activity decreased in soyabean oil fed animals whereas it is unaffected in cow ghee fed group. The study proved that cow ghee compared to soybean oil downregulates the enzyme activities responsible for carcinogen activation in liver and upregulates carcinogen detoxification activities in liver and mammary tissues (Rani and Kansal, 2012).

In a case study on a wound in a buffalo which did not respond to variable treatment that ranges from simple herbal preparation like turmeric to modern medicines (antibiotics) responded very well to the treatment by a formulation containing cow's ghee. Ghee contains several saturated and unsaturated fatty acids which are capable of taking part in metabolic processes involved in any wound healing. It seems therefore worthwhile that the cow's ghee is explored further as an effective component in wound healing formulation (Biyani *et al.*, 2011).

In a study to evaluate the cow ghee containing formulation of *Aloe vera* for wound healing potential, histological examinations revealed good keratinization, epithelization, fibrosis and collagenation indicative of good healing process. The results were comparable with framycetin sulphate cream (1% w/w). Incision wound for tensile strength, excision wound contraction and histological observations of regenerated tissues were used to investigate the healing potential of the formulation (Nandanwar *et al.*, 2010).

Cow ghee has lubricating property which may be useful in reducing the symptoms of computer vision syndrome (CVS) characterized by burning sensation, dryness, redness and itching in the eyes. Cow ghee contains Vitamin A 3500/100g, beta-carotene and Vitamin E. Vitamin A keeps the outer lining of eye ball moist and prevent blindness. Beta-carotene and Vitamin E are well known antioxidants. So Goghrita Eye drops (Aschyotana) can be used as alternative treatment in Computer Vision Syndrome (Mulik and Bhusari, 2013).

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Research Article

INTRAVENOUS AND ORAL PHARMACOKINETICS OF MARBOFLOXACIN IN LAYER BIRDS

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ABSTRACT

This is the report on pharmacokinetics of marbofloxacin administered via IV and oral route in layer chickens. Single intravenous and oral dose of marbofloxacin was administered in the six Rhode Island Red (RIR) layer birds at the rate of 2 $mg.kg^{-1}$ body weight to study pharmacokinetic parameters. Plasma concentrations of marbofloxacin were measured by High Performance Liquid Chromatography (HPLC) method. Following intravenous administration of marbofloxacin in birds, the mean values of elimination half-life [$t_{1/2}(\beta)$], volume of distribution of drug at steady-state [$V_{d(ss)}$] and total body clearance (Cl_B) were 4.25 h, 0.982 $L.kg^{-1}$ and 3.009 $ml.min^{-1}.kg^{-1}$, respectively. Following single dose oral administration, peak plasma concentration (0.982 $\mu g.ml^{-1}$) was observed at 1 h. The mean values $t_{1/2}(\beta)$, $V_{d(ss)}$, Cl_B and mean residence time (MRT) were 7.638 h, 2.195 $L.kg^{-1}$, 3.757 $ml.min^{-1}.kg^{-1}$ and 8.823 h, respectively. The calculated mean oral bioavailability of marbofloxacin was 76.22 per cent in layer birds.

Key Words: Marbofloxacin, IV and oral route, pharmacokinetics, layer birds, chicken

INTRODUCTION

Fluoroquinolones, the synthetic and broad-spectrum antimicrobial agents with bactericidal activity, are considered to have characteristics like large volume of distribution, low plasma protein binding, wide spectrum of bactericidal activity with low minimum inhibitory concentrations (MICs) against susceptible target micro-organisms and concentration-dependent effect against majority of bacteria (Brown, 1996; Dalhoff *et al.*, 1996). Marbofloxacin is a new fluoroquinolone developed exclusively for veterinary use. It has good activity against *Staphylococcus aureus*, *Staphylococcus intermedius*, *Pasteurella* spp., *Escherichia coli*, *Proteus mirabilis*, *Mannheima haemolytica* and also may be effective against species of Mycoplasma and Pseudomonas (Garcia-Montijano *et al.*, 2003; Carpenter *et al.*, 2006). It also has longer duration of bactericidal action with significant post-antibiotic effect (Spreng *et al.*, 1995). It is approved for use in dogs and cats in the United States, and in other species (cattle, swine and equine) outside the U.S. Infections treated with marbofloxacin include skin and soft tissue infections, bone infections, urinary tract infections, pneumonia and infections caused by intracellular organisms (Papich, 2016).

Thus, marbofloxacin is gaining popularity as an armament against the bacterial infections in veterinary medicine and extending its utility from pet animals and livestock species to avian species. In some countries, marbofloxacin is used as off-label drug under the supervision of a veterinarian to treat the respiratory diseases in chickens (Yang *et al.*, 2014). However, literature available on pharmacokinetics (PK) of marbofloxacin in avian species including broiler chickens, ducks, turkeys and Japanese quail is very scanty (Anadon *et al.*, 2002; Haritova *et al.*, 2006; Goudah and Hasabelnaby, 2011; Haritova *et al.*, 2013; Ding *et al.*, 2013). There were no such reports found in layer birds, hence, the present study was undertaken to investigate the pharmacokinetics of marbofloxacin in layer chickens following single dose IV and oral administration.

MATERIALS AND METHODS

Experimental birds

Six Rhode Island Red (RIR) layer birds of 24-25 weeks age with body weight range of 1400 - 1500 g were used for the present study. Layer birds were obtained from Government Poultry Farm, Palanpur, Gujarat. Birds were kept in cages at the Department of Pharmacology and Toxicology. The study was prior approved by the Institutional Animal Ethics Committee (IAEC), College of Veterinary Science and A.H., SDAU, Sardarkrushinagar. Standard housing and management practices were adopted and strictly followed to keep birds healthy and stress-free. Birds were given antibiotic free feed in manual feeders, whereas water was provided *ad lib*.

Drug and chemicals

Marbofloxacin 2% injectable solution (Marbocyl®,

Vetoquinol Animal Health Pvt. Ltd.) and marbofloxacin 80 mg tablet (Marbocyl®, Vetoquinol Animal Health Pvt. Ltd.) were used in the present study. All the chemicals and reagents used in the study were procured from S. D. Fine Chem. Ltd., Mumbai, India, and were of HPLC grade.

Experimental design and sample collection

Six RIR layer birds were given marbofloxacin @ 2 mg/kg body weight into wing vein and blood samples were collected from contra-lateral wing vein in heparinized centrifuge tubes. After washout period of one week, same birds were given same dose of marbofloxacin orally. Injectable marbofloxacin was used for intravenous administration whereas marbofloxacin tablet (80 mg) was dissolved in water and used for oral administration. Blood samples (about 1 ml) were collected at 0 time (before drug administration), 0.166 (10 minutes), 0.333 (20 minutes), 0.5 (30 minute), 1, 2, 4, 6, 8, 10, 12, 18 and 24 hours after both intravenous as well as oral administration of drug, and then plasma was separated and transferred to cryo-vials (2 ml capacity) and stored temporarily at -20°C until analyzed.

Sample preparation and HPLC assay

Plasma concentrations of marbofloxacin were measured by High Performance Liquid Chromatography (HPLC) method as described by Schneider *et al.* (1996) with some modifications as to make method suitable for layer birds' plasma. The optimized method was sensitive and reproducible, and assay linearity was observed from 0.01 to 50 µg/ml with R^2 (mean correlation coefficient) value of 0.9998.

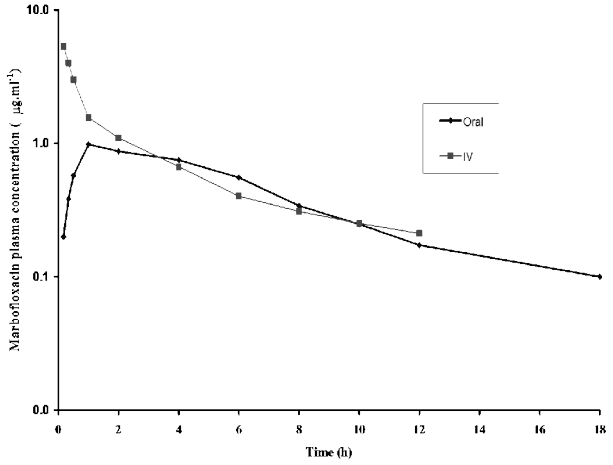

Figure 1
The Semi logarithmic plot of plasma marbofloxacin concentration versus time following IV and oral administration at the dose rate of 2.0 mg.kg^{-1} body weight in layer birds (Each point represents mean of six birds).

For drug extraction from samples, plasma proteins were precipitated by addition of a solution containing 0.8 M perchloric acid. Exactly, 500 µL of plasma and 50 µL of perchloric acid were mixed in a clean dry centrifuge tube. The mixture was then shaken on a vortex mixer for 1 min and centrifuged for 15 min at 4000 rpm speed. The clean supernatant was used for HPLC analysis of marbofloxacin.

The HPLC apparatus (Knauer, Germany) included isocratic solvent delivery pump (model K 501) and UV detector (model K 2501). Chromatographic separation was performed by using reverse phase C18 column (Zorbax, ODS; 25 cm x 46 mm ID) at room temperature. The mobile phase was consisted of a mixture of methanol: acetonitrile: buffer: acetic acid: triethylamine [10:2:86:1:1 (v/v)]. The buffer was prepared from a 0.4 % aqueous solution of diammonium hydrogen phosphate and adjusted to pH 2.7 using tetrabutyl ammonium hydrogen sulfate. Mobile phase was filtered with 0.22 µ filter and pumped into column at a flow rate of 1 ml/min at ambient temperature. The effluent was monitored at 295 nm wave length (λ_{max}).

PK analysis

Various PK parameters like elimination rate constant (β), elimination half-life ($t_{1/2\beta}$), apparent volume of distribution (Vd_{area}), volume of distribution at steady state (Vd_{ss}), area under curve (AUC), mean resident time (MRT), total body clearance (Cl_B) *etc*. were calculated by PK Solutions Ver. 2.0 software, USA, using non-compartmental model approach.

RESULTS

The graphical representation (semi logarithmic plot) of plasma marbofloxacin concentrations versus time after single dose intravenous and oral administration at the dose rate of 2.0 mg.kg^{-1} body weight in RIR layer birds is depicted in Figure 1.

The mean initial plasma marbofloxacin concentration observed following IV administration was 5.336 µg.ml^{-1} achieved at 0.166 h (10 min), which was rapidly declined to approximately one-fifth of its initial concentration (1.098 µg.ml^{-1}) at 2 h. Following single dose oral administration, the mean peak plasma concentration of marbofloxacin (0.982 µg.ml^{-1}) was observed at 1 h of drug administration. The concentrations of marbofloxacin in plasma could not be detected beyond 12 h (mean concentration at 12 h: 0.211 µg.ml^{-1}) after IV administration and beyond 18 h (mean concentration at 18 h: 0.100 µg.ml^{-1}) after oral dosing.

The PK parameters of marbofloxacin derived for layer birds following IV and oral administration are compared in the Table 1.

Table 1
Pharmacokinetic parameters (Mean± S.E.) of marbofloxacin after single dose intravenous and oral administrations in layer birds (n=6).

Pharmaco-kineticparameters	Unit	Intravenous	Oral
β	h^{-1}	0.16± 0.003	0.09±0.005
C_{max}	μg ml^{-1}	——-	0.98±0.39
T_{max}	h	——-	1.00±0.00
$t_{½}(\beta)$	h	4.25±0.08	7.64±0.43
AUC	μg h ml^{-1}	10.80±0.31	8.22±0.24
AUMC	μg h^2 ml^{-1}	51.45±1.78	72.85±5.24
MRT	h	4.76±0.08	8.82±0.41
$V_{d(area)}$	L kg^{-1}	1.14±0.04	2.45±0.16
$V_{d(ss)}$	L kg^{-1}	0.98±0.03	——-
Cl_B	L h^{-1} kg^{-1}	0.18±0.005	0.23±0.018
F	%	——-	76.22±2.19

β, elimination rate constant; C_{max}, Maximum drug concentration; T_{max}, observed time for C_{max}; $t_{½}(\beta)$, elimination half life; AUC, area under plasma drug concentration-time curve; AUMC, area under first moment of curve; MRT, mean residence time; $V_{d(area)}$, apparent volume of distribution; $V_{d(ss)}$, volume of distribution at steady state; Cl_B, total body clearance; F, bioavailability.

DISCUSSION

Fluoroquinolones have been employed in the avian medicine for the treatment of various bacterial infections and marbofloxacin, the veterinary exclusive drug, has great potential to be used in avian medicine (Urzua *et al.*, 2016). Various reports of PK of marbofloxacin following IV or oral or both routes are available in avian species like broiler chickens (Anadon *et al.*, 2002; Ding *et al.*, 2013; Yang *et al.*, 2014; El-Komy *et al.*, 2016; Urzua *et al.*, 2016; Atef *et al.*, 2017), macaws (Carpenter *et al.*, 2006), ostriches (de Lucas *et al.*, 2005), buzzards (Garcia-Montijano *et al.*, 2003), vultures (Garcia-Montijano *et al.*, 2011), Muscovy ducks (Goudah and Hasabelnaby, 2011), turkeys (Haritova *et al.*, 2006), Japanese quails (Haritova *et al.*, 2013; Lashev *et al.*, 2015) and pheasants (Lashev *et al.*, 2015). Most of these PK studies are performed at the dose rate of 2 or 5 mg/Kg b. wt. of the birds and the present study was done at the dose rate of 2 mg/kg b. wt. for the marbofloxacin in chickens.

Following IV administration of marbofloxacin in RIR layer birds, in the present study, at the dose rate of 2 mg/kg b. wt., the minimal detectable plasma level of 0.211±0.008 μg.ml^{-1} was measured at 12 h. Observed low value of mean elimination rate constant (0.16 h^{-1}) indicated that the drug which is known for rapid distribution has relatively slow elimination from the body of birds. Similar low elimination rate constants (0.13 h^{-1} and 0.10 h^{-1}) were also reported by Anadon *et al.* (2002) and Atef *et al.* (2017), respectively, in broiler chickens.

The mean elimination half-life [$t_{1/2(\beta)}$] following IV route in present study was 4.25 h, which was close (4.3 h) to that reported in macaws (Carpenter *et al.*, 2006) but a lower value (2.83 h) and very long value (12.51 h) were reported respectively in ducks (Goudah and Hasabelnaby, 2011) and vultures (Garcia-Montijano *et al.*, 2011). The corresponding values of $t_{1/2\beta}$ in healthy broiler chickens ranged from 5.2 to 6.8 h (Anadon *et al.*, 2002; El-Komy *et al.*, 2016; Urzua *et al.*, 2016; Atef *et al.*, 2017). The value of apparent volume of distribution (Vd_{area}: 1.14 L.kg^{-1}), which provides insight into the extent of penetration of drugs in body tissues, indicates good distribution of the drug in the body of birds and was comparable to that (1.33 L.kg^{-1}) reported in broiler chickens (Anadon *et al.*, 2002).

In the present study, mean value of total body clearance (Cl_B) was 0.18 L.h^{-1}.kg^{-1} after IV administration. Similar value (0.17 L.h^{-1}.kg^{-1}) was reported in broiler chickens (Anadon *et al.*, 2002) and ducks as 0.16 L.h^{-1}.kg^{-1} (Goudah and Hasabelnaby, 2011), however faster excretion rate was also reported in broiler birds as 0.41 L.h^{-1}.kg^{-1} (Urzua *et al.*, 2016) and in Japanese quails as 0.43 (Haritova *et al.*, 2013) and 0.34 L.h^{-1}.kg^{-1} (Lashev *et al.*, 2015). The reasons of faster clearance of marbofloxacin in birds may be due to low protein binding, high lipid solubility, and excretion of major portion as unchanged form and minimal tubular reabsorption.

In the present study, following single oral administration of marbofloxacin at the dose rate of 2 mg/Kg b. wt., the mean peak plasma marbofloxacin level (C_{max}) was observed as 0.98 μg.ml^{-1} at 1 h (T_{max}). For studies undertaken at same dose rate in avian species, C_{max} values of 0.67 (turkeys), 0.80 and 1.05 (broiler chickens), 1.97 (ducks) were reported by Haritova *et al.* (2006), Urzua *et al.* (2016), Anadon *et al.* (2002), and Goudah and Hasabelnaby (2011), respectively. Time to reach maximum concentration (T_{max}) value of 1 hour in present study suggest good oral absorption rate of marbofloxacin in layer birds, otherwise it was reported upto 2.53 h in broiler chickens (El-Komy *et al.*, 2016) and upto 5.7 and 6 h in pheasants and turkeys, respectively (Lashev *et al.*, 2015; Haritova *et al.*, 2006).

The mean elimination half-life [$t_{1/2(\beta)}$] following oral route was found to be 7.64 h in layer birds which is comparable with longer half-lives reported in broiler chickens as 7.63, 8.69 and 9.39 h, respectively by El-Komy *et al.* (2016), Anadon *et al.* (2002) and Atef *et al.* (2017), whereas lower value of $t_{1/2(\beta)}$ as 4.13 and 4.71 h were also reported in broiler chickens by Yang *et al.* (2014) and Urzua *et al.* (2016), respectively. In species like ducks (2.67 h) and Japanese quail (2.46 and 3.50 h) shorter half lives were observed (Goudah and Hasabelnaby, 2011; Haritova *et al.*, 2013; Lashev *et al.*, 2015).

A greater increase (1.8 times) was observed in the value of $t_{1/2\beta}$ after oral administration in the present study when compared with the value that was observed after IV administration of marbofloxacin. Also, more extensive distribution was observed after oral administration reflected by high $V_{d(area)}$ value of 2.45 $L.Kg^{-1}$) as compared to value observed after IV route kinetics (1.14 $L.kg^{-1}$). Marbofloxacin has large volume of distribution owing to its high lipid solubility and low plasma protein binding which results in extensive penetration in tissues as seen for other members of fluoroquinolones.

The mean AUC value of marbofloxacin given orally at the rate of 2 $mg.kg^{-1}$ of b. wt. in layer chickens was 8.22 $\mu g.h.ml^{-1}$ in the present study which was higher than the values of 6.71 and 4.7 $\mu g.h.ml^{-1}$ but lower than the values of 16.81 and 16.75 $\mu g.h.ml^{-1}$ reported in broiler chickens by different researchers (Anadon *et al.*, 2002; El-Komy *et al.*, 2016; Urzua *et al.*, 2016; Atef *et al.*, 2017).

Following oral marbofloxacin administration, the mean residence time was calculated to be 8.82 h in the present study indicating that marbofloxacin drug molecules remain for sufficient time in the body of layer birds. Total body clearance of marbofloxacin following single dose oral administration was calculated to be 0.23 $L.h.Kg^{-1}$, which shows good clearance rate of the drug following oral administration in layer birds. Oral bioavailability of marbofloxacin in the present study was found in good range of 71.15 to 83.00 % with an average of 76.22 %. In various avian species, oral bioavailability of marbofloxacin has been reported ranging from 50.1 % in Japanese quail to 118.5 % in pheasants (Lashev *et al.*, 2015).

Fluoroquinolones are active against bacterial pathogens in a concentration-dependent manner and PK-PD indices like AUC/MIC and C_{max}/MIC ratio have been associated with the optimum antibacterial effect (Papich and Riviere, 2001). MIC_{50} of marbofloxacin ranges from 0.016 to 0.24 $\mu g.ml^{-1}$ for most of the common pathogens except *Pseudomonas aeruginosa* (Spreng *et al.,* 1995). Considering higher value for calculations, value of AUC/MIC and C_{max}/MIC in the present study for oral dosing will be 4 and 34.25, respectively. Such values may provide therapeutic goals but to consider prevention of development of resistance against fluoroquinolones (Haritova *et al.*, 2006), the applied dose should provide an AUC/MIC ratio of >125, therefore higher dose of marbofloxacin (>2 mg/Kg b.wt.) needs to be considered for effective bacterial cure in layer chickens.

In conclusion, there exists the large inter-avian species variation in PK of marbofloxacin. However, the differences in pharmacokinetics of marbofloxacin in layer and broiler chicken birds are minimal. Marbofloxacin with rapid and extensive tissue distribution, high plasma concentrations, longer half-life, higher AUC value and oral bioavailability, and good clearance, makes it a good choice as antibacterial drug to be used orally in layer chickens.

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Research Article

ACUTE ORAL TOXICITY OF TOLTRAZURIL IN RATS

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ABSTRACT

The present study was conducted to assess acute oral toxicity of Toltrazuril in rats. The rats (Wistar, 2 to 2.5 months) were divided into five groups of six rats in each as Control, Test Group I (Toltrazuril @ 12.5 mg/kg, po), Test Group II (25 mg/kg po), Test Group III (50 mg/kg po) and Test Group IV (100 mg/kg po). At the end of the study, no significant changes were found in Hb, PCV, TEC and TLC of test groups I, II, and III as compared to control group. However, there were significant (P<0.01) decrease in Hb (11.47±0.18) and PCV (35.63±0.77) values of rats of group IV as compared to control group. Rats of test group IV showed significant (P<0.01) increase in AST (76.35±1.40) and ALT (28.56±0.43) values as compared to control group. The data obtained from the acute oral toxicity study of Toltrazuril in rats indicates that Coxuril® (Toltrazuril 2.5% oral solution) is safe for clinical use at therapeutic dose (7 $mg.kg^{-1}$ b.wt.).

Keywords: Toltrazuril, acute oral toxicity, rats.

INTRODUCTION

Toltrazuril [TZR, 1-methyl-3-{3-methyl-4-[4 (trifluoromethylthio) phenoxy]phenyl}-1,3,5-triazine-2,4,6 (1H,3H,5H)-trione], is a triazine-based drug that is widely used in the prevention and treatment of coccidiosis in poultry and mammals. Toltrazuril after absorption is rapidly converted to the short-lived intermediary metabolite toltrazuril sulfoxide (TZR-SO), and then metabolized to the reactive toltrazuril sulfone (TZR-SO_2) (Lim *et.al.* 2010). Efficacy of a toltrazuril metabolite, toltrazuril sulfone has been reported for inhibiting merozoite production of *Sarcocystis neurona* in cell cultures (Lindsay *et al.*, 2000). Toltrazuril is active against both sexual and asexual stages of coccidia by inhibiting nuclear division of schizonts, microgamonts and the wall-forming bodies of macrogamonts. Toltrazuril interferes with the enzymes of the respiratory chain of coccidian parasite and also inhibits the pyrimidine synthesis (Mundt *et al.*, 2006). Toltrazuril has been found effective *in vivo* against *Eimeria* species in avians, intestinal and hepatic coccidiosis in rabbits and *in vitro* against *Toxoplasma gondii.* (Peters & Geeroms, 1986; Chapman, 1987; Ricketts & Pfefferkorn, 1993). The present study was conducted to observe the effect of single dose administration of Toltrazuril in rats.

MATERIALS AND METHODS

Wistar rats, weighing between 150 to 200 gm and 2 to 2.5 months of age were procured from Laboratory Animal Resource Centre, IVRI, Izatnagar. The animals were kept in plastic cages and were acclimatized for two weeks in the experimental laboratory animal shed of College of Veterinary and Animal Sciences, Pantnagar, under standard managemental conditions. The animals were reared as per IAEC guidelines. Standard rat feed and water were provided *ad libitum* throughout the experimental period. All the experimental animals were kept under constant observation during entire period of study.

Rats were randomly divided into five groups (one control and four test groups) with 6 rats in each group. An initial dose of 12.5 mg/kg b.wt orally (Test group I, 1/ 160^{th} of LD_{50}) as per OECD guidelines was used for toxicity testing in the present study. The dose of Coxuril® (Toltrazuril 2.5 % w/v oral solution, M/s. Montajat Veterinary Pharmaceuticals Ltd.) was serially increased to 25 mg/kg (Test group II, $1/80^{th}$ of LD_{50}), 50 mg/kg (Test group III, $1/40^{th}$ of LD_{50}), 100 mg/kg (Test group IV, $1/20^{th}$ of LD_{50}) b.wt. Rats were kept under observations for consecutive 14 days and examined regularly for any clinical sign or mortality due to acute toxicity. On 15^{th} day all the rats were sacrificed. The viscera and internal organs were examined for the gross lesions and the blood samples were collected to evaluate haematological and biochemical parameters.

For assessment of haematological parameters, 0.5 ml of blood was collected from each rat in heparinized microcentrifuge tube and hematological parameters like Total erythrocytic count (TEC), Total leucocyte count (TLC), (Natt and Herrie,1952) Packed cell volume (PCV%), Haemoglobin concenteration (Hb%) (Jain, 1986) were estimated immediately after collection of blood sample.

For estimation of biochemical parameters, 2 ml of blood sample was collected from all the rats in non heparinized test tubes and serum was harvested by keeping tubes in slant position for 3-4 h at room temperature (18-20^0C). Oozed out serum was collected

by micropipette in a microcentrifuge tube. It was further centrifuged at 4^0C for 20 minutes at 4000 rpm in a refrigerated centrifuge. The top layer of serum was collected in another microcentrifuge tube and stored at -20^0C in deep freezer. The biochemical parameters were estimated within 72 hr of blood collection.

Total protein, Albumin, Globulin, Albumin:Globulin ratio, Creatinine, Alanine Aminotransferase, Aspartate Aminotransferase, Glucose, Urea were estimated in serum using commercial kits.

The data obtained in the present study were finally analysed using analysis of variance (ANOVA) (Snedecor and Cochran, 1969).

RESULTS AND DISCUSSION

No significant changes were observed as compared to control group in the Hb, PCV, TEC and TLC of test groups I, II, and III after receiving single oral dose of Toltrazuril @ 12.5, 25.0 and 50 mg.kg^{-1} b.wt., respectively. There were significant (P<0.01) decrease as compared to control group in Hb (11.47±0.18) and PCV (35.63±0.77) in rats of test group IV but no significant (P<0.05) changes were observed in the TEC and TLC of test group IV as compared to control group. Haemolysis and shrinkage in RBC might be the reason for decline in cell count (TEC, TLC) and Hb in treated rats of groups at high dose of Toltrazuril (Singh *et al.*, 2004). Pooling of the circulating blood cells in the spleen or other reservoirs secondary to decreased sympathetic activity explains the decrease in Hb and PCV (Mohammed *et al.*, 2001).

No significant changes were observed in the protein profile (Total protein, Albumin, Globulin and Albumin: Globulin) of test groups as compared to control group. The values of AST (76.35±1.40) and ALT (28.56±0.43) in rats of group IV showed significant (P<0.01) increase as compared to control group. The increase in activities of serum AST and ALT is known to be the indicator of hepatic damage (Cornelius, 1989). A significant increase in the activity of serum AST and ALT in treated groups might be due to hepatic damage caused by Toltrazuril in this study. The rise in serum levels of AST and ALT activity has been attributed to the

Table 1
Effect of Coxuril® (Toltrazuril, 2.5% w/v oral solution) on hematological profile following oral administration in rats (Mean± S.E.; n = 6)

Groups	Dose rate(mg/kg)	Hb (g/dl)	PCV (%)	TEC(10^6/µl)	TLC(10^3/µl)
Control	NIL	12.50±0.15	39.26±0.46	8.45±3.46	11.28±0.32
Test group I	12.5	12.20±0.08	34.95±0.60	8.17±3.34	11.23±0.12
Test group II	25	12.16±0.20	35.27±1.07	7.75±3.17	11.11±0.07
Test group III	50	12.36±0.19	35.29±0.44	8.17±3.34	11.21±0.31
Test group IV	100	11.47±0.18**	35.63±0.77**	7.91±3.24	11.12±0.21

** Significant (P< 0.01) as compared to Control group within the same column

Table 2
Effect of Coxuril® (Toltrazuril, 2.5% w/v oral solution) on hepatic function following oral administration in rats (n=6).

Groups	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G	AST (U/L)	ALT (U/L)
Control	6.81±0.32	4.00±0.09	2.80±0.18	1.46±0.12	68.16±2.38	24.935±1.18
Test group I	6.62±0.48	4.32±0.11	2.29±0.23	2.01±0.26	64.65±2.44	24.08±0.40
Test group II	6.25±0.45	3.77±0.16	2.25±0.25	1.75±0.16	69.18±1.71	24.31±0.24
Test group III	6.25±0.90	4.01±0.25	2.24±0.29	1.99±0.36	68.73±2.63	24.13±0.46
Test group IV	6.31±0.60	4.06±0.24	2.25±0.18	1.87±0.20	76.35±1.40**	28.56±0.43**

** Significant (P< 0.01) as compared to Control group within the same column

Table 3
Effect of Coxuril® (Toltrazuril, 2.5% w/v oral solution) on glucose, urea and creatinine levels following oral administration in rats (n=6).

Groups	Glucose (mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Control	79.81±2.40	36.70±1.22	0.49±0.02
Test group I	79.50±0.34	35.77±1.21	0.51±0.01
Test group II	80.16±0.47	37.85±1.24	0.46±0.10
Test group III	80.33±0.42	37.62±1.44	0.50±0.10
Test group IV	81.51±1.37	38.37±1.15	0.55±0.03

** Significant (P< 0.01) as compared to Control group within the same column

loss of structural integrity of hepatocytes as these enzymes are located in the cytoplasm and are released into circulation after cellular damage (Ahmad and Khater, 2001). No significant changes were observed in level of glucose, urea and creatinine of test groups as compared to control group.

It is concluded from the acute toxicity study of Toltrazuril in rats that Toltrazuril produces significant hemotoxic, and hepatotoxic effect at the higher dose level of 100 $mg.kg^{-1}$ b.w. but it does not produce any significant toxicity at the dose rate of 12.5, 25 and 50 $mg.kg^{-1}$ b.wt. The results of the present study and the pharmacokinetic study conducted by the author in Rhode Island Red poultry birds (Bisht *et al.*, 2018) indicate that Coxuril® (Toltrazuril 2.5% oral solution) is safe for clinical use at the therapeutic dose of 7 $mg.kg^{-1}$ b.wt.

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Research Article

EVALUATION OF PROTECTIVE EFFECTS OF *ERYTHRINA VARIEGATA* AND *SPONDIA SPINNATA* ON CLINICO-HEMATOLOGICAL PROFILE OF RAT EXPOSED TO SUB-CHRONIC CADMIUM INTOXICATION.

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ABSTRACT

This investigation was undertaken to evaluate ameliorative efficacy of the bark powder of *E. variegata* (EVBP) and *Spondia spinnata* (SPBP) against cadmium chloride induced toxic effects in a 90 days trial in terms of body weight gain, clinical signs and hematology in Wistar albino rats. Forty-two, six-month-old, albino Wistar rats, weighing 170-200gm, were randomly and equally allocated into seven groups. For 90 days trial, group I served as control. Groups II, III, IV, V, VI and VII were given cadmium chloride @100mg/L of drinking water. Group II served as negative control. Simultaneous treatment was given as silymarin@100mg/kg bwt in group III and EVBP and SPBP @ 1 and 2% in groups IV, V, VI and VII, respectively, in feed for 90 days. No clinical sign was observed except curved spine or lordosis in Cd treated group II. Change in body weight, clinical sings and haematological parameters were examined at day 0, 30, 60 and 90. There was a significant ($P<0.05$) decline in body weight, haematological parameters Hb, PCV, TEC, TLC and DLC in Cd treated groups whereas a significant elevation in these parameters was observed in treatment groups as compared to Cd treated group II. SPBP and EVBP significantly ameliorated haematological and prevented any clinical signs and body weight. It is concluded from this study that EVBP and SPBP @1 and 2 % in feed ameliorated the cadmium chloride, given @ 100 ppm in drinking water, induced hemo-toxicity and prevented clinical signs and body weight decline, following simultaneous oral administration for 90 days in rats.

Key words: Cadmium, haematology, *Spondia spinnata,* , Wistar rats

INTRODUCTION

Cadmium (Cd) is a soft, silver white, redox-inactive non-essential metal known to be most toxic environmental and industrial pollutant, present in soil, air, water and cigarette smoke and even in food. It is listed by the USEPA as one of 126 priority pollutants and is claimed as a human carcinogen. Cd belongs to the group of transition elements, it almost adopts oxidation state of 2+, and thus, in most chemical reactions it behaves like a main-group metals. Cadmium does not induce production of ROS through a Fenton-like reaction (Stohs *et al.*, 2001). Since past two centuries, surge in anthropogenic and industrial activities have led to high emissions of Cd into the environment. It is estimated that about 25,000 to 30,000 tons of cadmium is released into the environment each year, about half from the natural sources mainly from the weathering of rocks, forest fires and volcanoes. Release of cadmium from human activities is estimated at from 4,000 to 13,000 tons per year. Major contributors are mining activities and burning of fossil fuels. The major resources of consumption of Cd are Ni-Cd batteries (79%), Cd pigment (11%), Cd stabilizer (2%), Cd coating (7%), Cd alloys and miscellaneous (1%) (Kumar *et al.*, 2009). Cadmium is one of the major hazardous metals of concerns for India in terms of their environmental load due to zinc smelting, waste batteries, e-waste, paint sludge, incinerations and fuel combustion (Indian National Science Academy, 2011). In addition, agrochemicals particularly the fertilizers often contain some cadmium that enters in to the soil and water, subsequently enter in to food chain comprising fish, plants and animals and then ultimately to the tissue in human being. The half-life of cadmium in humans is estimated to be between 15 and 20 years.

Cadmium exposure is linked with various chronic diseases. Prolonged cadmium disturbs calcium metabolism leading to osteomalacia and painful fracture, called as "ouch-ouch" (Itai-itai) disease in Japan. Cadmium is also known to be carcinogenic, and have been associated with cancers of the lungs and prostate. Cd induces multiple renal injuries, evident by tubular dysfunction, marked reduction of renal energy metabolism, altered essential mineral composition, and many trans-membrane transport abnormalities. In addition, following chronic and acute Cd exposure, it causes ultra-structural changes primarily in hepatocytes sinusoidal endothelial cells, depending on type and duration of exposure.

The permissible level of Cd in inland water is <2ppm and <0.01ppm for drinking water (ISI, 1991). The use of chemo-preventive agents has been employed for reversing the toxic effect and therapy against Cd induced

toxicity including man and animals however; they are most often associated with undesirable side effects. Therefore, years of attention has been directed towards safer amelioration and treatment with herbal remedies (Pari and Murugavel, 2005). As per estimate, 70% of the population around the world rely upon traditional medicines derived from plants. A range of plants, vegetables and synthetic compounds have been screened to ascertain their potential in the prevention and cure of heavy metal induced toxic effects (Desai *et al.*, 2012). In the past decade, several naturally occurring dietary or non-dietary constituents, and parts of several species of medicinal plants like *Eclipta alba*, *Emblica officinalis*, *Withania somnifera, Aloe vera, Tinospora cordiflora, Phyllanthus fraternus, Spondia spinnata etc.* have been reported to possess various pharmacological activities.

Despite many folklore practices and therapeutic uses of *Erythrina variegata* and *Spondia spinnata,* there is paucity of data on its ameliorating potential of heavy metals intoxication. Keeping in view of the above fact, the present investigation was undertaken with the objectives to evaluate protective effect of stem bark powder of *Erythrina variegata*and *Spondia spinnata* following sub chronic intoxication of cadmium in rats.

MATERIALS AND METHODS

The plant specimen of *Erythrina variegata* (EV) and *Spondia spinnata*(SP) was collected from Santhal Pargana region of Jharkhand during mid-June, 2012.The plant were taxonomically identified vide CHN/64/2012/ Tech-II dated 12.10.2012, BSI, Howrah 711-103, as *Erythrina variegata* and *Spondia spinnata* (L. f.) Kurz. The raw bark was gathered, chopped fine, shade dried, ground in electric grinder to fine homogenous powder. *Erythrina variegat*a bark powder (EVBP) and *Spondia spinnata* bark powder (SPBP) werestored in sealed polyethylene pouch. This EVBP and SPBP were used to feed the animals under study along @ of 1 and 2% with the steamed standard computed ration

This plan of work has been approved by the Institutional Ethical Committee vide approval number IAEC/VPT/CVASc/129 dtd. 06.11.2013.

Forty-two Wistar albino rats weighing about 170 to 200 gm were procured from Lab animal unit IVRI, Izatnagar. The rats were maintained at Lab Animal Shed, C.V.A.Sc., Pantnagar under standard management practice and randomly allocated into seven groups of 6 rats each. After a fortnight adaptation period, drinking (tap) water (*ad lib*) was replaced with deionised water for group I and mixed with Cadmium chloride @ 100mg/ L for group II to VII. The rats of group I and group II served as positive and negative control, respectively. After every 30 days blood was withdrawn from each rat for study of different parameters.

All the chemical includingdisodium EDTA, methanol, Drabkin's reagent, Hayem's fluid, Leishmann's stain, Giemsa stain and cadmium chloridewere procured from Himedia, India.

Experimental design

Forty-two albino Wistar rats weighing 170-200 gm were considered for the study. Group I served as control and was given deionized water *ad libitum*. Cadmium chloride @100mg/L in deionised drinking water were provided *ad libitum* to group II to VII, silymarin @ 100mg/kg b.wt. in feed to Group III in addition. Group IV & V received ration containing EVBP @1% and 2% respectively. The animals in group VI and VII were catered with ration along with SPBP @ 1% and 2% respectively. The experiment continued for ninety days. All the groups were maintained under standard management condition throughout the study. The experimental design as shown in the table 1a. Blood samples were collected antioxidant parameters at 0, 30, 60 and 90-days interval.

Collection and processing of blood samples

Blood samples 0.5ml were collected from retro-orbital sinus of Wistar-rats in fliptop tubes,containing heparin (@ 5-10 IU/ml) under partial inhalational anesthesia, on every 30th day using capillary tubes and used for analysis of hematological parameters.

Haematology

Haematological parameters such as Total Erythrocyte Count (x 10^6/µl blood) and Total Leukocyte Count (x 10^3/µl blood) wereestimated as mentioned Chauhan (1998). Packed Cell Volume (PCV %) was estimated by capillary/microhematocrit method and hemoglobin (gm/dl) was estimated by Drabkin's (cyanmet-hemoglobin) method. Differential Leukocyte

Table 1a
Experimental design

Groups	Treatment	Duration
I	Control- *ad lib* de-ionized water	90 days
II	Cadmium chloride@ 100mg/L in de-ionized water.	90 days
III	Cadmium chloride@ 100mg/L in de-ionized water + Silymarin @100mg/kg.b.wt. in feed	90 days
IV	Cadmium chloride@ 100mg/L in de-ionized water + *E. variegata* bark powder @ 1% in feed.	90 days
V	Cadmium chloride@ 100mg/L in de-ionized water + *E. variegata* bark powder @ 2% in feed.	90 days
VI	Cadmium chloride@ 100mg/L in de-ionized water + *S. pinnata* bark powder @ 1% in feed.	90 days
VII	Cadmium chloride@ 100mg/L in de-ionized water + *S. pinnata* bark powder @ 2% in feed.	90 days

Count (DLC %) was done by preparing thin blood smear from a drop of blood without anticoagulant. The smear was air dried and stained using Leishman stain. The leucocytes were counted by zig-zag method and recorded on percent basis.MCV (fl), MCH (Pg), and MCHC (%) were determined by the methods as described by Benjamin (2004).

STATISTICAL ANALYSIS

Statistical analysis was done using two-way analysis of variance (ANOVA) to determine the significance ($P < 0.05$) of efficacy of EVBP and SPBP treatment between two means based on individual observations of different parameters (Snedecor and Cochran, 1975).

RESULTS AND DISCUSSION

The investigation was undertaken to evaluatethe ameliorative efficacy of the bark powder of *E. variegata* (EVBP) and *Spondia spinnata* (SPBP) in term of its impact over gain in body weight, clinical signs and haematology in cadmium chloride exposure in Wistar albino rats.

The animals of groups II and VI showed significantly ($P<0.05$) less sign of weight gain as compared to treatments and control groups on day 30 (Table 1). Group I attained significantly ($P<0.05$) higher weight gain than group V, VII, III, IV, VI and II on day 90 of the study. Treatment group V attained significantly greater weight as compared to group IV and were at par with control group I, which seems to be a dose related response of EVBP. However, SPBP did not exhibit dose dependent effect on weight gain. Silymarin treated group III had better gain in weight as compared to group II, like group IV and VI but was less than control. It can be inferred from the Table 1 that weight gain were significantly less in group II in comparison to control and treatment group due to exposure of Cd, however the animals of group V, fed with EVBP @2% maintained the growth rate *at par* with control.

Initially up to 30 days of the study, no apparent clinical signs were noticed. Later, on day 60 onward, reduced water intake, emaciation and apparent curving of the spine were visible in group II and were more prominent by 90 days. Such effects were also reported in Cd toxicity in rats and termed as lordosis (Itokawci,*et al.*, 1973), may be due to the disturbance in Ca-P homeostasis. This phenomenon of spine deformity was not noticed among any other group, which indicated the protective effect of the EVBP and SPBP in Cd intoxicated rats. The water intake was slightly reduced. Rest of the groups apparently did not show any undesirable signs or symptoms during study.

Haematology

A significant reduction in Hb% was observed in rats of group II (Table 1) at 30, 60 and 90 days in

Table 1
Effect of *E. variegata* (EVBP) and *S. pinnata* (SPBP) on body weight, Hb and PCV following exposure of cadmium chloride daily for 90 days in rats (n=6).

Group	Treatments	Weight (gm)				Hb (gm/dL)				PCV (%)			
		0 day	30 day	60 day	90 day	0 day	30 day	60 day	90 day	0 day	30 day	60 day	90 day
I	Control	181.83± 2.57^{1a}	204.16± 2.14^{2b}	219.67± 1.20^{2c}	229.33± 2.00^{4d}	11.50± 0.33^{1a}	11.48± 0.45^{2a}	11.54± 0.33^{2a}	11.59± 0.28^{2a}	46.66± 1.451^{a}	47.16± 2.28^{1a}	46.16± 1.24^{1a}	46.50± 1.43^{1a}
II	Cadmium chloride @ 100mg/L in water	182.66± 5.66^{1a}	186.5± 5.05^{1a}	193.33± 5.52^{1a}	192.67± 4.72^{1a}	11.72± 0.19^{1b}	9.66± 0.30^{1a}	9.31± 0.25^{1a}	8.90± 0.24^{1a}	47.50± 1.381^{a}	47.33± 1.33^{1a}	42.83± 1.22$^{123\,b}$	41.00± 1.18^{3b}
III	Cadmium chloride @ 100mg/L+ Silymarin @100mg/Kg b.wt. in feed	182.50± 6.89^{1a}	192.34± 6.29^{12ab}	197.83± 6.24^{1ab}	206.50± 6.42^{123b}	11.35± 0.68^{1a}	12.05± 0.16^{2a}	11.89± 0.29^{2a}	11.70± 0.30^{2a}	47.66± 0.951^{a}	45.83± 0.47^{1a}	43.66± 0.49^{123b}	42.33± 0.42^{23b}
IV	Cadmium chloride @ 100mg/L + 1% EVBP in feed	180.00± 5.77^{1a}	190.50± 4.07^{12ab}	198.83± 4.83^{1bc}	205.83± 3.22^{12c}	11.13± 0.28^{1a}	11.41± 0.15^{2a}	11.33± 0.19^{2a}	11.24± 0.19^{2a}	46.83± 0.70^{1a}	46.16± 0.70^{1a}	45.66± 0.42^{12a}	44.83± 0.65^{12a}
V	Cadmium chloride @ 100mg/L + 2% EVBP in feed	182.16± 5.67^{1a}	197.66± 5.40^{12b}	205.83± 4.93^{1bc}	219.83± 4.81^{34c}	11.85± 0.37^{1a}	11.60± 0.41^{2a}	11.40± 0.38^{2a}	11.27± 0.21^{2a}	46.50± 0.84^{1a}	44.16± 0.94^{1ab}	42.33± 1.74^{23a}	41.66± 1.35^{23b}
VI	Cadmium chloride @ 100mg/L+ 1%	182.67± 4.12^{1a}	185.67± 3.24^{1ab}	196.33 ±3.48^{1bc}	202.50± 3.84^{12bc}	11.68± 0.35^{1a}	11.35 ±0.24^{2a}	11.25± 0.18^{2a}	11.17± 0.41^{2a}	47.50± 0.42^{1a}	44.00± 1.21^{1b}	42.00± 0.96^{3bc}	40.66± 0.88^{3c}
VII	Cadmium chloride @ 100mg/L + 2% SPBP in feed	182.17± 4.77^{1a}	190.50± 4.80^{12ab}	201.17± 4.74^{1b}	210.33± 4.79^{23c}	11.69± 0.35^{1a}	11.42± 0.21^{2a}	11.31± 0.25^{2a}	11.27± 0.36^{2a}	47.66± 0.84^{1a}	46.66± 0.95^{1a}	44.83± 1.01^{123ab}	43.33± 1.05^{123b}

Mean values in a column bearing different numeral superscript i.e. 1, 2, 3, 4 and 5 differ significantly ($p<0.05$) when compared vertically. Mean values in a row bearing different alphabetic superscript i.e. a, b, c, d and e differ significantly ($p<0.05$) when compared horizontally.

Table 2
Effect of *E. variegata* (EVBP) and *S. pinnata* (SPBP) on TEC, TLC and Neutrophil, following exposure of cadmium chloride daily for 90 days in rats (n=6).

Groups	Treatments	TEC ($X10^6$/µL)				TLC ($X10^3$/µL)				Neutrophil (%)			
		0 day	30 day	60 day	90 days	0 days	30 day	60 days	90 days	0 days	30 day	60 days	90 days
I	Control	7.19± 0.07^{1a}	7.18± 0.10^{1a}	7.20 ±0.07$^{1\ a}$	7.20± 0.06^{1a}	8.36± 0.04^{1a}	8.40± 0.04^{1a}	8.35± 0.04^{1a}	8.35± 0.03^{1a}	20.83± 1.03^{1a}	21.33± 1.35^{1a}	20.66± 1.05^{1a}	21.66± 0.67^{1}
II	Cadmium chloride @ 100mg/L in water	7.17± 0.07^{1a}	6.76± 0.07^{2b}	6.66± 0.07^{2b}	6.59 ±0.06^{3b}	8.35± 0.04^{1a}	8.62± 0.03^{3b}	8.94± 0.02^{3c}	9.12± 0.01^{4d}	22.33± 1.08^{1a}	35.00± 1.15^{3b}	39.50± 0.76^{4c}	45.83± 1.44^{4d}
III	Cadmium chloride @ 100mg/L+Silymarin @100mg/Kg b.wt. in feed	7.18± 0.18 ^{1a}	7.14± 0.13^{1a}	7.15± 0.09^{1a}	7.11± 0.09^{12a}	8.36± 0.02^{1a}	8.47± 0.02^{12b}	8.62± 0.03^{2c}	8.68± 0.03^{2c}	21.83± 1.13^{1a}	27.16± 0.70^{2b}	30.00± 0.93^{2c}	33.16± 0.87^{2d}
IV	Cadmium chloride @ 100mg/L+1% EVBP in feed	7.18± 0.01 ^{1a}	7.18± 0.08^{1a}	7.15± 0.09^{1a}	7.14± 0.03^{12a}	8.36± 0.02^{1a}	8.50± 0.02^{2b}	8.67± 0.02^{2c}	8.76± 0.02^{23d}	21.50± 0.76^{1a}	27.50± 0.76^{2b}	31.83± 0.90^{2c}	34.00± 0.93^{2c}
V	Cadmium chloride @ 100mg/L+2% EVBP in feed	7.20± 0.10 ^{1a}	7.10± 0.01^{1a}	7.08± 0.09^{1a}	6.99± 0.04^{2a}	8.36± 0.02^{1a}	8.45± 0.02^{12b}	8.64± 0.02^{2c}	8.70± 002^{2c}	21.66± 0.80^{1a}	26.33± 0.67^{2b}	29.00± 0.85^{2b}	32.00± 1.23^{2c}
VI	Cadmium chloride @ 100mg/L+1% SPBP in feed	7.20± 0.04 ^{1a}	7.10± 0.04^{1b}	7.04 ±0.01^{1b}	7.02± 0.03^{12b}	8.36± 0.03^{1a}	8.52 ±0.04^{2b}	8.71± 0.02^{2c}	8.83 ±0.03^{3d}	21.83± 1.10^{1a}	28.00± 1.06^{2b}	35.00± 1.15^{3c}	39.67± 0.67^{3d}
VII	Cadmium chloride @ 100mg/L+2% SPBP in feed	7.19± 0.05 ^{1a}	7.11± 0.03^{1a}	7.09± 0.04^{1a}	7.06± 0.06^{12a}	8.36± 0.01 ^{1a}	8.52± 0.01^{2b}	8.69± 0.01^{2c}	8.76± 0.02^{23d}	21.67± 0.71^{1a}	27.83± 0.94^{2b}	31.33± 0.88^{2c}	35.33± 1.45^{2d}

Mean values in a column bearing different numeral superscript i.e. 1, 2 and 3 differ significantly (p<0.05) when compared vertically. Mean values in a row bearing different alphabetic superscript i.e. a and b differ significantly (p<0.05) when compared horizontally

Table 3
Effect of *E. variegata* (EVBP) and *S. pinnata* (SPBP) on Lymphocyte, Monocytes and Eosinophil following exposure of cadmium chloride daily for 90 days in rats (n=6).

Group	Treatments	Lymphocyte (%)				Monocyte (%)				Eosinophil (%)			
		0 day	30 day	60 day	90 days	0 days	30 day	60 days	90 days	0 days	30 day	60 days	90 days
I	Control	74.50± 0.95^{1a}	74.34 ±1.45^{1a}	74.00 ±1.23^{1a}	73.33 ±1.40^{1a}	3.67± 0.34^{1a}	3.67± 0.42^{1a}	4.16± 0.30^{1a}	3.67± 0.33^{1a}	1.00± 0.36^{1a}	0.67± 0.33^{1a}	1.16± 0.30^{1a}	1.16± 0.54^{1a}
II	Cadmium chloride @ 100mg/L in water	73.16± 1.44 ^{1a}	60.50± 0.84^{3b}	55.83± 1.13^{4c}	50.83± 1.16^{4d}	3.67± 0.21^{1b}	2.50± 0.42^{2b}	2.83± 0.30^{2ab}	2.16± 0.30^{2b}	0.83± 0.30 ^{1a}	2.00± 0.36^{2a}	1.83± 0.65^{12a}	1.50± 0.42^{1a}
III	Cadmium chloride @ 100mg/L+Silymarin @100mg/Kg b.wt. in feed	74.50± 1.47 ^{1a}	66.50± 0.80^{2b}	64.16± 1.10^{2bc}	61.66± 0.66^{2c}	3.16± 0.30 ^{12a}	4.00± 0.36^{1a}	3.67± 0.21^{12a}	3.16± 0.47^{12a}	0.50± 0.22^{1a}	2.33± 0.21^{2b}	2.16± 0.30^{12b}	2.00± 0.25^{1b}
IV	Cadmium chloride @ 100mg/L+1% EVBP in feed	74.66± 0.49 ^{1a}	67.34± 0.67^{2b}	63.33± 0.80^{2c}	60.83± 1.16^{2d}	3.16± 0.16 ^{12a}	3.33± 0.33^{12a}	3.33± 0.21^{12a}	2.83± 0.30^{12a}	0.67± 0.21^{1a}	1.83± 0.16^{2ab}	1.5± 0.34^{12ab}	2.33± 0.42^{1b}
V	Cadmium chloride @ 100mg/L+2% EVBP in feed	74.34± 1.11 ^{1a}	68.16± 0.54^{2b}	65.83± 0.65^{2bc}	62.50± 1.80^{2c}	2.67± 0.33 ^{2a}	3.50± 0.22^{12a}	3.50± 0.22^{12a}	3.16± 0.70^{12a}	1.16± 0.47^{1a}	2.00± 0.25^{2ab}	1.67± 0.21^{12ab}	2.33± 0.42^{1b}
VI	Cadmium chloride @ 100mg/L+1% SPBP in feed	74.16± 1.13^{1a}	66.83± 0.94^{2b}	59.50± 1.45^{3c}	55.83± 0.79^{3d}	3.00± 0.25 ^{12a}	3.50± 0.22^{12a}	3.00± 0.44^{2a}	3.00± 0.36^{12a}	1.00± 0.25^{1a}	1.67± 0.21^{2b}	2.50± 0.22^{2b}	1.50± 0.34^{1a}
VII	Cadmium chloride @ 100mg/L+2% SPBP in feed	74.83± 1.07 ^{1a}	67.50± 0.71^{2b}	64.34± 0.80^{2c}	59.50± 1.20^{2d}	2.67± 0.21^{2a}	3.50± 0.22^{12ab}	3.16± 0.16^{2ab}	3.67± 0.42^{1b}	0.83± 0.30^{1a}	2.00± 0.25^{2ab}	1.16± 0.30^{1ab}	1.50± 0.22^{1ab}

Mean values in a column bearing different numeral superscript i.e. 1, 2, 3 and 4 differ significantly (p<0.05) when compared vertically. Mean values in a row bearing different alphabetic superscript i.e. a, b, c and d differ significantly (p<0.05) when compared horizontally

comparison to control and treatment groups which indicates protective effect of silymarin, EVBP and SPBP in respective group.

Significant (P <0.05) decline in the PCV (Table 1) in II, III, V, VI group was evident on day 60 and 90 compared to control. Silymarin treated group III, exhibited significant (P <0.05) fall in PCV during the study. There was no significant difference between control and treatment group when compared vertically in the table. However, on viewing horizontally, there is a decline in PCV from 60 day onward within group in all the groups except Group I. This decline in PCV may be an indicator of decline in haem synthesis by reducing the absorption of iron from GIT as observed by Rous (2000) and

Table 4
Effect of *E. variegata* (EVBP) and *S. pinnata* (SPBP) on MCV, MCH and MCHC following exposure of cadmium chloride daily for 90 days in rats (n=6).

Group	Treatments	MCV (fl)				MCH (pg)				MCHC (%)			
		0 day	30 day	60 day	90 day	0 day	30 day	60 day	90 day	0 day	30 day	60 day	90 day
I	Control	64.88± 2.22^{1a}	65.73± 3.44^{12a}	64.16 ±2.09^{1a}	64.55± 2.05^{1a}	15.97± 0.34^{1a}	15.94± 0.44^{2a}	16.00± 0.31^{1a}	16.09± 0.30^{1a}	24.77± 1.07^{1a}	24.64± 1.68^{1a}	25.13± 1.16^{1a}	25.11± 1.17^{1a}
II	Cadmium chloride @ 100mg/L in water	66.24± 2.17^{1ab}	70.04 ±2.04^{2ab}	64.30± 2.03^{1ab}	62.22± 1.73^{12a}	16.33± 0.12^{1a}	14.28± 0.30^{3b}	13.96± 0.26^{2bc}	13.49± 0.23^{2c}	24.79± 0.89^{1a}	20.47± 0.75^{2b}	21.82± 0.85^{2b}	21.77± 0.73^{2b}
III	Cadmium chloride @ 100mg/L+Silymarin @100mg/Kg b.wt. in feed	66.64± 2.76^{1a}	64.27± 1.33^{12ab}	61.05± 1.05^{1b}	59.57± 1.21^{12b}	15.72± 0.54^{1a}	16.89± 0.23^{1b}	16.60± 0.24^{1ab}	16.44± 0.22^{1ab}	23.97± 1.89^{1a}	26.315± 0.44^{1ab}	27.27± 0.83^{1ab}	27.69± 0.91^{1b}
IV	Cadmium chloride @ 100mg/L+1% EVBP in feed	65.34± 1.80^{1a}	64.37± 1.57^{12a}	63.92± 1.33^{1a}	62.75± 0.95^{12a}	15.49± 0.21^{1a}	15.89± 0.06^{2ab}	16.26± 0.20^{1bc}	16.70± 0.20^{1c}	23.82± 0.89^{1a}	24.76± 0.64^{1ab}	25.49± 0.61^{1ab}	26.67± 0.68^{1b}
V	Cadmium chloride @ 100mg/L+ 2% EVBP in feed	64.67± 1.79^{1a}	62.19± 1.13^{1a}	59.68± 1.82^{1a}	59.60± 2.04^{12a}	16.44± 0.29^{1a}	16.31± 0.39^{12a}	16.08± 0.36^{1a}	16.11± 0.23^{1a}	25.57± 1.14^{1a}	26.28± 0.80^{1a}	27.01± 0.64^{1a}	27.20± 1.05^{1a}
VI	Cadmium chloride @ 100mg/L+ 1% SPBP in feed	65.98± 0.93$^{c\,1a}$	61.91± 1.61^{1b}	59.58± 1.31^{1bc}	57.88± 1.14^{2c}	16.22± 0.24^{1a}	15.97± 0.24^{2a}	15.96± 0.19^{1a}	15.88± 0.50^{1a}	24.63± 0.71^{1a}	25.89± 0.79^{1ab}	26.84± 0.57^{1ab}	27.50± 1.03^{1b}
VII	Cadmium chloride @ 100mg/L+ 2% SPBP in feed	66.32± 1.40^{1a}	65.59± 1.15^{12a}	63.21± 1.21^{1ab}	61.37± 1.50^{12b}	16.24± 0.38^{1a}	16.05± 0.21^{12a}	15.94± 0.26^{1a}	15.95± 0.38^{1a}	24.58± 0.94^{1a}	24.50± 0.38^{1a}	25.24± 0.41^{1a}	26.07± 0.98^{1a}

Mean values in a column bearing different numeral superscript i.e. 1 and 2 differ significantly ($p<0.05$) when compared vertically.
Mean values in a row bearing different alphabetic superscript i.e. a, b and c differ significantly ($p<0.05$) when compared horizontally

(ATSDR, 2000).

The decline in TEC in negative control group (group II) was significant ($P<0.05$) from days 30, 60 and 90 (Table 2) in comparison to the rest of the groups. The decrease in TEC was more obvious in the group II later as compared to its day zero data. Similarly, the TEC declined significantly in group VI on day 60th onward. There was no significant ($P<0.05$) difference in TEC among the rest of the treatment groups indicating the ameliorative effect of the EVBP and SPBP on Cd induced reduction in TEC.

Cd inhibit hepatic δ-Aminolevulinate dehydratase (δ-ALA-D) like lead: a sulfhydryl-containing enzyme that catalyses the asymmetric condensation of two δ-aminolevulinic acids (δ-ALA) molecules yielding porphobilinogen, a haem precursor (Jaffe, 1995). Consequently, δ-ALA-D inhibition may perturb haem-dependent metabolic pathway (Maciel *et al.*, 2000) and can result in the accumulation of 5-aminolevulinic acid, which has some pro-oxidant activity (Yusof *et al.*, 1999). The exposure of Cd leads to decrease in the levels of erythrocyte adenosine triphosphate (ATP) and 2,3-diphospho-glycerate (2,3-DPG) as well as increase the oxidative stress that initially altering membrane skeleton of erythrocyte, followed by deformation of the cell, thus promoting haemolysis, and resulting in decreased level of haemoglobin and anaemia (Klapcinska *et al.*, 2000**)**.

In addition, earlier reports revealed that Cd exposure induces anemia along with decline in erythrocytes' counts. It induces oxidative damage and lipid peroxidation in RBC. Cd intoxication mediated anemia might be due to either suppression of the activity of hematopoietic tissues, impaired erythropoiesis, and accelerated erythroclasia as a result of the altered RBCs membrane permeability, increased RBCs mechanical fragility, and/or defective iron metabolism (Yuan *et al.*, 2014).

Thus, the usual hemopoietic pathway blocked by Cd might have been rectified to certain degree by EVBP and SPBP interfering absorption of Cd in gut, and thereby improved the absorption of iron. As such both the plants have good antioxidant attributes which might have helped stabilize the hemopoietic system and reduced fragility of RBC by reducing lipid peroxidation and scavenging oxidant radicals.

As observed in Table 2, there was a significant increase in TLC among group II from day 30 onward, as compared to rest of the groups *viz.* III, IV, V, VI and VII. The result is in accordance to the earlier reports (Onwuka *et al.,* 2010). The increased production and/or release of neutrophils from bone marrow might account for their rise in animals (Kataranovski *et al.*, 2009).

A significant increase in neutrophil on DLC was observed in group II in comparison to control and treatment groups on day 30 onward till 90 days. The treatment with silymarin and both the concentration of EVBP and SPBP treatment brought nonsignificant improvement in the neutrophil count showing their limited ameliorative effects (Table 2). This neutrophilia was also observed in descending order among group VI, VII, IV,

III and V during the study period. Neutrophilia has also been reported by Ashour (2014) among Cd exposed albino Wistar rats. The increased production and/or release of neutrophils from bone marrow might account for their rise in animals (Kataranovski *et al.*, 2009). A higher responsiveness to exogenous (PMA) stimulation, i.e. neutrophil priming is in agreement with the data that demonstrated a higher peripheral blood neutrophil count after systemic inflammation evoked by traumatic tissue injury among rats (Deitch *et al.*, 2006). This can be stated that Cd can induce in pro-inflammatory mode as the increased number of immature neutrophils and accelerated regeneration of granulocyte-macrophage progenitor cells in irradiated mice received cadmium (Fedorocko *et al.*, 1996).

Mobilization of neutrophils from the marginal into the circulating pool due to altered cytokines release contributes to the rise of neutrophils (Suwa et al., 2000). Amelioration by EVBP and SPBP as observed in this study, thus, might have been due to reduced systemic inflammation induced by Cd by reducing the cytokines.

There was a significant ($P<0.05$) lymphocytopaenia on DLC on day 30 onward in cadmium treated group II as compared to remaining groups (Table 3). The lymphocytes count on day 90 in ascending order was observed as group VI, VII, IV, III and group V. So it can be inferred that EVBP, SPBP and silymarin however did not normalize the lymphocyte count but it was significantly ($P<0.05$) higher than Cd treated group which indicated good potential to protect against immunotoxic capacity of Cd.

The level of monocytes was on a down side turn among the cadmium treated group II on day 30 (Table 3). However, the monocytes were at par to rest of the treatment and positive control groups on 30, 60 and 90 days which indicate the ameliorative effects of the treatment. Monocytes help other white blood cells remove dead or damaged tissues. Cadmium is apparently brought into the vascular wall by Cd-laden monocytes which differentiate into foam cells (Steffensen *et al.,* 1994).

As such the mean value (Table 3) for eosinophil was significantly raised ($P<0.05$) among all the treatment groups except the control group (1.00± 0.36) on day 30th. The value of eosinophil was least in group I and VII which was significantly lesser as compared to groups II, III, IV and V on day 60th. Group VI had significantly higher value as compared to rest of the groups on day 60th. On day 90 the eosinophil was significantly up in group IV and V in comparison to the control, but at par to the rest of the treatment groups. Therefore, it can be interfered that the treatment with EVBP and SPBP made least impact on eosinophilia. Anemia and eosinophilia have been associated with cadmium intoxication, and accumulation in kidneys leads to nephropathy and proteinuria (Martelli *et al.*, 2006).

The mean values for mean corpuscular volume of group II were significantly elevated (Table 4) in comparison to treatment groups like group V and VI and was *at par* to rest of groups *viz.* group I, III, V and VII on day 30th. However, later this difference was disappeared on day 60 and 90. This trend of MCV value assumes at par in all groups except the group VI on day 90, which remained least and at par to group II, III, IV, V and VII but significantly different to group I at $p<0.05$. However, the result in current study regarding the impact of Cd over MCV is in accordance to the earlier study (Ashour, 2014). From the result this can be inferred that the treatment of EVBP, SPBP (at 1% and 2%) and silymarin normalized the MCV among the treatment groups.

The mean value of mean corpuscular hemoglobin were found to decline as compared to group I, IV, VI on day 30 (Table 4). Group II, V and VII were showing raised value for MCH significantly higher in comparison to but were *at par* to the control group on day 30. However, the mean MCH values of group II remained significantly lesser as compared to the control group on day 90. The rest of the treatment groups were showing values similar to control group. Thus, it can be inferred that the treatment of EVBP and SPBP has restorative effect over MCH.

There was a significant decline in MCHC (Table 4) in group II as compared to control group on day 30. This trend remained during day 60 as well as day 90. The MCHC values for the treatment group under silymarin, EVBP (1 & 2%) and SPBP (1 & 2%) exhibited value close to the control group. The reduction in the MCHC values is usually observed in cases of hypochromic microcytic anemia. Exposure to Cd induces anemia associated with decrease in RBCs counts and induction of oxidative damage and lipid peroxidation in blood and RBCs. Moreover, the fall in the values of MCV, MCH, and MCHC in Cd group can also indicate the further ability of Cd intoxication to induce microcytic hypochromic anemia (Yuan *et al.*, 2014).

It is concluded on the basis of hematological parameters that Cd induced hemotoxic effects were ameliorated by EVBP and SPBP in 90 days.

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Research Article

IDENTIFICATION OF PHYTOCOMPOUNDS OF *PHYLLANTHUS NIRURI* AND CHARACTERIZATION BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY ANALYSIS

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ABSTRACT

Phyllanthus niruri is an important species of the *Euphorbiaceae* family and the leaves act as magnificent reservoirs of bioactive compounds. To detect the bioactive compounds in the *P.niruri* leaves, a study was conducted by qualitative and quantitative phytochemical analysis and also by GC-MS analysis. The qualitative phytochemical analysis of aqueous and alcoholic extracts of *P.niruri* leaves revealed the presence of alkaloids, carbohydrates, flavonoids, phenols, saponins, tannins and terpenoids. The quantitative phytochemical screening revealed the total quantity of alkaloids, flavonoids and phenol compounds in *P.niruri* extract. The GC-MS analysis detected thirty phytocompounds in methanolic extracts of *P.niruri* leaves. The present study forms the basis for biological characterization and phyto pharmaceutical significance of the identified compounds. The presence of various bioactive compounds justifies the usage of *P.niruri* leaf as an herbal choice for treating various diseases in animals.

Keywords: *P.niruri*, Qualitative analysis, Quantitative phytochemical analysis and GC-MS.

INTRODUCTION

Phyllanthus niruri has a long history in herbal medicine systems such as Indian Ayurveda, Traditional Chinese Medicine and Indonesian Jamu. The plant was useful for treating hepatotoxicity, hepatitis B, hyperglycaemia and viral and bacterial diseases (Kaur *et al.*, 2016). In South India, the herb was called Bhumyamalaki, and believed to treat constipation, gonorrhoea and syphilis (Kaur *et al.*, 2016). In northern India, this herb is locally known as 'pitirishi' and it has gained a reputation as a household remedy for asthma, bronchitis and even tuberculosis (Putri, *et al.*, 2018).The whole plant was used as remedy for many conditions such as dysentery, influenza, vaginitis, tumours, diabetes, jaundice, kidney stones and dyspepsia (Mao *et al.*, 2016). An herb contains a wide variety of biologically active compounds whose concentration depends upon the variety, season and climate (Chauhan *et al.*, 2016). Hence the identification of active constituents of locally available variety of *P.betle* herb will be useful to signify their use in the herbal medicine. Since Gas Chromatography and Mass Spectrometry (GC-MS) analysis could able to detect and quantify the compounds of herbs, the present study was conducted in locally available, *P.niruri* leaves by GC-MS analysis.

MATERIALS AND METHODS

P. niruri herb was collected from different regions of Namakkal District, South India and authenticated by the Botanical Survey of India (No.BSI/SRI/5/23/2017/Tech/1921) Coimbatore, Tamil Nadu.

Preparation of the leaf extract

Freshly collected leaves of *P.niruri* were shade dried and the size reduced to powder with the use of mechanical grinder. 10 grams of the pulverized material were soaked in 100 mL of methanol and kept on a rotary shaker for 24 hrs. The extract was then filtered through Whatman No. 1 filter paper and the process was repeated till the extraction of all soluble compounds. The extract was concentrated in a rotary evaporator under reduced pressure. The dried material was collected and stored in refrigerator for further experimental procedures.

Qualitative phytochemical analysis

The qualitative phytochemical screening of aqueous and alcoholic extracts of *P.niruri* was done by using the method of Harborne (1998) at the laboratory of Ethno Veterinary Herbal Research Centre for Poultry, Veterinary Clinical Complex Campus, Namakkal, Tamil Nadu.

Five gram of dried aqueous and alcoholic extracts of both the plants were added with 50 mL of distilled water and heated below 50°C for 1 – 2 minutes and utilized for the detection of various phytochemicals.

Quantitative estimation of phytochemicals

The estimation of total alkaloids, total phenols and total flavonoids in *P. niruri* was carried out to findout the quantity of individual phytoconstituents present in the herb.

Estimation of total alkaloids

The total alkaloids content was measured as

per the method of Harborne (1998). Briefly, 40 mL of 10 % acetic acid in ethanol was added to 1 gram of powdered sample of *Piper betle* allowed to stand for 4 hours. The filtrate was then concentrated on water bath to one fourth of its original volume. Concentrated ammonium hydroxide was added drop wise to the extracts until the precipitation was complete. The whole solution was allowed to settle and collected precipitate was washed with diluted ammonium hydroxide and then filtered. The residue was dried and weighed to findout the total alkaloids content.

Estimation of total phenol

The total phenolic content was determined by the Folin-Ciocalteu assay described by Patel *et al.* (2010). Fifty microliter of alcoholic extract of *P.niruri* was mixed separately with 250 µl of 10 % Folin-Ciocalteu solution followed by addition of 750 µl of 7.5 % (w/v) sodium carbonate and then the solution was incubated at room temperature for 2 hours in the dark. The absorbance was measured at 750 nm using a double beam UV-Visible spectrophotometer. A calibration curve was obtained using gallic acid as standard for the concentration ranging from 25 to 250 µg/ml as standard. The total phenolic content was expressed in terms of the milligrams of the gallic acid equivalent per gram of the dry mass (mg GAE/g).

Estimation of total f`lavonoid

The total flavonoid content in the extracts of *P.niruri* was estimated by the aluminium chloride colorimetric method (Patel *et al.,* 2010). Briefly, 0.25 ml extract of *P.niruri* herb (10mg/ml) was separately mixed with 0.75 ml of ethanol, 0.05 ml of the 10% aluminium chloride, 0.05 ml of the 1M potassium acetate and 1.4 ml of the distilled water. The reaction mixture was incubated for 30 minutes at room temperature. The absorbance of the mixture was measured at 415 nm using double beam UV-Visible spectrophotometer. A calibration curve was obtained using rutin as standard with concentration ranging from 10 to 160 µg/ml. The total flavonoid content was expressed in terms of the milligram of rutin equivalent per gram of the dry mass (mg RU/gm).

Gas Chromatography – Mass Spectrometry (GC-MS) analysis

Gas Chromatography - Mass Spectrometry (GC-MS) was used in the present study to identify the bioactive components present in the alcoholic extracts of *P.niruri*. The analysis was performed using GC-MS 5975 C Agilent System and Gas chromatograph interfaced to a mass spectrometer (GC-MS) equipped with elite – 1 fused silica capillary column.

For GC - MS detection, an electron ionization energy system with ionization energy of 70eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1.51 ml/min and an injection volume of 1 ml (100 mg of extract/mL) was employed (Split Ratio: 10). The Injector and Ion source temperature were 240°C and 200°C, respectively. The oven temperature was programmed from 70°C (isothermal for 2 min.), with an increase of 300°C for 10 minutes. Mass spectra were taken at 70eV; a scan interval of 5 minutes with scan range of 40 – 1000 m/z. Total GC running time was 30 minutes. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas.

Turbo Mass software was adopted to handle mass spectra and chromatograms (Adams, 2007). Interpretation of GC - MS results was carried out using Dr. Duke's phytochemical and ethno botanical database which is having more patterns on phytocomponents. The spectrum of the unknown components of the *P.niruri* was compared with the spectrum of the known components stored in National Institute of Standards and Technology (NIST) library and identified.

RESULTS AND DISCUSSION

The results of qualitative phytochemical analysis of aqueous and alcoholic extracts of *P.niruri* leaves were shown in table 1. The qualitative phytochemical analysis of aqueous and alcoholic extracts of *P.niruri* leaves revealed the presence of alkaloids, carbohydrates, flavonoids, phenols, saponins, tannins and terpenoids.

The quantitative analysis of *P.niruri* showed the total alkaloid content of 81.00± 0.57 mg/g, total phenol content of 110.00± 1.00 mg of GAE (Gallic acid equivalent)/g and total flavonoid content of 7.43± 0.40 mg of RU (Rutin)/g of extract). The quantitative analysis of *P.niruri* extracts revealed the higher content of alkaloids, phenols and substantial quantity of flavonoids.

Table 1.
Phytochemical constituents of *P.niruri leaves* extracts identified by qualitative screening

Phytochemicals	Aqueous	Alcoholic
Alkaloids	Present	Present
Amino acids and Proteins	Absent	Absent
Carbohydrates	Present	Present
Cardiac glycosides	Absent	Absent
Flavonoids	Present	Present
Glycosides	Absent	Absent
Hydrolysable Tannins	Absent	Absent
Phenol	Present	Present
Phylobatannin	Absent	Absent
Saponin	Present	Present
Tannin	Present	Present
Terpenoids	Present	Present
Vitamin C	Absent	Absent
Volatile oil	Absent	Absent

Table 2
Phytoactive Compounds detected in GC-MS analysis of P.niruri leaves extract

S. No	Name of the Compound	Retention time (minutes)	Area detected (percentage)	Phyto compounds concentration (mg/100mg)
1	a. Phytol b. Phytol c. Isophytol	18.827	1.87	1.87 mg
2	a. 9,12,15-Octa decatrienoic acid, ethyl ester, (Z,Z,Z) b. 2-Methyl-Z, Z-3,13 octadecadienol c. 9,12,15-Octa decatrienoic acid, ethyl ester, (Z,Z,Z)	19.315	1.41	1.41 mg
3	a. 9-Octa decadienal, (Z) b. 1,2-15, 16 – Diepoxy hexadecane c. 2-Methyl-Z,Z-3,13-octa decadienol	21.981	5.53	5.53 mg
4	a. Benzenamine, N – 2 - (3,4- dimethoxyphenyl) ethyl 2 - nitro. b. 2 (3H) - Oxazolone , 3- (3- 4- dimethoxy phenyl) methyl - 4,5 - diphenyl. c. Benzeneethamine N- (3,4- dimethoxy phenyl) methyl - 3,4- dimethoxy	25.029	31.04	31.04 mg
5	a. Beta tocoperol, b. Isoquinoline,6,7 dimethoxy- 1- methyl 4- (3- 4-dimethyl phenyl) c. 5, 5'-Bis (2- (4- amino phenyl) –1H-1,3- benzimidazol)	25.1	4.92	4.92 mg
6	a. Vitamin E b. Silane, dimethyl (4- chloro benzyl oxy) - tridecyloxy- c. Beta tocopherol, O-methyl	25.528	13.18	13.18 mg
7	a. 3H-Imidazo (4 – 5 – b - Pyridine), 2 (2-ethyl hexyl sulianyl) b. Benzimidazol - 2 - amine, N - (2,4- dimethoxy benzyl) c. Butyl dimethyl silyl oxybenzene	25.982	15.54	15.54 mg
8	a. 1,4- Dimethoxy,2,3 dimethyl benzene b. Phenol, 4- methoxy,2,3,6 – trimethyl c. Phenol, 3- methoxy,2,4,6 – trimethyl	26.063	18.45	18.45 mg
9	a. Hexesterol b.1,4 benzenediol, 2-5-bis (1,1- dimethyl ethyl) c. Methoxy-2,1,5-trifluro benzoic acid, nonadecyl ester.	26.135	1.83	1.83 mg
10	a. Amidocarb b. 3- (3-4- Dimethoxy phenyl) propylamine c. (R) - (-) - Alpha - methyl - 4 - nitro - benzylmine.	26.295	2.58	2.58 mg
11	a. Vitamin E b. Vitamin E c. Alpha tocopherol	26.557	1.25	1.25 mg
12	a. Tris (tert-bytyl dimethylsilyloxy) arsane b. Benzo(h)quinoline, 2,4- dimethyl- c.1,1,1,3,5,5,5- Hepta methyl trisiloxane	28.126	2.4	2.40 mg

The results of GC-MS analysis of *P.niruri* extract was presented in table 2. Thirty phytocompounds were identified in *P.niruri by* GC-MS analysis of herbal extract. The phytocompounds present in the *P.niruri* was proven for various biological activities. The phytocompound phytol showed antioxidant, anti-inflammatory, antimicrobial and diuretic activity (Krishnamoorthy and Subramaniam, 2014). The phenol,4-methoxy,2,3,6-trimethyl compound showed antioxidant and anti-inflammatory activity (Hadi *et al.*, 2016) and 9-octa decadienal, had antimicrobial activity (Krishnamoorthy and Subramaniam, 2014).

Isoquinoline, an alkaloid compound detected in *P.niruri* leaves howed antimicrobial and anti-inflammatory effects (Shanmugapriya and Kalavathi, 2012). Also the 9,12,15-octa decatrienoic acid and ethyl ester, (Z, Z, Z) showed anti-inflammatory and hepatoprotective activity (Krishnamoorthy and Subramaniam, 2014).

2 methyl - Z, Z-3,13-octadecadienol, 1,2-15,16-diepoxy hexadecane and vitamin E were commonly detected in GC-MS analysis; Of which the diepoxy hexadecane showed anti-inflammatory (Hameed *et al.*, 2016) effect, Vitamin E showed antioxidant, anti-inflammatory and hepatoprotective activity (Traber and

Atkinson, 2007). The results revealed that the methanolic leaf extract of *P. niruri* has number of bioactive phytoconstituents, which are responsible for numerous therapeutic activities. The compounds identified by GC-MS are medicinally valuable and possess wide variety of pharmacological applications. Further testing of individual phytoconstituents by *in vivo* experiments will validate the biological activities of *P.niruri* herb.

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Research Article

QUALITATIVE PHYTOCHEMICAL SCREENING AND *IN VITRO* ANTIOXIDANT POTENTIAL OF GERMINATED SEEDS OF *HORDEUM VULGARE*

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ABSTRACT

A good antioxidant balance in a biological system helps in maintaining normal physiological homeostasis. Deficiency of these compounds leads to oxidative stress resulting in inflammation, heart disease, diabetes, genotoxicity and cancer. The phytochemicals present in the plants are proved to have potential to produce antioxidant effect. Therefore, the present study was undertaken to evaluate the phytochemicals and to assess the *in vitro* antioxidant activity of methanol extract of germinated seeds of *Hordeum vulgare.* Qualitative phytochemical screening showed the presence of steroids, glycosides, saponins, diterpenes and triterpenes. Significant concentration-dependent antioxidant activity was produced by the extract in both DPPH and superoxide anion radical scavenging assays. The concentration of 320 µg/mL produced maximum inhibition of DPPH and superoxide radical with an inhibitory per cent of 65.39±1.21 and 57.73±1.64 respectively. The IC_{50} concentration of the extract was found to be 112.75±5.74 and 28.90±1.85 µg/mL for DPPH and superoxide radical scavenging assays respectively. Thus, the methanol extract of germinated seeds of *Hordeum vulgare* showed the presence of majority of phytochemicals which are proved to possess potent antioxidant activity in scavenging free radicals.

Keywords: *Hordeum vulgare,* Barley, germinated seeds, DPPH, superoxide, qualitative phytochemical screening, IC_{50}, free radical scavenging

INTRODUCTION

Majority of the physiological effects produced by plants in the body are due to the presence of phytochemicals in them. Phytochemicals present in the plants play a major role in maintaining and improving the health status of living beings. Different phytochemicals have different added advantages in biochemical reactions that occur in the body.

Antioxidants are the agents that protect biological systems against oxidative stress. The harmful free radicals which are known as radical derivatives of oxygen (ROS) are produced during normal aerobic respiration by macrophages, polymorphonuclear leukocytes and peroxisomes that cause extensive damage to the tissue leading to pathological conditions such as inflammation, genotoxicity and cancer (Kourounakis *et al.,* 1999; Gülçýn *et al.,* 2003; Oktay *et al.,* 2003). Pollutants, pesticides, organic solvents and smoking are few exogenous causative agents that produce free radicals. Superoxide anion, H_2O_2, reactive hydroxyl (-OH) radicals, peroxyl (ROO-) radicals, peroxynitrite anion (ONOO-) and nitric oxide are the most commonly occurring ROS species. The free electrons present on the outer shell of these species is highly reactive and unstable which causes destruction of major macromolecular components of cell like nucleic acid, proteins and lipids.

Antioxidants maintain balance between ROS and antioxidants by donating an electron to a free radical without making themselves unstable. Many of the plants contain phytochemicals that have potent natural antioxidant activity. Flavonoids, polyphenols and terpenoids are few such phytochemicals that possess antioxidant potential thereby preventing the damage produced by free radicals in the biological system.

Hordeum vulgare commonly known as barley, belongs to Poaceae family. It is generally used as animal fodder and in alcoholic breweries and distilleries. Traditionally, it has been used as anticholesteremic, immunomodulatory, cognitive enhancing and detoxifying agent. Barley is also known to have several medicinal properties such as antioxidant (Madhujith *et al.* 2006), anticancer (Czerwonka *et al.* (2017), antiproliferative (Madhujith and Shahidi, 2007), antidiabetic (Minaiyan *et al.* 2014), anti-inflammatory (Gul *et al.* 2014), antimicrobial, antihypertensive (Lee *et al.* 2010),

antiurolithiatic (Shah *et al.* 2012) and cardioprotective properties (Gul *et al.* 2014).

Thus the present study was designed to identify the phytochemicals present in the methanol extract of germinated seeds of *Hordeum vulgare* and its *in vitro* antioxidant activity using DPPH and superoxide radical scavenging assay.

MATERIALS AND METHODS

Collection of plant samples

The seeds of *Hordeum vulgare* were collected from Hebsur herbal store, Hubballi, Karnataka in January, 2018. The seeds were authenticated by Raw Material Herbarium & Museum, CSIR-National Institute of Science, Communication and Information Resources, New Delhi with authentication number NISCAIR/RHMD/ Consult/2018/3232-33 and voucher specimen with accession number HERB/VPT/CVASMTY/5/2019 was deposited in the Department of Veterinary Pharmacology & Toxicology, College of Veterinary and Animal Sciences, Mannuthy

Processing of plant sample: The seeds of *H. vulgare* were soaked in water for 24 h followed by spreading them on a plate at a thickness of 1 cm and sprayed with water every 4 h in order to keep them moist. The seeds were harvested on sixth day of germination and were thoroughly dried in hot air oven at 50 °C for 36 h. The germinated seeds were coarsely powdered using an electric pulveriser. The powdered form was stored in an airtight container until further use.

Preparation of methanol extract of germinated seeds of *H. Vulgarecollection* of plant samples

Approximately, 100 g of powdered germinated seeds of *H. vulgare* was subjected to hot extraction using Soxhlet apparatus and extraction was performed at 67^{0}C. The methanol extract was concentrated using a rotary evaporator under reduced pressure (230 mbars) and temperature (40^{0}C) for evaporating the solvent. The obtained extract was kept under refrigeration in airtight glass container until further use.

Qualitative phytochemical screening

Phytochemical analysis of the methanol extract of germinated seeds of *H. vulgare* for various phytoconstituents like steroids, alkaloids, glycosides, phenolic compounds, diterpenes, triterpenes and saponins was performed as per the method proposed by Harborne (1991).

In vitro antioxidant activity

Assessment of *in vitro* antioxidant activity of methanol extract of germinated seeds of *H. vulgare* was performed using DPPH and superoxide anion radical scavenging assays

DPPH radical scavenging activity

2, 2-Diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity was measured using the method of Karthishwaran and Mirunalini (2012) with few modifications. Four millilitres of the reaction mixture containing one mL of DPPH (0.1 mM in methanol), 3 mL of methanol extract of germinated seeds of *H. vulgare* at 2.5, 5, 10, 20, 40, 80, 160 and 320 µg/mL concentrations of plant extract was incubated at 37^{0}C for 30 min and absorbance of the resulting solution was measured at 517 nm using UV/VIS/NIR Spectrophotometer (Lambda 750, Perkin Elmer, Singapore). Ascorbic acid was used as the reference standard. The per cent inhibition of DPPH radical was calculated by comparing the absorbance of the test with those of control using the following equation:

Per cent inhibition = (1- AT/AC) x 100

AC= Absorbance of control,

AT= Absorbance of plant extract/standard

The half-maximal inhibitory concentration (IC_{50}) values were calculated using the Graphpad Prism Version 5.

Superoxide anion radical scavenging assay

Superoxide anion free radical scavenging activity was measured according to the method of Robak and Gryglewski (1988) with some modifications. All the solutions were prepared in 100 mM phosphate buffer (pH 7.4). One millilitre of nitroblue tetrazolium (NBT, 156 µM), 1 mL of reduced nicotinamide adenine dinucleotide (NADH, 468 µM) and 3mL of test solution at 2.5, 5, 10, 20, 40, 80, 160 and 320 µg/mL concentrations of plant extract was mixed. The reaction was initiated by adding 100 µL of phenazine methosulphate (PMS, 60µM). The reaction mixture was incubated at 25^{0}C for 5 min followed by measurement of absorbance at 560 nm using UV/ VIS/NIR Spectrophotometer (Lambda 750, Perkin Elmer, Singapore). Ascorbic acid was used as the reference standard. The IC_{50} values were calculated using the Graphpad Prism Version 5. The per cent inhibition was calculated by using the equation

Per cent inhibition = (1- AT/AC) x 100

AC= Absorbance of control, AT= Absorbance of plant extract/ standard

STATISTICAL ANALYSIS

All results were expressed as Mean± SE with 'n' equal to the number of replicates. The IC_{50} values of the extract were calculated using the Graphpad Prism Version 5. All the statistical analysis was conducted using SPSS software version 24. Analysis of variance (ANOVA) in a completely randomized design followed by Duncan's multiple range tests was used to compare any significant differences among various concentrations of the extract and independent sample t-test was performed to compare the IC_{50} concentration of the extract and the standard ascorbic acid.

RESULTS AND DISCUSSION

Qualitative phytochemical screening

The phytochemical analysis of methanol extract of germinated seeds of *H. vulgare* yielded steroids, diterpenes, triterpenes, glycosides and saponins (Table 1). Rajesh and Mita (2015) performed qualitative phytochemical screening in husked barley seeds and detected glycoside, alkaloid, phytosterol, tannins, flavonoids, saponin, reducing sugar, proteins and fats. Hamli *et al.* (2017) reported the occurrence of flavonoids, tannins, glycosides, terpenoids, saponins, coumarins and phenols in methanol extract of *H. vulgare*. Nepal *et al.* (2018) evaluated the antioxidant, antimicrobial and phytochemical assessment of methanol extract of leaves of *H. vulgare* and detected alkaloids, sterols, saponins, flavonoids, coumarins, reducing sugars and phlobatannins. The variations in the results of phytochemical screening obtained from the studies conducted by different authors could be due to various factors such as part of the plant extracted, climatic conditions, geographical area, type of soil, stress factors, weather conditions and seasonal variations.

In vitro antioxidant activity

2-Diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity

The per cent inhibition of DPPH radical by methanol extract of germinated seeds of *H. vulgare* at concentrations ranging from 2.5 to 320 µg/mL are presented in Table 2 and Fig. 1. A concentration dependent inhibition of DPPH radical was produced by the extract. The inhibitory concentration 50 (IC_{50}) of the extract was found to be 112.75 µg/mL. Significant ($p<0.05$) difference in IC_{50} values was observed between the plant extract and ascorbic acid. The IC_{50} value of ascorbic acid was found to be 21.98 µg/mL. The extract produced concentration-dependent inhibition of DPPH radical and the highest extract concentration of 320 µg/mL produced 65.39±1.21 per cent inhibition of DPPH radicals.

Similar findings were obtained by Lee *et al.* (2010) who performed a comparative study of antioxidant activity of ethanol extract of whole barley, milled barley and barley by-products by DPPH assay and found that the extracts at 500 µg/mL produced inhibition of DPPH radical by 65.43, 30.84 and 54.62 per cent respectively. Anwar *et al.* (2010) reported an increased antioxidant effect of aqueous methanol (20:80 v/v) extract of barley

Table 1.
Phytochemical analysis of methanol extract of germinated seeds of *H. vulgare*

Phytochemical test	*Hordeum vulgare*
Steroids	
• Salkowski's test	+
• Liebermann Burchardt test	+
Alkaloids	
• Dragendroff's test	-
• Mayer's test	-
• Wagners's test	-
• Hager's test	-
Glycosides	
• Sodium hydroxide test	+
• Benedict's test	+
Tannins	
• Ferric chloride test	-
• Gelatin Test	-
Flavonoids	
• Lead acetate test	-
• Ferric chloride test	-
Phenolic compounds	
Saponins	**+**
• Foam test	+
Diterpenes	+
Triterpenes	
• Salkowski's test	+
• Liberman Burchardt test	+

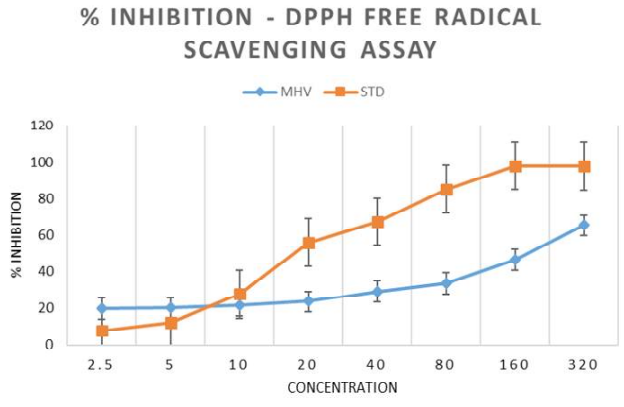

Fig. 1.
The per cent inhibition of DPPH free radical generation by methanol extract of germinated seeds of *H. vulgare*

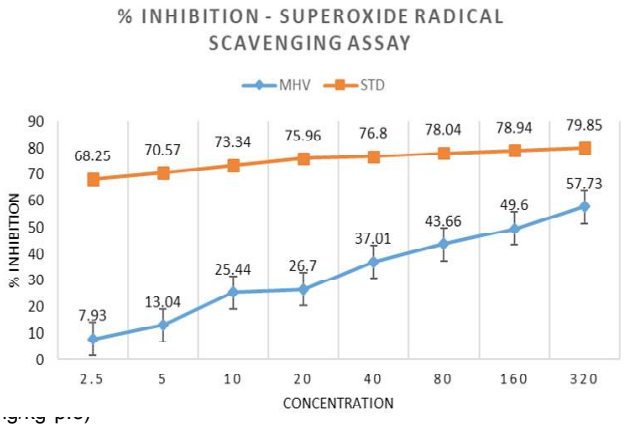

Fig. 2.
The per cent inhibition of superoxide free radical generation by methanol extract of germinated seeds of *H. vulgare*

Table 2
The per cent inhibition of DPPH free radical generation by methanol extract of germinated seeds of *H. vulgare*

Concentrations (µg/mL)	% inhibition of DPPH free radical	
	H. vulgare	Ascorbic acid (Standard)
2.5	19.94±1.26^{a}	7.77±1.03^{a}
5 20.26±0.98^{a}	12.45±1.39^{a}	
10	21.59±1.02^{ab}	27.81±1.35^{b}
20	23.70±0.80^{b}	55.99±2.41^{c}
40	29.28±1.28^{c}	67.35±3.00^{d}
80	33.61±0.84^{d}	85.15±1.60^{e}
160	46.59±1.17^{e}	97.87±0.34^{f}
320	65.39±1.21^{f}	97.71±0.44^{f}
IC_{50} (µg/mL)	**112.75±5.74^{A}**	**21.98±0.54^{B}**

Values are expressed as Mean±SE (n=6). Means bearing the different superscript (a-f in columns) and (A-B in row) vary significantly at p<0.05

Table 3.
The per cent inhibition of superoxide free radical generation by methanol extract of germinated seeds of *H. vulgare*

Concentrations (µg/mL)	% inhibition of DPPH free radical	
	H. vulgare	Ascorbic acid (Standard)
2.5	7.93±1.03^{a}	68.25±1.36^{a}
5	13.04±1.01^{b}	70.57±1.39^{ab}
10	25.44±1.67^{c}	73.34±1.24^{bc}
20	26.70±1.23^{c}	75.96±1.12^{cd}
40	37.01±1.21^{d}	76.80±1.15^{cde}
80	43.66±1.59^{e}	78.04±0.94^{de}
160	49.60±2.02^{f}	78.94±0.94^{de}
320	57.73±1.64^{g}	79.85±0.98^{e}
IC_{50} (µg/mL)	**28.90±1.85^{A}**	**14.17±0.22^{B}**

Values are expressed as Mean±SE (n=6). Means bearing the different superscript (a-h in columns) and (A-C in row) vary significantly at p<0.05

seeds in a concentration-dependent fashion by performing DPPH assay with IC_{50} values ranging from 90.7 to 168.6 µg/mL. Nepal *et al.* (2018) performed DPPH radical scavenging assay using methanol extract of leaves of *H. vulgare* and found an IC_{50} value of 135.62 µg/mL.

Superoxide anion free radical scavenging activity

The per cent inhibition of superoxide radical by methanol extract of germinated seeds of *H. vulgare* at concentrations ranging from 2.5 to 320 µg/mL are presented in Table 3 and Fig. 2. The extract produced concentration-dependent inhibition of superoxide radicals and the IC_{50} values of the extract was found to be 28.90 µg/mL whereas the IC_{50} value of ascorbic acid which was found to be 14.17 µg/mL. Ascorbic acid showed significantly (p<0.05) higher superoxide anion scavenging action when compared with the extract. The highest extract concentration of 320 µg/mL produced 57.73±1.64 per cent inhibition of superoxide radicals.

Omwamba *et al.* (2013) investigated antioxidant activity of roasted barley grain extract and found that the extract inhibited generation of superoxide radical in concentration-dependent manner with 3.26±0.15 µg/mL as its EC_{50} value. The authors also found significant increase in the levels of superoxide dismutase and glutathione in liver and brain tissue homogenate of mice when treated with 200 mg/kg of the extract indicating a potent antioxidant effect of barley *in vivo*.

It has been stated that the terpenoids act by quenching singlet oxygen, hydrogen or electron transfer. Some terpenoids are also reported to react with peroxyl radical leading to rapid termination of oxidative chain reactions thereby reducing the number of reactive radicals being produced (Grassmann *et al.,* 2005). Thus it may be concluded that the presence of diterpenes and triterpenes could contribute to the significant antioxidant activity of the extract. Further studies in the isolation of these terpenoid compounds are warranted. Thus, the qualitative phytochemical screening of methanol extract of germinated seeds of *Hordeum vulgare* yielded potent phytochemicals such as steroids, diterpenes, triterpenes, glycosides and saponins. The extract proved to have a significant concentration dependent antioxidant activity in DPPH and superoxide radical scavenging assay.

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Research Article

SIMULTANEOUS DETERMINATION OF TRIMETHOPRIM AND SULPHAMETHOXAZOLE IN BUFFALO MEAT USING LIQUID CHROMATOGRAPHY

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ABSTRACT

Trimethoprim and sulphamethoxazole combinations have been used extensively as for treatment of bovine infections. Residues of these antimicrobial agents are harmful to the consumer's health and could induce resistance in pathogenic microbes. A liquid chromatographic method for simultaneous trace determination of trimethoprim and sulphamethoxazole in buffalo meat matrix was established for the quantification purpose. The analytes were co-extracted using liquid-liquid extraction approach. The chromatographic separation was performed using a C18 column with a mobile phase comprised of ammonium phosphate buffer and acetonitrile (85:15, v/v) in an isocratic elution mode. The determination is carried out by liquid chromatography using PDA detector at a wavelength of 270 nm. Linearity, limits of detection (LOD) and quantification (LOQ), recovery, precision, and selectivity and recoveries were determined. The assay was linear in the concentrations ranging from 25 to 200 µg/kg. Relative standard deviations of the recoveries were less than 5% within the same day and less than 10% between days. The LOQ for trimethoprim and sulfamethoxazole were 42 and 68 µg/Kg, respectively. This RP-HPLC method has highly applicability for routine analysis of residual trimethoprim and sulphamethoxazole in buffalo meat below established MRL.

Key words: tissue residues, trimethoprim, sulphamethoxazole, buffalo meat, HPLC

INTRODUCTION

Trimethoprim and sulfamethoxazole or co-trimoxazole is an synthetic antimicrobial used extensively in bovine therapy for variety of bacterial, fungal and protozoal infections in a ratio of 1 part trimethoprim to 5 parts sulfamethoxazole. These antimicrobials inhibit the *de novo* folate biosynthesis in a synergistic fashion. Trace residues of antimicrobials could prop up in edible tissues whenever, there is incomplete observation of prescribed withdrawal times and off label use. Besides emergence of drug-resistant microbes (Haller *et al.*, 2002) these residues could cause possible health problems like GI discomfort, hypersensitivity and urticarial rashes (Yu *et al.*,2011).As a consequence, to ascertain food safety to consumers regulatory agencies have laid down maximum residue level (MRL) of 100 µg/kg for sulphonamides (EU EMEA/ MRL/026/95;CAC 32th session, 2010) and 50 µg/kg for trimethoprim in animal derived foods. A number of methods for quantitative analysis were reported for sulphonamides in various matrices (Yu *et al.,* 2011; Lopes *et al.*,2011 and Li *et al.,* 2013) using LC-MS or LC-MS/MS which is quite expensive and not always available for routine monitoring purpose. Some reports are available on quantification of sulphonamides by HPLC using fluorescence detection which involves complex derivatisation step (Stoev & Michailova., 2000; Kao *et al.,* 2001; Pecorelli *et al.,* 2004 and Patyra *et al.*, 2019).Also, limited HPLC methods using UV/DAD were also being reported in meat (Biswas *et al* 2007; Kishida *et al.*, 2007; Won *et al.*, 2011 and Mokhtar *et al.*, 2019) for quantification of sulphonamide/trimethoprim residues. The increased attention to this residue issues marks the need to have a simple and accurate techniques for detection and quantification of antimicrobials in foods of animal origin. Therefore, the present study describes a liquid chromatographic method using photo diode array detector for simultaneous quantitation of trimethoprim and sulfamethoxazole in buffalo meat in compliance with current MRL.

MATERIALS AND METHODS

Chemicals and reagents

The analytical standards trimethoprim and sulfamethoxazole were obtained from Sigma-Aldrich (St. Louis, MO, USA.HPLC-grade acetonitrile (ACN) from Merck (Darmstadt, Germany), were used. All the reagents used were of analytical grade. HPLC purity water obtained from Milli-Q purification unit (Millipore, Bedford, MA, USA) was used. Solvents were filtered through 0.22µm nylon membrane filter before use.

Preparation of Standard and working drug solutions

Analytical grade standard of sulfamethoxazole (Vetranal lot # SZBC 124 XV) and trimethoprim (Vetranal lot # SZBD 010 X) procured from Sigma-Aldrich, USA having 99.9 and 99.7% of purity were used for this study. Stock solutions of sulfamethoxazole and trimethoprim at a concentration of 1 mg/ml were prepared in HPLC grade acetonitrile. From this stock solution 25,50,75, 100,150 and 200 ng /ml concentrations of working

standard solutions were prepared by diluting with acetonitrile. All stock and working standard solutions kept at 4°C under refrigerator.

Extraction Procedure

The buffalo meat samples were extracted using liquid-liquid extraction (LLE) method. To 5 gram of meat homogenate 1ml ammonium phosphate buffer was added, vortexed and given an 8 minutes contact time. Thereafter 2 ml acetonitrile was added and vortexed for 2 minutes followed by centrifugation at 4000 rpm for 20 minutes. Finally, the supernatant was filtered through a 0.25 µ nylon membrane filter, 20 µl of this filtrate was then injected into the column for HPLC analysis.

High- performance liquid chromatographic assay

The High-Performance Liquid Chromatography (HPLC) system (Shimadzu, Kyoto, Japan) comprised of a quaternary gradient pump with an autoinjector and a photodiode array (PDA) detector was employed. The HPLC system consisting of a Gradient pump (LC-10AT vp), Diode array detector (SPD-H10Avp), Column oven (CTO-10ASvp) and an autoinjector (SIL-10ADvp). The separation of two analytes of our interest was accomplished using a reverse phase Hypersil BDS C18 column (Thermo Scientific, 250mm x 4.6mm with the particle size of 5µm) as a stationary phase. The mobile phase consisting 0.05M ammonium phosphate buffer and acetonitrile (85: 15, v/v) was used for elution process in an isocratic mode. A flow rate of 0.6 $ml.min^{-1}$ was used and column oven temperature was maintained at 30° C. PDA detector wave length was adjusted to have an λmax of 270 nm.

Calibration curve

Calibration curves were prepared by adding to blank samples, the corresponding volume of working solution to obtain the concentrations of trimethoprim and sulfamethoxazole in the range 25 to 200 µg/Kg levels in buffalo meat samples which correspond to 0.25 to 2 times MRL of sulphonamides and 0.5 to 4 times MRL of trimethoprim respectively. Trimethoprim and sulfamethoxazole were quantified in µg/Kg based on peak area measurements using external calibration method.

STATISTICS

The recovery and precision data were evaluated with an in-house statistical software program making use of Snedecor and Cochran (1989).

RESULTS

The method entails a simple liquid-liquid extraction of buffalo meat followed by liquid chromatographic determination of trimethoprim and sulfamethoxazole using PDA detector. These antimicrobials were detected using PDA detector at 270 nm wavelength. Chromatographic separation between trimethoprim and sulfamethoxazole was achieved using 0.05M ammonium phosphate buffer and acetonitrile (85: 15, v/v) as the mobile phase (Table 1). The method allows identification and quantification of the analytes of our interest in a single analytical run within a total run time of 15 minutes. The retention time (RT) of trimethoprim and sulfamethoxazole were 11 and 13.5 minutes, respectively.

Representative chromatogram of control muscle samples showed that there were no interfering compounds at the retention times of analytes of our interest (Fig 1). The assay was linear from 25 to 200 µg/kg (Fig 2). The coefficients of determination (R^2) values of the calibration curves were 0.99. Detection and quantification limits were evaluated as the concentration corresponding to 3 and 10 times the standard deviation of baseline noise. The limit of quantification (LOQ) of trimethoprim and sulfamethoxazole were 42 and 68 µg/kg, respectively (Table 2).

As summarised in Table 2, the recoveries were investigated at three concentration levels around MRL (50,100,150 µg/kg) and determined by comparing amounts in the sample with the total spiking amounts. The percentage recovery from buffalo meat ranged from 82-84% (trimethoprim) and 85-87% (sulfamethoxazole). As listed in Table 3, the inter-day and intraday variation are less than 5% and 10% respectively.

Table 1

Analytical Conditions

System	HPLC
Column	Reverse phase Hypersil BDS C_{18} 4.6 X 250 mm 5µ
Injection volume	20 µl
Oven temperature	30 °C
Mobile phase	0.05M ammonium phosphate buffer & Acetonitrile (85:15, v/v)
Flow rate	0.6ml/min
Detector	PDA λmax 270nm

Table 2: Performance parameters of sulfamethoxazole and trimethoprim

S. No	Antimicrobials	LOD (µg/ kg)	LOQ (µg/kg)	Recovery (%)	Linearity Range (µg/kg)	Correlation Coefficient	selectivity
1.	Sulfamethoxazole	20	68	85-87	25-200	0.999	No Interference was found
2.	Trimethoprim	15	42	82-84	25-200	0.999	No Interference was found

Table 3
Inter-day and Intra-day variation sulfamethoxazole and trimethoprim in buffalo meat.

S. No.	Antimicrobials	Concentration MRL level (µg/kg)	Intra –day variation (RSD%)	Inter-day variation (RSD%)
1.	Sulfamethoxazole	100	4.93	8.31
2.	Trimethoprim	50	2.53	7.27

DISCUSSION

A simple RP-HPLC method displaying high specificity and sensitivity has been obtained with good recoveries and linearity to quantitate simultaneously, sulfamethoxazole and trimethoprim in buffalo meat matrix. The method described proved to be specific in the sense that there is total absence of interference with endogenous substance at the same retention time as that of analytes which can be seen in the LC chromatogram (Fig.1). The calibration curve was found to be linear over the range 25 to 200 µg/kg studied which was centralised around the maximum permittable limits. Also, the correlation coefficient was established with R2 above 0.98 indicating good correlation and in compliance with the guidelines proposed in Commission Decision (CD 2002/657/EC). Furthermore, the limit of quantification (LOQ) of trimethoprim and sulfamethoxazole in our study were 42 and 68 µg/kg, which is in agreement with that of Biswas *et al.* (2007). However, the LOQ levels in our study were considerably lower than the established MRL for trimethoprim (50 µg/kg) and sulfamethoxazole (100 µg/kg), respectively (EMEA/MRL/026/95).

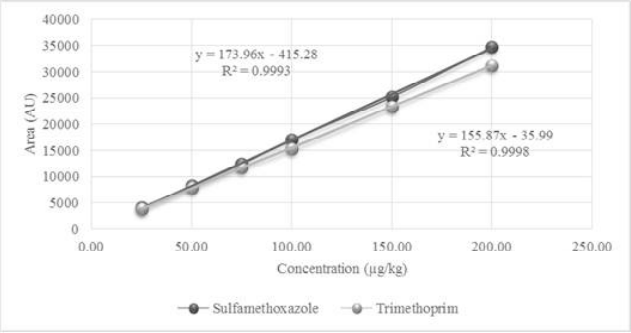

Fig. 1
Representative optimized chromatogram spiked with 100 µg/Kg of sulfamethoxazole and trimethoprim.

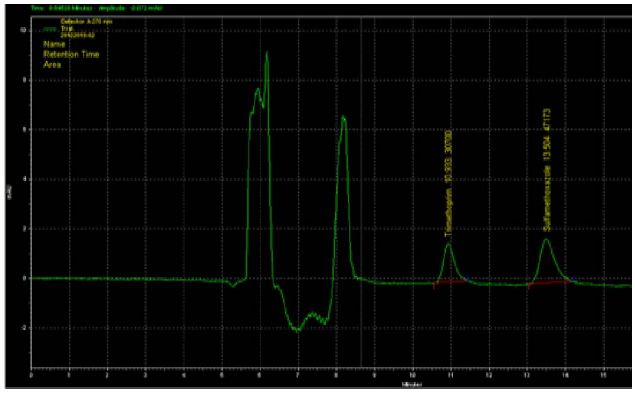

Fig.2
Calibration curve in the concentration range 10-200 µg/Kg of sulfamethoxazole and trimethoprim

The results for the recovery assays, are shown in Table 3. The average percentage recovery from buffalo meat in our study ranged from 82-84% (trimethoprim) and 85-87% (sulfamethoxazole). Biswas *et al.* (2007) presented a similar HPLC-PDA method in the same matrix with slightly different method for extraction and used Bond-Elute C_{18} cartridge for clean-up that yielded them a higher recovery up to 108% whereas our study using simple LLE yielded a slightly lesser recovery. However, the recovery percentage of our study were consistent with the acceptable range specified by the Codex Alimentarius (Codex Alimentarius Commission, 2009) (between 70 and 120%). The intraday and inter-day variation result was below 10 % RSD and fell within the acceptable limit mentioned in Codex guidelines for residue analysis RSD < 20% for an analyte concentration of <0.1 mg/kg (Codex Alimentarius Commission, 2009). The usefulness of this method for simultaneous monitoring of trimethoprim and sulfamethoxazole was successfully demonstrated in real samples. This method can therefore be used for quantitative determination of trimethoprim and sulfamethoxazole, below the prescribed MRL in buffalo meat samples.

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Research Article

ATTENUATING POTENTIAL OF LEAF EXTRACTS OF *ALSTONIA SCHOLARIS* ON ALTERED GLYCEMIC INDEX, LIPID, HEPATIC AND RENAL PARAMETERS IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Alstonia scholaris (Apocynaceae) is an important constituent of numerous and widely used therapeutic formulations of Ayurveda, the traditional Indian system of medicine. Present study was aimed to determine the potential of leaf extracts of *A. scholaris* administration in attenuating the glycemic index [mean blood glucose (MBG), per cent glycosylated haemoglobin fraction (HbA1c)], lipid profile [total cholesterol (TC), triglycerides (TG), low and high density lipoproteins (LDL and HDL)], hepatic [transaminases, phosphatases, dehydrogenases] and renal indices [plasma proteins, blood urea nitrogen (BUN), creatinine (CR), uric acid (UA)] in streptozotocin (STZ) induced diabetic rats. Administration of STZ increased ($P<0.05$) the levels of MBG, HbA1c fraction which indicated the induction of diabetes in rats. Enhanced ($P<0.05$) TC, TG, LDL, total oxidant status (TOS), oxidative stress index (OSI), malondialdehyde (MDA) levels, hepatic and renal indices were observed in blood of diabetic rats. However, levels of HDL, protein profile, total antioxidant status (TAS), glutathione (GSH), total thiols (TTH) and activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-s-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PDH) were significantly reduced in diabetic rats. Repeated oral administration of ethanolic leaf extract of *A. Scholaris* reduced the levels of MBG, HbA1c and TC in diabetic rats. While it restored OSI, TTH, GSH, CAT, SOD, GST and MDA levels; it also increased activities of G6PDH and GPx in diabetic rats compared to untreated diabetic rats. These findings indicated that *A. Scholaris* leaf extracts possess antidiabetic potential against STZ induced diabetes in rats. Overall, the ethanolic leaf extract of *A. Scholaris* displayed greater antidiabetic capacity than its aqueous counterpart.

Key words: *Alstonia scholaris*, malondialdehyde, glycemic index, antioxidant, diabetes, rat.

INTRODUCTION

Plant *Alstonia scholaris* L. (Family: Apocynaceae) commonly known as Saptaparna is incorporated as a key constituent in many traditional ayurvedic formulations. Bark, leaves, flowers, fruits, root, areal part of the plant are rich sources of diverse phytochemical ingredients viz. terpenoids, alkaloids, glycosides, flavonoids, steroids, tannins, etc. (Verma *et al.,* 2015). Triterpenes and sterols like stigmasterol, betulin, betulinic acid and alpha-amyrin acetate were isolated from the hexane fraction of the alcoholic extract of leaves of *A. Scholaris* (Desoky *et al.,* 2000). Most of monoterpenoidindole alkaloids *viz*., alschomine, isoaslchomine, scholarine andscholaricine originate from the condensation of tryptophan with secologanin. In Ayurveda, bark of the plant is used as immuno-stimulant, cardio-tonic, antipyretic, anthelmintic, astringent and hypoglycemic agent. The aqueous bark extract of *A. scholaris* at a lower dose stimulated cellular immune response while at a higher dose it inhibited the delayed type of hypersensitivity reaction (Iwo *et al.,* 2000). Different parts of *A. scholaris* also have anti-inflammatory, analgesic, antitussive, anti-asthmatic, expectorant (Shang *et al.,* 2010), antidiarrhoeal (Abdul *et al.,* 2010), antidiabetic, antihyperlipidemic (Sinnathambi *et al.,* 2010), antioxidant (Verma *et al.,* 2015), hepatoprotective (Verma *et al.,* 2016) and nephroprotective potential (Verma *et al.,* 2019).

Herbal preparations have recently attracted a lot of attention as alternative medicines useful for treatment and prevention of a wide variety of disorders induced by exposure to chemicals and/or stress. Therefore, the present study was planned to determine the effect of aqueous and ethanolic leaf extract of *A. scholaris* on altered glycemic index, lipid, hepatic and renal parameters in streptozotocin induced diabetic rats.

MATERIALS AND METHODS

Collection and preparation of extracts

Leaves of *A. scholaris* were collected from University campus of Jammu (India). Taxonomic identification of the plant was done Taxonomists in Department of Botany, University of Jammu (AU-2875). Sufficient fresh flowers were collected and air-dried in shade (temp not exceeding 40°C) for 3-4 weeks. Air dried flowers were pre-crushed and later pulverized into a fine powder using electric blender. The aqueous extract was prepared by soaking dry powder in a 1:10 ratio in distilled water for 72 h with intermittent shaking. After 72 h of soaking, the contents were filtered through filter paper

(0.45 µm) and filtrate was concentrated under reduced pressure using rotatory evaporator (temp 50-55°C, 10-15 rpm). The ethanolic extract was prepared using ethyl alcohol in extract container of soxhlet apparatus according to standard method. The dried, aqueous and ethanolic floral extracts of *A. scholaris* were stored in air tight containers. The extracts were reconstituted in 0.1 % carboxy methyl cellulose (CMC) before administration by oral gavage to Wistar rats.

Chemicals and experimental animals

The chemicals used in the study were of analytical grade. 42, Wistar rats were procured form Indian Institute of Integrative Medicine, Jammu, INDIA. Animals were acclimatized in the laboratory conditions for a period of 3 weeks prior to the start of experiment. Animals were provided standard pelleted ration and *ad libitum* drinking water under standard managemental conditions (22±3°C 50-60% relative humidity and 12 h light-dark cycles) and were kept under constant observation during entire period of study. All experimental rats received humane care in accordance with National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996).The experimental protocol was approved by Institutional Animal Ethics Committee (FVSc/C-11/2456-68).

Experimental design

Animals were divided into seven groups of 6 rats each. Rats in Group I, II and III, received 1ml/day CMC, aqueous and ethanolic leaf extracts of *A. scholaris,* respectively. Diabetes was induced in 24 rats of Group IV, V, VI and VII by single intra-peritoneal injection (55 mg/kg) of streptozotocin (STZ), freshly dissolved in 0.1 M cold citrate buffer (pH 4.5) as described by Pandit *et al.* (2010). Five days after STZ injection, fasting blood glucose of the animals was estimated and the rats having blood glucose above 225 mg/dl were considered as diabetic and included in the experimental trial. Group IV rats served as diabetic control and Group V diabetic rats received glibenclamide (10 mg/kg, orally), a standard antidiabetic drug. Group VI and VII diabetic rats received 300 mg/kg BW aqueous and ethanolic leaf extracts of *A. scholaris,* respectively. All treatments were administered daily for 21 days in the morning between 10-11AM.

Collection and processing of samples

Blood samples were collected directly from heart in sterilized tubes containing heparin from all rats on 21stday of the experiment. Blood glucose level was determined immediately using glucometer (Contour® TS, Bayer Pharmaceuticals Pvt. Ltd. India). A part of blood sample was used for the determination of reduced blood glutathione (GSH), haemoglobin (Hb) and Glycosylated fraction of haemoglobin (HbA1c). The remaining blood was centrifuged at 4000 rpm for 10 min; plasma was collected in glass vials for the estimation of hepatic and renal biomarkers. Glycosylated haemoglobin (A1c) fraction was determined via ion exchange method using by assay kit (Transasia Bio-Medicals Ltd, India) using UV-visible spectrophotometer (UV-1601, Shimadzu).

Assaying of hepatic and renal biomarkers

The plasma activities of G6PDH was assessed based upon the ability of enzyme to catalyze the conversion of Glucose-6-phosphate and $NADP^+$ to 6-phosphogluconolactone and NADPH (reduced nicotinamide adenine dineucleotide phosphate) as per the method described by Deutsch (1978). Other hepatic biomarkers like activities of aspartate and alanine aminotransferase (AST, ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT) and levels of plasma proteins, albumin, blood urea nitrogen (BUN), creatinine (CR) and uric acid (UA) were determined by standard kits (Transasia Bio-Medicals Ltd, India) using Chemistry Analyzer (CHEM-7, ERBA, Mannheim).

Assaying of antioxidant parameters

Total antioxidant status (TAS) was determined spectrophotometrically by using ABTS according to the method described (Re *et al.,* 1999); final TAS values were expressed as mM of ascorbic acid equivalents in the plasma. Similarly, TOS level in plasma was measured using a novel automated method developed by Erel (2005) and results were expressed in terms of µmol H_2O_2 Equiv. L^{-1}. The percent ratio of TOS to TAS level was oxidative stress index (OSI) (Aycicek *et al.,* 2006). The enzymatic parameters viz. catalase (CAT) and glutathione peroxidase (GPx) were determined as described by Aebi (1983) and Hafeman *et al.* (1974), respectively; the activities of superoxide dismutase (SOD) and glutathione-S-transferase (GST) were determined as per the method described by Marklund and Marklund (1974) and Habig *et al.* (1974), respectively. Total thiols (TTH) level was determined in plasma as per the standard protocol and the concentration of total thiol (mM) was expressed using reduced glutathione as a standard (Motchnik *et al.,* 1994). Similarly, malondialdehyde (MDA) levels in plasma (nmole of MDA formed/Hb/h) were determined to estimate the membrane lipid peroxidation (Shafiq-ur-Rehman, 1984). The levels of reduced blood glutathione (GSH) and plasma ceruloplasmin (CP) were determined as per the standard method (Beutler, 1975; Sunderman and Nomoto, 1970).

Statistical analysis

The values are presented in mean ± standard error. Biochemical and oxidative stress parameters were analyzed by analysis of variance (ANOVA) in completely randomized design (CRD) using the Duncan Multiple Range Test at 5 % level of significance.

RESULTS

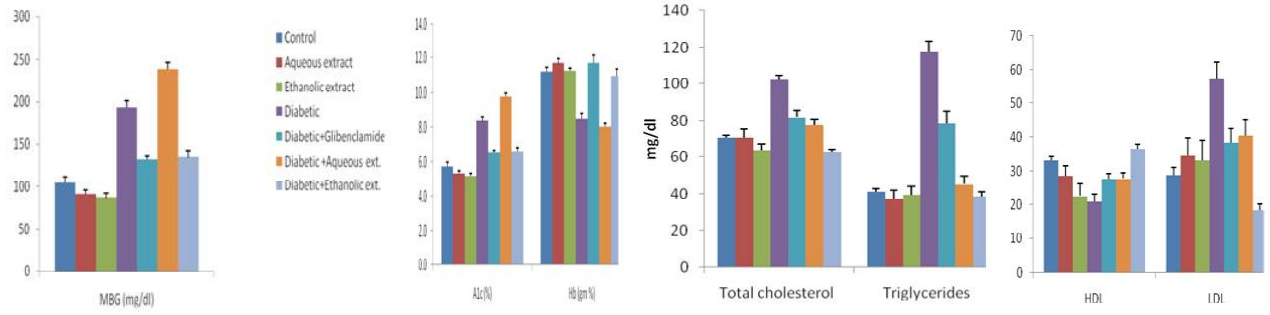
Biochemical parameters

Table 1 depicts the mean values of blood glucose, Hb, glycosylated Hb (HbA1c) and lipid profile in different groups treated with either aqueous or ethanolic leaf extracts of *A. scholaris*. STZ @ 55 mg/kg after single intra peritoneal (IP) injection in overnight fasted Wistar rats significantly (P<0.05) increased the mean blood glucose (MBG) level as compared to normal control group rats. Administration of glibenclamide in diabetic rats daily for 21 days significantly (P<0.05) reduced the MBG level as compared to diabetic control but the values were significantly (P<0.05) higher compared to normal control group. Similar reduction in MBG was observed after administering ethanolic extract of *A. scholaris* to diabetic rats for 21days. However aqueous extract of *A. Scholaris* failed to restore the level of MBG. Significant (P<0.05) reduction in Hb and significant increase in per cent glycosylated Hb was observed in diabetic rats as compared to sham group. Treatment with aqueous extract of *A. scholaris* for 21 days in diabetic rats could not restore the Hb levels, whereas, Hb levels in diabetic rats treated with ethanolic extract of *A. Scholaris* were significantly (P<0.05) reversed to the extent that they became non-significantly different from control group. Treatment with either aqueous or ethanolic extract of *A. scholaris* in diabetic rats significantly (P<0.05) reduced the per cent HbA1c as compared to diabetic rats but the values remained significantly higher than the control group.

Induction of diabetes with the single IP administration of STZ to rats significantly (P<0.05) increased levels of total cholesterol, triglycerides, LDL and significantly decreased level of HDL when compared to normal control rats. Glibenclamide treatment in diabetic rats significantly (P<0.05) reduced the levels of total cholesterol, triglycerides and LDL as compared to diabetic control rats but these values were still significantly (P<0.05) higher than the normal control animals. Glibenclamide administration in diabetic rats enhanced HDL levels although this increase was non-significant. Treatment with either aqueous or ethanolic leaf extract of *A. scholaris* in diabetic rats significantly (P<0.05) reduced the levels of total cholesterol, triglycerides and LDL. A greater reduction in these indices was observed after treatment with ethanolic extract than after treatment with the aqueous extract. The HDL level with ethanolic extract treatment was significantly (P<0.05) higher than the corresponding levels in the diabetic control animals as well as aqueous extract treated diabetic rats.

The effect different *A. scholaris* leaf extracts in modulating plasma hepatic biomarkers alterations in diabetic rats is presented in figure 2. Significantly (P<0.05) reduced levels of total bilirubin and indirect bilirubin were observed in diabetic rats as compared to normal control group. Glibenclamide treatment restored the levels of total and indirect bilirubin but direct bilirubin remained significantly (P<0.05) lower than values of normal control group. Treatment with either extract failed to correct the altered values of total, direct and indirect bilirubin in diabetic rats.

Activities of AST, ALT, LDH and ALP were increased in diabetic rats as compared to normal control group. Repeated treatments with glibenclamide although significantly (P<0.05) reduced the activities of AST, ALT, ALP and LDH compared to diabetic rats but such values excepting the values of ALT were still significantly (P<0.05) higher from the normal control group. Aqueous leaf extract of *A. scholaris* in diabetic rats significantly (P<0.05) reduced ALP and LDH activities compared to diabetic control rats but this extract failed to rejuvenate the activities of AST and ALT to the levels of normal control group. Similarly, ethanolic extract of *A. Scholaris* administration in diabetic rats restored activities of AST, ALP and LDH but not ALT as compared to diabetic control



A) B) C)

Figure 1:
Effect of aqueous and ethanolic leaf extract of *A. scholaris* on (A) mean blood glucose (MBG), (B) glycosylated Hb & Hb levels and (C) lipid profile in plasma of diabetic rats.

group (Figure 2).

The results of effect of aqueous and ethanolic leaf extracts of *A. Scholaris* treatments on biochemical parameters specific to kidney functions are presented in figure 3. BUN, CR and uric acid levels were increased significantly (P<0.05) in diabetic rats. Glibenclamide treatment significantly (P<0.05) reduced the BUN and CR levels when compared to diabetic rats but these values were significantly higher than the normal control. The levels of plasma uric acid in diabetic rats were reduced on treatment with glibenclamide but only non-significantly. Aqueous extract of *A. scholaris* significantly (P<0.05) reduced BUN, CR and uric acid in diabetic rats and these values except for BUN, became non significantly (P<0.05) different from the normal control group. Likewise, treatment with ethanolic extract of *A. scholaris* in diabetic animals significantly (P<0.05) reduced CR levels whereas levels of BUN and uric acid remained significantly higher as compared to the normal control rats. In diabetic rats, there was a significant reduction in total plasma proteins and globulin content as compared to animals of normal control group. Administration of glibenclamide significantly (P<0.05) increased total plasma proteins as compared to diabetic rats but these values were still significantly (P<0.05) lower than the normal control group. However no such alterations were recorded in the levels of albumin and globulin after glibenclamide administration. Repeated administration of aqueous extract of *A. scholaris* in diabetic rats failed to restore the values of total plasma proteins, albumin and globulin whereas ethanolic extract administration restored the levels of total plasma proteins, albumin and globulin in diabetic rats when compared to the corresponding to normal control animals (Figure 3).

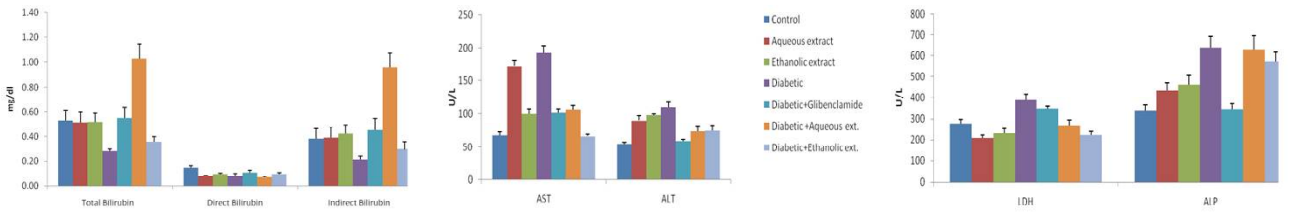
Antioxidant parameters in blood

The levels of non-enzymatic components of antioxidants in blood of different groups of rats are depicted in table 1. Significantly (P<0.05) increased levels of TOS and OSI and significantly (P<0.05) reduced TAS levels indicative of derangements in antioxidant system were seen in diabetic rats. Daily treatment with glibenclamide for 21days restored the levels of TAS, TOS and OSI in diabetic rats. Repeated oral exposure of aqueous extract of *A. scholaris* for 21 days in diabetic rats significantly (P<0.05) increased the TAS and restored the TOS and OSI when compared to values of normal group. However, treatment with ethanolic extract significantly increased TAS but failed to restore the levels of TOS and OSI in diabetic rats when compared with the corresponding values of normal control group.

GSH and TTH were significantly (P<0.05) reduced in diabetic rats as compared to sham group. Glibenclamide treatment significantly (P<0.05) increased the GSH and TTH values in rats with diabetes though the value of TTH but not GSH were still significantly (P<0.05) lower from sham control. Treatment with aqueous leaf extract of *A. scholaris* in diabetic rats also restored TTH but not the GSH level which remained significantly (P<0.05) lower than the sham control (Fig.10C). Similarly treatment with ethanolic leaf extract of *A. scholaris* significantly (P<0.05) increased GSH as compared to diabetic rats but the levels of TTH were significantly higher from the normal group. Ceruloplasmin level was significantly elevated in diabetic rats as compared to control rats. Glibenclamide treatment significantly (P<0.05) reduced the CP level in diabetic rats but these values were still significantly (P<0.05) higher than the normal control rats. Repeated administration of either aqueous or ethanolic extract of *A. scholaris* in diabetic rats didn't replenish CP levels.

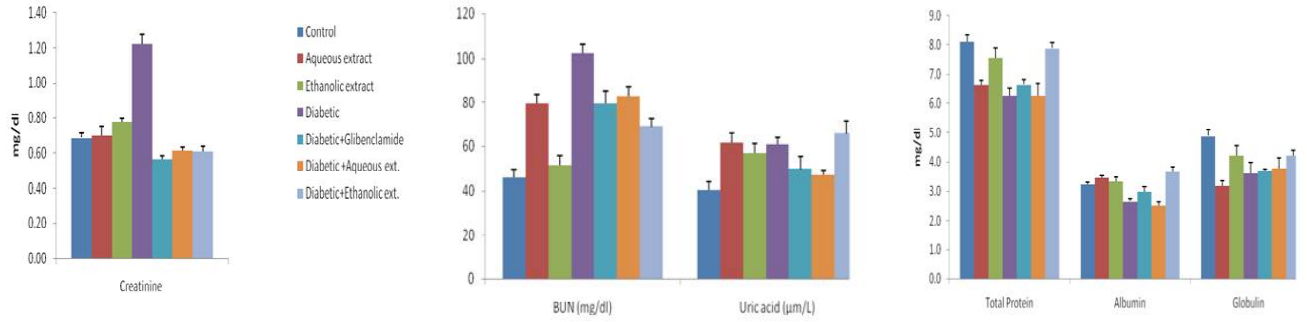
MDA level was significantly (P<0.05) high in diabetic rats as compared to sham control. Repeated treatments with glibenclamide or aqueous leaf extract of *A. scholaris* in diabetic rats significantly (P<0.05) reduced the MDA level but these values were still significantly (P<0.05) higher from the sham control. Repeated treatment of diabetic rats with ethanolic but not aqueous leaf extract of *A. scholaris* restored MDA levels and the decreased levels were non-significantly different from sham group.

The activities of CAT, SOD, GPx, GST and



A) B) C)

Figure 2:
Effect of aqueous and ethanolic floral extracts of *C. officinalis* treatments (A) bilirubin profile, (B) aminotransferases and (C) dehydrogenase & phosphatase in plasma of diabetic rats.



A) **B)** **C)**

Figure 3:
Effect of aqueous and ethanolic leaf extract of *A. scholaris* treatment on (A) creatinine, (B) BUN, uric acid and (C) protein profile in plasma of diabetic rats.

G6PDH were significantly (P<0.05) reduced after induction of diabetes as compared to control. Daily treatment with glibenclamide in diabetic rats not just significantly (P<0.05) restored the GPx activity and but surprisingly its activity was found to be greater than even the normal rats. Activities of CAT SOD and GST after glibenclamide treatment in diabetic rats were significantly elevated but did not differ from normal rats. Treatment of diabetic rats with either aqueous or ethanolic leaf extract of *A. scholaris* restored the activities of CAT and GST but GPx activity significantly was (P<0.05) higher and SOD activity was significantly (P<0.05) lower as compared to control. Similarly, activities of G6PDH were restored on treatment with ethanolic extract of *A. scholaris* whereas aqueous extract failed to do so.

DISCUSSION

Biochemical parameters

Haemoglobin is a complex protein within red blood cells that transports oxygen to all the tissues in

Table 1:
Effect of aqueous and ethanolic leaf extracts of *A. scholaris* treatment on non-enzymatic components of antioxidant system in the plasma/blood of diabetic rats.

Groups	TAS	TOS	OSI	TTH	GSH	CP
Normal control	1.17^{bc}±0.05	2.56^{bc}±0.24	0.218^{bc}±0.014	0.124^{b}±0.007	3.50^{d}±0.27	0.176^{a}±0.010
Aqueous extract	1.19^{bc}±0.04	2.99^{c}±0.33	0.255^{c}±0.032	0.116^{b}±0.004	$4.10^{d\pm}$0.24	0.104^{a}±0.008
Ethanolic extract	1.11^{b}±0.02	1.64^{a}±0.04	0.148^{ab}±0.005	0.119^{b}±0.009	3.14^{d}±0.29	0.202^{ab}±0.015
Diabetic control	0.74^{a}±0.06	3.95^{d}±0.26	0.555^{d}±0.062	0.093^{a}±0.006	1.36^{a}±0.15	0.465^{c}±0.060
Diabetic+Glibenclamide	1.25^{bc}±0.05	2.18^{ab}±0.13	0.176^{abc}±0.013	0.117^{b}±0.008	2.53^{bc}±0.20	0.298^{b}±0.029
Diabetic+Aqueous extract	$1.56^{d\pm}$0.03	2.47^{bc}±0.20	0.159^{ab}±0.013	0.116^{b}±0.004	1.70^{ab}±0.19	0.438^{c}±0.053
Diabetic+Ethanolic extract	1.29^{c}±0.04	1.64^{a}±0.04	0.128^{a}±0.006	0.153^{c}±0.008	2.63^{bc}±0.20	0.609^{c}±0.047

Values are given as mean±SE of 6 animals unless otherwise statedValues having different superscripts (a, b, c) in a column are statistically different from one another at 5 % level of significanceValues of TAS (Total antioxidant status), TOS (total oxidant status), TTH (total thiols), GSH (blood glutathione) are expressed in mM Values of plasma CP (ceruloplasmin) are expressed in g/l

Table 2:
Effect of aqueous and ethanolic leaf extracts of *A. scholaris* treatment on enzymatic components of antioxidant system in the erythrocytes of diabetic rats.

Groups	CAT	SOD	GPx	GST	G6PDH	MDA
Normal control	29.38^{bc}±2.13	60.11^{c}±1.55	1.04^{b}±0.13	0.285^{cd}±0.016	3047.77^{b}±150.69	0.76^{a}±0.09
Aqueous extract	33.20^{cd}±3.35	24.47^{a}±1.35	0.71^{b}±0.04	0.115^{a}±0.024	2400.17^{a}±135.83	1.67^{a}±0.27
Ethanolic extract	$38.76^{d\pm}$2.43	25.12^{a}±0.50	1.64^{c}±0.14	0.145^{ab}±0.017	4245.83^{c}±364.28	0.97^{a}±0.18
Diabetic control	17.52^{a}±1.61	25.73^{a}±0.94	0.62^{a}±0.10	0.201^{b}±0.020	2334.19^{a}±88.83	3.64^{d}±0.16
Diabetic+Glibenclamide	23.37^{ab}±1.99	55.51^{c}±4.10	1.55^{c}±0.08	0.272^{c}±0.014	3039.67^{b}±343.22	1.08^{b}±0.12
Diabetic+Aqueous extract	26.79^{bc}±3.11	50.28^{b}±1.21	1.35^{c}±0.15	0.291^{cd}±0.023	2080.42^{a}±127.51	2.83^{c}±0.11
Diabetic+Ethanolic extract	31.09^{bc}±2.67	40.33^{b}±3.30	1.73^{c}±0.08	0.338^{d}±0.022	3646.80^{bc}± 245.81	0.79^{a}±0.07

Values are given as mean±SE of 6 animals unless otherwise statedValues having different superscripts (a, b, c& d) in a column are statistically different from one another at 5 % level of significanceValues of CAT (Catalase) are expressed in µmol H_2O_2 decomposed/ min/ mg Hb.Values of SOD (Superoxide dismutase) and GPx (glutathione peroxidase) are expressed in Unit/mg of Hb.Values of GST (glutathione S transferase) are expressed in µmol of CDNB conjugate formed/ min/mg Hb.Values of G6PDH (glucose 6 phosphate dehydrogenase) are expressed in U/L.Values of MDA (malondialdehyde) level are expressed in nmoles MDA produced / mg of Hb/ hr.

body for aerobic respiration to fulfil their metabolic needs. When blood glucose levels are persistently raised, glucose combines with Hb resulting in the formation of glycosylated haemoglobin (HbA1c). Glycosylated haemoglobin is elevated in the patients with uncontrolled or poorly controlled diabetes mellitus and this increase in glycosylated haemoglobin is directly proportional to the level of hyperglycaemia. Evidence shows that glycation itself may induce the formation of oxygen-derived free radicals in diabetic condition, and the level of HbA1c is considered as a marker for determining degree of oxidative stress in cases of diabetes mellitus (Verma *et al.,* 2016). Also, the measurement of HbA1c is regarded as a sensitive index for assessment of extent of glycemic control. In the present study, significant increase in HbA1c level was observed in STZ induced diabetic rats indicating persistent hyperglycaemia in these animals. Also, administration of STZ caused increased MBG levels in diabetic animals. Decreased levels of MBG and HbA1c observed after repeated treatments with ethanolic extract of *A. scholaris* in diabetic rats showcased its antidiabetic potential. The results are in agreements with the reports of Anurakkun *et al.* (2007) and Bandawane *et al.* (2010) who too have reported similar reductions in MBG and HbA1c after repeated administration of bark and leaf extracts of *A. scholaris* in diabetic rats. The leaf extracts of *A. scholaris* have been found to have significant α-glucosidase inhibitory activity which may also account for the observed hypoglycemic activity in this study(Sinnathambi *et al.,* 2010).

The exact mechanisms of lowering MBG and per cent HbA1c by leaf extracts of *A. scholaris* is not clear but plant extracts producing hypoglycemic effects may do so either by restoring functioning of pancreatic tissues resulting in an increase in insulin output or by decreasing the intestinal absorption of glucose (Anurakkun *el al.,* 2007). In addition, reduced blood glucose levels after plant extract intake may also be attributed to an extra pancreatic hypoglycemic action involving the stimulation of peripheral glucose utilization or enhancing glycolytic and glycogenic processes with a concomitant decrease in glycogenolysis as well as gluconeogenesis (Luzi *et al.,* 1997). In a similar fashion, extracts of various plants like *Allium cepa*, *Ficus bengalensis* and *Syzygium cumini*, *Calendula officinalis* exhibited antidiabetic properties through release of insulin from pancreatic tissue and also by extra-pancreatic mechanisms (Jung *et al.,* 2006). In the present study, administration of ethanolic extract of *A. scholaris* reduced the elevated HbA1c levels in STZ induced diabetic rats further reiterating its potential for the long term control of hyperglycemia (Bandawane *et al.,* 2010).

In the present study repeated oral administration of leaf extracts of *A. scholaris* in diabetic rats also restored the total cholesterol, triglycerides, LDL and HDL levels illustrating hypolipidemic potential of the extract. In our study it was observed that ethanolic extract of *A. scholaris* was less effective in normalizing lipid profile in diabetic rats when compared to the potential of aqueous extract. Reduction in bad and increase in good cholesterol is beneficial and may help to check various diabetic complications like subsequent development of cardiovascular disorders (CVD). Similar results were also obtained by others from the use of different parts of *A. scholaris* plant (Anurakkun *et al.,* 2007; Bandawane *et al.,* 2010). The hypocholesterolemic effect of the plant extract may be due to the overall inhibition of fatty acid synthesis. The significant reduction of LDL levels in *A. scholaris* treated rats may be due to the activation of LDL receptors in hepatocytes thus reducing the serum LDL level or may be due to the inhibition of cholesterol synthesis pathway. Increase in total cholesterol, triglycerides and LDL, with decrease in HDL levels in diabetic conditions is also a common finding in people with coronary artery disease (Guerci *et al.,* 2001). Apart from classical risk factors like dyslipidemia, elevated HbA1c is now being regarded as an independent risk factor for CVD in subjects with or without diabetes. In the present study, restoration of lipid profile and reduction in HbA1c per cent by extracts of *A. scholaris* indicate diminished risk of progressive development of diabetic complications most notably CVD.

Significant reduction in total plasma proteins, albumin and globulin in diabetic rats observed in present study may be due to increased catabolic activities or decreased plasma protein synthesis in liver. Increased BUN and CR in plasma of diabetic rats reflects increased catabolic activity. Additionally, significantly increased dehydrogenase, aminotransferases and phosphatase activities are also indicative of altered liver functions and liver damage which can lead to overall reduction in plasma protein synthesis by liver. Many other studies have also reported that STZ caused alterations in hepatic functions as was reflected by altered activities of AST, ALT, ALP, etc (Ahmed *et al.,* 2017).

In blood, bilirubin circulates in two main forms either as conjugated or as unconjugated bilirubin, which can be detected by a direct or indirect Van den berg reaction respectively. *A. scholaris* extracts restored all liver function tests except direct bilirubin levels. In liver, hydrophobic bilirubin (unconjugated bilirubin) is converted to bilirubin diglucuronide (conjugated) by UDP glucuronyl transferase to facilitate its excretion into the bile. Supplementation of aqueous and ethanolic extracts of *A. scholaris* restored the total and indirect bilirubin but values of direct bilirubin remained significantly lower,

which indicate reduced conjugation by UDP glucuronyl transferase (Verma *et al.,* 2016). Previous studies have revealed that STZ induced diabetes in rodent results in development of kidney disease similar to early stage clinical diabetic nephropathy. STZ by itself does not cause any long term direct effects on the kidney excluding those that are initiated after development of diabetes mellitus. Thus adverse diabetic renal complications are mainly due to increased levels of glucose which glycate proteins leading to increased formation of advanced glycation end products (AGEs) (Vlassara and Palace, 2001). The glycation of proteins such as transporters and enzymes impacts their functioning. Elevated blood glucose level also increases the work load on kidney due to hyperglycemia induced increased osmotic pressure of blood. Therefore, increased work load and decreased transporter protein function (due to formation of AGEs) together put kidney at a high risk for developing nephropathy in diabetic patients which may interfere with normal excretion of waste product and ultimately cause elevated plasma creatinine and BUN levels in diabetics (Katyal *et al.*, 2014). Previous studies have shown that increase in BUN and CR in diabetic rats indicates progressive renal damage because high sugar levels impair renal function (Anjaneyulu and Chopra, 2004). Repeated administration of either aqueous or ethanolic leaf extracts of *A. scholaris* significantly decreased BUN and CR levels in diabetic rats. Thus extract administration effectively protected kidney from developing diabetes related renal complications as indicated by significant improvements in altered renal functions tests. Similarly previous researchers have demonstrated presence of nephroprotective properties in extracts derived from different kinds of plants (Verma *et al.,* 2013; Ahmad *et al.,* 2017).

Uric acid is another well known low molecular weight water soluble plasma antioxidant. The concentration of uric acid significantly increased in diabetic rats. Aqueous leaf extracts of *A. scholaris* administration restored uric acid level whereas ethanolic leaf extract of *A. scholaris* was ineffective in doing so. Various studies have suggested that plasma level of uric acid may alter in physiological and metabolic disorders (Maesaka and Fishbane, 1998). Increased levels of uric acid also contribute to plasma antioxidant potential thus introducing another possible confounding factor in the measurement of plasma total antioxidant capacity (Maxwell *et al.,* 1993).

Antioxidant biomarkers

Oxidative damage occurs from an increase in the production of free radicals by exogenous or endogenous sources such as inflammation, the respiratory burst and xenobiotic killing. Mammals have evolved to acquire complex antioxidant strategies to utilize oxygen and at the same time to minimize the noxious effects of it partially reduced species (Halliwell and Gutteridge, 1990). Antioxidants within cells, cell membranes and extracellular fluids can be up-regulated and mobilized to neutralize excessive and inappropriate ROS formation. For the maintenance of redox balance in oxidant conditions like chronic inflammation and diets poor in antioxidants and/or rich in pro-oxidants, blood plays a central role because it transports and redistributes antioxidants to every part of the body. Increased blood glucose, AGEs and free fatty acid levels increase the amount of free radicals/ROS which builds up oxidative stress. Oxidative damage to different body tissues are important events in the pathogenesis of diabetes associated complications (West, 2000). Apart from this, AGEs accumulate in tissues and interfere with normal cellular and tissue functioning (Brownlee*,* 2001). In addition, AGEs also bind to specific macrophage receptors on endothelial cells leading to endothelial dysfunction and increased vascular permeability. AGEs also act on nucleic acids and histones and cause mutations and altered gene expression (Evans *et al.*, 2002).

Equilibrium between plasma total antioxidant and oxidant status is paramount for the proper functioning of antioxidant system and maintenance of physiological redox homeostasis in mammals. Oxidative stress is a common event in the pathogenesis of secondary diabetic complications . In diabetic animals, phenomenon such as oxidation of LDL, glycosylation of Hb and other proteins, spur ROS genesis which inflicts oxidative damage in divergent tissues. The ROS interacts with polyunsaturated fatty acids of cell membrane and causes peroxidation of membrane lipids leading to loss of membrane integrity which jeopardises cellular structure and functions. Evidence from earlier research states that excessive ROS production and reduced antioxidant defences, which act as triggers for membrane lipid peroxidation and protein oxidation, are common phenomenon among diabetic subjects (Bravi *et al.,* 2006). These reactive molecules also interact with carbohydrates and nucleic acids causing further cytotoxicity and organ dysfunction (Maritim *et al.*, 2003). In the present study, significant decrease in TAS and significantly increased TOS disturbed OSI level in diabetic rats and created an environment of oxidative stress. Increased TOS along with decreased TAS and total thiols levels also contribute significantly in pathogenesis of different disease conditions in mammals (Oncel *et al.,* 2014). Aqueous extract of *A. scholaris* in diabetic rats significantly increased the TAS and restored the TOS and OSI values suggesting the presence of inherent antioxidant capabilities in the extract. Also,

treatment with ethanolic extract significantly increased TAS even though it was unable to restore the levels of TOS and OSI in diabetic rats. These increased TAS values indicate that extracts bolstered antioxidant defences either by direct scavenging of the free radicals or indirectly by elevating the levels of free thiols which serve as the main scavengers of soluble free radicals (Verma *et al.,* 2016).

The significantly increased MDA level in diabetic rats in the present study, suggest that diabetes associated oxidative stress induced lipid peroxidation. Since MDA is the terminal end product of lipid peroxidation, thus the concentration of MDA is a good estimate of the extent of lipid peroxidation and cell membrane damage in cases of diabetes mellitus (Ahmad *et al.,* 2014, 2017). Similar increased lipid peroxidation has also been reported in clinical and experimentally induced diabetic conditions (Ahmad *et al.,* 2014; Verma *et al.,* 2016; Ahmad *et al.,* 2017).

Dysregulation of enzymatic and non-enzymatic components of antioxidant system in diabetic rats are major contributing factors for reduction in TAS and increased levels of ROS/RNS/free radicals (Halliwell and Gutteridge, 1990). In the present study significantly reduced activities of enzymatic components like CAT, SOD, GPx, GST and G6PDH in diabetic rats point towards decreased ROS/RNS/free radicals scavenging capability along with existence of a state of oxidative stress in these animals. Repeated treatments with either aqueous or ethanolic leaf extracts of *A. scholaris* in diabetic rats restored the activities of most of these enzymatic components. Our findings are in accordance with the results of others who have used different parts of *A. scholaris* in their research work with similar effectiveness (Anurakkun *et al.,* 2007; Bandawane *et al.,* 2010). The normalization of activities of these enzymatic components was more pronounced after ethanolic leaf extract treatment as against medication with aqueous extract in diabetic rats. The superior protective effects of ethanolic extract may be due to presence of significantly higher content of phenolic, flavonoid, ß-carotenes, lycopene in this extract as compared to aqueous extract (Verma *et al.,* 2015).

G6PDH is the rate limiting enzyme of the pentose phosphate pathway. The primary function of the pathway is to generate reducing power in the cytoplasm in the form of reduced nicotinamide adenine dineucleotide phosphate (NADPH) (Zhang *et al.,* 2000). Cytosolic NADPH is a critical cofactor for CAT as it maintains the enzyme in an active state (Felix *et al.,* 2003). Also, NADPH serves as a cofactor for glutathione reductase which catalyzes oxidized glutathione to its reduced form (GSH), a major free radical scavenger. Therefore, a decrease in G6PD activity leads to decreased intracellular NADPH levels and makes the cell extremely vulnerable to oxidant damage. In the present study G6PDH was significantly diminished in diabetic rats, which likely resulted in reduced level of NADPH and exposure of cells to oxidative environment (Zhang *et al.,* 2000). Repeated administration of ethanolic extracts in diabetic rats restored the level of G6PDH which may also be responsible for the normalization of the activity of CAT and GSH levels in these animals due to the sustenance of an optimum cytosolic concentration of NADPH.

GST isoenzymes, which are ubiquitously distributed throughout body, catalyze the conjugation of reduced glutathione with compounds that contain an electrophilic centre through the formation of a thio-ether bond between the sulphur atom of GSH and the substrate. GSTs also play regulatory roles in many cellular processes in ways that are not usually directly related to their catalytic activity. Frequently they directly interact with other crucial enzymes and modulate cellular responses to oxidative stress, cellular proliferation, differentiation, as well as apoptosis (Cho *et al.,* 2001). In our study reduction in GST activity in diabetic rats may be due to depletion of GSH or increased levels of reactive free radicals. Repeated administration of either aqueous or ethanolic extract of *A. scholaris* reinstated the GST activity in diabetic rats thus improving free radical scavenging ability in these animals. Consistent with our findings restoration of activities of GST in diabetic rats after administration of different plant extracts have also been observed by other workers (Jain *et al.,* 2010; Ahmad *et al.,* 2014).

Ceruloplasmin is an acute phase response protein which is the chief transporter of copper. CP also acts as a scavenger of free radicals particularly superoxide ions, and a modulator of inflammatory responses, therefore, synthesis and secretion of CP can be markedly increased during inflammation, infection, and in diseases such as diabetes, cancer, and cardiovascular diseases (Uriu-Adams and Keen, 2005; Ahmad *et al.,* 2017). In the current study also, ceruloplasmin levels were significantly increased in diabetic rats and repeated treatment with either aqueous or ethanolic extracts of *A. scholaris* in diabetic rats failed to restore its levels.

Protein and non-protein thiols (Pr-SHs) are low molecular weight molecules such as reduced glutathione, cysteine, and protein bound sulfhydryl groups which protect cellular macromolecules from reactive radicals. Likewise, thiols (-SH) also protect macromolecules by counteracting ROS and other free radicals. In the present study, significant reduction in total thiols levels including the decrease in blood concentration glutathione (GSH) in diabetic rats signified

either their increased utilization or decreased production (Kyle *et al.,*1990), a scenario expected with presence of high quantities of oxidative stress generating molecules in diabetic animals. Treatment with either aqueous or ethanolic extracts of *A. scholaris* in diabetic rats refurbished the reduced levels of total thiols and GSH in diabetic rats. Rejuvenation of thiols including that of GSH may reflect increased synthesis of total thiols by modulating the activity of γ-glutamylcysteine synthase by polyphenolic compounds present in the extracts (DiMonte *et al.,* 1984). It is concluded from this investigation that the ethanolic extract proved to be more effective than the aqueous extract in countering hyperglycemia and other adverse effects of STZ induced diabetes in Wistar rats.

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Research Article

A MULTIPLE ONCE DAILY DOSE PHARMACOKINETIC OF AMIKACIN IN COW CALVES FOLLOWING INTRAVENOUS ADMINISTRATION

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ABSTRACT

A multiple once daily dose pharmacokinetic of amikacin (@ 10 mg/kg, i.v.) was carried out in healthy cow calves weighing between 80-100 kg. Concentrations of amikacin in blood plasma were estimated by microbiological assay techniques as well as various kinetic parameters were calculated by using two compartment open model for amikacin. Serial blood samples were taken on days 1 and 5 of treatment and at predose, 1 and 6 h on days 2, 3 and 4. Attempts were made to calculate the rational dosage regimens of amikacin on first and last dosing, on the basis of kinetic data and maintenance of therapeutic concentrations in plasma. Amikacin was administered separately in each of four healthy calves by intravenous (i.v) route once daily for five days. The drug was detectable up to 10 h. The minimum therapeutic concentration (≥ 1.0 μg/ml) of amikacin was maintained up to 2 h in both 1st and 5th day of amikacin administration. Significantly higher plasma concentrations of the drug appeared from 0.042 to 6 h except 0.25 h in 5th day of amikacin administration as compared to 1st day amikacin administration. Mean trough amikacin plasma concentrations were 0.3, Significantly higher values of extrapolated zero time concentration of the drug during distribution phase (A), theoretical zero time concentration (C_p^o), area under curve (AUC), area under first moment curve (AUMC), mean residential time (MRT) and elimination of drug from central compartment (Kel) while significantly lower value of elimination rate constant (β) and total body clearance (Cl_B) are observed in 5th day of amikacin administration as compared to 1st day of amikacin administration. All other kinetic parameters differ non-significantly between 1st and 5th day of amikacin administration. It is concluded that once-daily administration of amikacin may provide adequate plasma levels to treat most susceptible gram-negative infections in cow calves.

Key Words: Multiple Dose, Pharmacokinetics, Amikacin, Cow calves

INTRODUCTION

Amikacin, a recent aminoglycoside antibiotic derived from kanamycin by the process of acetylation, has proved its effective and greater clinical advantages over other aminoglycosides due to its high antibacterial spectrum against wide range of bacteria which are resistant to other aminoglycosides. It is most commonly used drug for the treatment of mixed bacterial infections which are unresponsive to other routine antibiotics, in medical and veterinary clinical practices. The aminoglycoside antibiotics have concentration-dependent bactericidal activity and the peak drug concentration (C_{max}) to minimum inhibitory concentration (MIC) ratio (C_{max}/MIC) is the pharmacokinetic/ pharmacodynamic parameter best correlated with clinical efficacy (Blaser *et al.*, 1987). High drug concentrations of no less 10 fold the MIC of the bacterial pathogen are associated with successful treatments. However, because of its adverse effect i.e. ototoxicity and nephrotoxicity, plasma concentration must fall below a specific level before administering another dosage (Prins *et al.*, 1996). Thus, safe and effective dosage regimens must be carefully determined.

Once-daily administration of aminoglycosides may allow high peak levels, resulting in enhanced bacterial killing and post antibiotic effects and less nephrotoxic low trough levels, due to an extended interval between doses. Once-daily administration is currently applied in humen clinical practice in order to reduce aminoglycosides renal toxicity (Prins *et al.*, 1996). Hence, the aim of the present study was to determine the pharmacokinetics of repeated doses of amikacin administered once daily in cow calves following intravenous route. However, no literatures are available for the pharmacokinetics of repeated doses of amikacin administered once daily in cow calves following intravenous route.

MATERIALS AND METHODS

The present study was undertaken to determine the pharmacokinetics of repeated doses of amikacin administered once daily in cow calves following intravenous route.

Experimental animals

In the present study, four clinically healthy female cow calves of non-descript breed between 4 to 6 months of age and 80 - 100 kg body weight were used. The animals were maintained at the Instructional Farm, College of Veterinary Science and Animal Husbandry, Mhow, Madhya Pradesh. They were kept under constant observation for fifteen days prior to commencement of the experiment. During this period they were subjected

to clinical examination in order to exclude the possibility of any disease. The animals were then housed in separate pen and were provided standard ration as per the farm schedule. Water was provided *ad libitum.* Fifteen days before the start of experiment deworming carried out with broad spectrum anthelmintics. All necessary managemental procedures were adopted to keep the animals free from stress. The experimental protocol for general procedure and use of animals for conducting the present study has been reviewed and approved by the Institutional Animal Ethics Committee (IAEC).

Administration of drugs

Amikacin (AMIDAC®) - an injectable commercial preparation containing amikacin equivalent to 250 mg/ml, marketed by ZYDUS AHL., India was used. Amikacin was administered at the dose rate of 10 mg/kg body weight by i.v. route in each of four healthy calves. Intravenous injection of the drug was given through a jugular vein. Amikacin was administered separately in each of four healthy calves by intravenous (i.v) route once daily for five days.

Collection of blood samples

Blood samples (approx.1 ml) were withdrawn from jugular vein into heparinized glass centrifuge tubes on days 1 and 5 of treatment: at 0, 2.5, 5, 10, 15, 20, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 10, 12 and 24 h after administration of the drug. On days 2, 3 and 4 blood samples were taken 1 and 6 h after drug administration. Plasma was separated by centrifugation at 3,000 r.p.m. for 15 min at room temperature and kept at -4°C until analysis, which was usually done within two days of collection of samples.

Estimation of amikacin

The concentration of amikacin in plasma was estimated by a rapid, specific microbiological assay technique using *Escherichia coli* as the test organism (Paul *et al.*, 1971).

Pharmacokinetic analysis

The plasma concentration-time profile of amikacin for each animal was used to determine the pharmacokinetics. The data of amikacin was subjected to two compartment open model. Kinetic parameters were calculated on the basis of Baggot (1977) and Gibaldi and Perrier (1982). Based on kinetic parameters, dosage regimen was derived using the following formula.

For calculation of D* (loading or priming dose) and D_0 (maintenance dose) the following formulae of Baggot (1977) were used

$D^* = C_P (min). Vd_{area} (e^{\beta.\tau})$

$D_0 = C_P (min). Vd_{area} (e^{\beta.\tau}-1)$

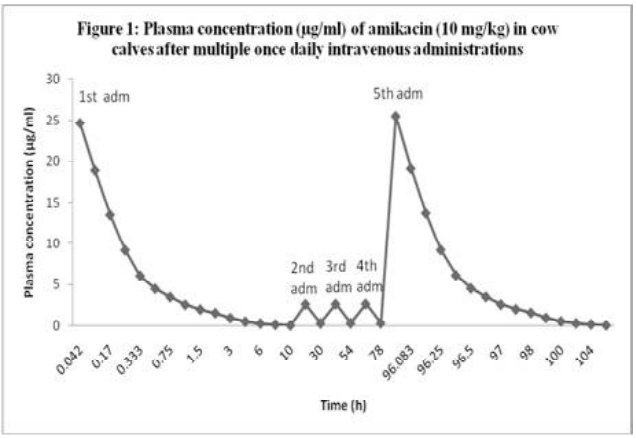
Where, C_P (min) = minimum therapeutic plasma drug concentration, Vd_{area} = volume of distribution based on total area under the plasma drug concentration versus time curve, β = elimination rate constant, τ = dosage interval, e = base of natural logarithm

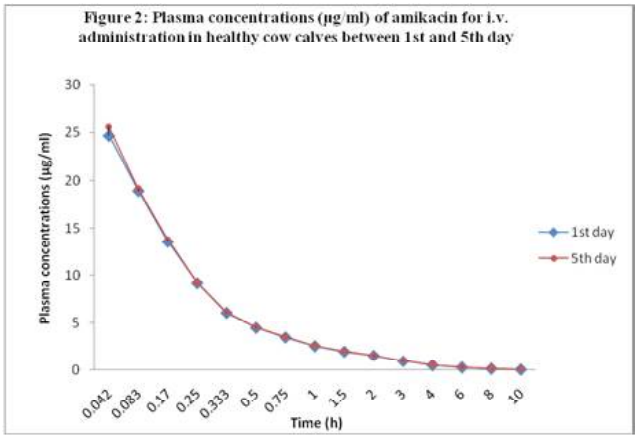
Statistical analysis

Comparison of concentrations of the drugs in plasma, various kinetic parameters and dosage regimen of amikacin on first and last doses after multiple i.v. once daily dose administration in cow calves were compared by using paired 't' test (Snedecor and Cochran, 1994).

RESULTS

Concentrations of amikacin in plasma at various time intervals following its multiple intravenous (i.v.) injection at the dose rate of 10 mg/kg body weight have been shown in Figure 1. On first dosing, the mean plasma concentration of the drug at 0.042 h was found to be 24.69 ± 0.011 μg/ml and the values ranged from 24.75 to 24.64 μg/ml. The drug was detectable in all the four animals up to 10 h and the mean concentration at 10 h was noted to be 0.08 ± 0.002 μg/ml however, the effective therapeutic concentration (≥1 μg/ml) of amikacin was maintained up to 2 h of administration. While, on last dosing the mean plasma concentration of the drug at 96.042 h was found to be 25.56 ± 0.097 μg/ml and the values ranged from 25.79 to 24.98 μg/ml. The drug was





detectable in all the four animals up to 10 h (i.e 106 h) and the mean concentration at 106 h was noted to be 0.09 ± 0.002 μg/ml however, the effective therapeutic concentration (≥1 μg/ml) of amikacin was maintained up to 2 h of administration.

Comparative plasma concentrations of amikacin in healthy cow calves after i.v administration between 1st and 5th days have been shown in Figure 2. The drug was detectable up to 10 h. The minimum therapeutic concentration (≥ 1.0 g/ml) of amikacin was maintained up to 2 h in both 1st and 5th day of amikacin administration. Significantly higher plasma concentrations of the drug appeared from 0.042 to 6 h except 0.25 h in 5th day of amikacin administration as compared to 1st day amikacin administration.

Table 1 reveals the comparison of kinetic parameters of amikacin in healthy calves. Significantly higher values of extrapolated zero time concentration of the drug during distribution phase (A), theoretical zero time concentration (C_p^o), area under curve (AUC), area under first moment curve (AUMC), mean residential time (MRT) and elimination of drug from central compartment (Kel) while significantly lower value of elimination rate constant (β) and total body clearance

Table 1:
Comparison of kinetic parameters of amikacin for i.v. administration in healthy cow calves between 1st and 5th day

Parameter (Unit)	1st day	5th day
A (μg/ml)	15.439±0.008	15.71±0.023**
B (μg/ml)	3.23±0.007	3.20±0.012
C_p^o (μg/ml)	18.67±0.002	18.92 ± 0.03*
β (h^{-1})	0.378±0.002	0.362±0.001*
α (h^{-1})	3.812±0.005	3.81±0.012
$t_{1/2}$ α (h)	0.182±0.002	0.181±0.005
$t_{1/2}$ β (h)	1.834±0.012	1.91±0.010
AUC (μg/ml.h)	13.3±0.051	13.67±0.042**
AUMC (μg/ml.h^2)	22.7±0.266	24.57±0.229**
MRT (h)	1.7±0.020	1.82±0.012*
K_{12} (h^{-1})	1.735±0.002	1.770±0.005
K_{21} (h^{-1})	0.972±0.004	0.947±0.004
Kel (h^{-1})	1.48±0.005	1.458±0.005*
Vd_{area} (L/kg)	1.99±0.007	2.02±0.007
Cl_B (ml/kg/h)	754.66±2.68	732.994±2.187**

A = The extrapolated zero time concentration of the drug in plasma during distribution phase, B = The extrapolated zero time concentration of the drug in plasma during elimination phase, Cp^0 = The theoretical zero time concentration, α = The distribution rate constant, $t_{1/2\alpha}$ = The distribution half-life, β = The elimination rate constant, $t_{1/2\beta}$ = The elimination half-life, AUC = The area under curve in plasma, AUMC = The area under first moment curve, MRT = The mean residence time, K_{12} = The average rate of transfer of drug from central to peripheral compartment, K_{21} = The average rate of transfer of drug from peripheral to central compartment, K_{el} = The elimination from central compartment, Vd_{area} = The volume of distribution during area under curve, and Cl_B = The total body clearance.

Table 2:
Comparison of calculated dosage regimens for amikacin for i.v. administration in healthy cow calves between 1st and 5th day

C_P^∞ min (μg/ml)	τ (h)	Dose (mg/kg)	Amikacin on 1st day	Amikacin on 5th day
1	8	D*	2.21±0.01	2.29 ±0.13
		D_0	0.21±0.03	0.12 ±0.01*
	12	D*	3.01±0.006	2.97±0.01*
		D_0	1.02±0.02	0.95±0.01**
2	8	D*	4.42±0.03	4.58±0.26
		D_0	0.43±0.05	0.25±0.03*
	12	D*	6.03±0.01	5.94±0.02**
		D_0	2.04±0.04	1.89±0.03**

D* =Priming or Loading dose, D_0 = Maintenance dose, τ = Dosage interval, C_P^∞ min =Minimum therapeutic concentration in plasma

(Cl_B) are observed in 5th day of amikacin administration as compared to 1st day of amikacin administration. All other kinetic parameters differ non-significantly between 1st and 5th day of amikacin administration.

Comparison of calculated dosage regimens of amikacin for different therapeutic levels (C_p^∞ min = 1.0 and 2.0 μg/ml) and different dosage intervals (τ) of 8 and 12 h have been shown in Table 2. All calculated data for loading (D*) and maintenance (D_0) doses for different therapeutic levels at different dosage intervals (τ) were noted to be significantly lower except loading (D^*) at dosage interval of 8 h in 5th day of amikacin administration as compared to 1st day of amikacin administration.

DISCUSSION

The semi logaritham plot of plasma levels of amikacin as a function of time after its multiple once daily dose i.v. administration of amikacin exhibited two distinct phases on 1st and 5th day of drug administration and the data obtained were adequately described by two compartment open model in the present study. Following multiple once daily intravenous dose of amikacin in healthy cow calves, mean peak plasma concentration at 2.5 min was 24.69 ± 0.011 μg/ml (1 day) & 25.56 ± 0.097 μg/ml (5 day) and amikacin was detected up to 10 h with a mean plasma concentration of 0.08 ± 0.002 μg/ml (1 day) & 0.09 ± 0.002 μg/ml (5 day). This value is comparatively higher in cross-bred bovine calves after a single intravenous administration of amikacin at dose rate of 10 mg/kg at 1 min. The concentration of amikacin in the plasma was 116.9±3.16 μg/ml and the minimum therapeutic concentration was maintained for 8 h (Saini and Shrivastava, 1998). The study conducted by Orsini *et al.* (1985) indicated that doses of amikacin at the rate of 4.4, 6.6 and 11.0 mg/kg show the concentrations 30.3 ± 0.3, 61.2 ± 6.9 and 122.8 ± 7.4 μg/ml, respectively at 15 min following i.v. injection.

Sumano *et al.* (2005) determined the pharmacokinetic variables of amikacin in cows after administration of amikacin sulphate either intravenously (i.v.) or intramuscularly (i.m.) at a dose of 25 mg/kg per day for three days and amikacin concentrations at time zero and maximum serum concentrations were found as 240.8 µg/mL and 122.53 µg/mL, respectively. According to Edward and Richard (1993), amikacin administration resulted in peak values of 27.3 ± 6.9 µg/ml in study of the pharmacokinetic properties of gentamicin and amikacin in the cockatiel (*Nymphicus hollandicus*), a small (approximate body weight = 100 g) psittacine bird, utilizing treatment regimens developed in larger parrot species.

In the present study, plasma concentrations of amikacin *versus* time disposition curves after intravenous administration were best fitted to a two compartment open model. Similarly, (Kathryn *et al.*, 1995) reported two compartment open model after the intravenous dose of 5.8 mg/kg of amikacin in scimitar-horned oryx, also reported in dogs (Baggot *et al.,* 1985), in cats (Jernigan *et al.*, 1988) and in goats (Uppal *et al.*, 1992). Likewise first compartment model was fitted in mice according to Zhou *et al.* (1997).

The minimum therapeutic concentration of amikacin in plasma ranges from 1- 4 µg/ml (Leroy *et al.,* 1978). According to Brown *et al.* (1984) and Orsini *et al.,* (1985), for most of the susceptible bacteria, the therapeutic level of amikacin is 1-2 µg/ml maintained at time interval of 8 and 12 h. Therefore the Keeping in view the influence of certain unavoidable factor *in vivo*, the MIC of ($\geq$ 1 µg.ml^{-1}) of amikacin has been maintained up to 6 h.

The elimination half-life ($t_{1/2}\beta$) is the time taken for plasma concentration in the body to be reduced by its half (50 per cent). Half-life provides a good indicator of time which is required to reach steady state after initiation of dosage regimen. The elimination half-life of amikacin in cow calves following multiple once daily intravenous administration in the present study was 1.834 ± 0.012 h (1 day) and 1.91 ± 0.010 h (5 day). The elimination half-life of amikacin in cow calves is more or less similar to 3.09 ± 0.27 h in bovine calves (Saini and Shrivastava, 1998), in lactating sheep 1.64 ± 0.06 h (Haritova, 2004) and 2.16 h for goats (Uppal *et al.,* 1992).

The distribution half life ($t_{1/2}\alpha$) of amikacin in cow calves following multiple once daily intravenous administration in the present study was 0.182 ± 0.002 h (1 day) and 0.181 ± 0.005 h (5 day). These values are more or less similar to values reported in goat as 0.24 h (Uppal *et al.,* 1997), 0.36 h in calves and 0.43 h in sheep (Carli *et al.,* 1990).

The high values of AUC and AUMC reflect that most of the body area is covered with the drug concentrations. The AUC values of amikacin in cow calves following multiple once daily intravenous administration in the present study was 13.3 ± 0.051 µg/ml.h (1 day) and 13.67 ± 0.042 µg/ml.h (5 day), but significantly higher in goats 73.18 µg/ml.h (Agrawal *et al.,* 2001), in lactating sheep 94.09 ± 6.95 µg/ml.h (Haritova, 2004) and in Greyhounds dogs 79.97 h·µg/ml (Kukanich and Coetzee, 2007). Similarly, the AUMC values of amikacin in cow calves following multiple once daily intravenous administration in the present study was 22.7 ± 0.266 µg/ml.h^2 (1 day) and 24.57 ± 0.229 µg/ml.h^2 (5 day). The mean residence time (MRT) of amikacin in cow calves following multiple once daily intravenous administration in the present study was 1.7 ± 0.02 h (1 day) and 1.82 ± 0.012 h (5 day), which is more in goat 4.67 ± 0.19 h (Agrawal *et al.,* 2001) and almost same in oryx 2.27 h (Kathryn *et al.*, 1995). This is in contrast to Witchel *et al.* (1992) who reported the increased values of AUC, AUMC and MRT in diseased foals after amikacin administration.

The volume of distribution (Vd_{area}) values of amikacin in cow calves following multiple once daily intravenous administration in the present study was 1.99 ± 0.007 l/kg (1 day) and 2.02 ± 0.007 l/kg (5 day). This value in healthy cow calves is higher than in bovine calves 0.40 ± 0.03 L/kg (Saini and Shrivastava, 1998), in man 0.27 ± 0.04 L/kg (Bauer and Blouin, 1983) and in Beagles dog 234.0 ml/kg (Kukanich and Coetzee, 2007). This is reflecting good penetration of amikacin into various body fluids and tissues of cow calves. A very high value of Vd_{area} obtained in the present study may be attributed to wide distribution of amikacin in the body because of its polar organic base nature (Carli *et al.*, 1990). The total body clearance (Cl_B) values of amikacin in cow calves following multiple once daily intravenous administration in the present study was 754.66 ± 2.68 ml/kg/h (1 day) and 732.994 ± 2.187 ml/kg/h (5 day). It was observed that elimination of amikacin was altered by first and last dose, which plays an important role in the decrease of body clearance of drugs, including amikacin which is widely eliminated by the renal route. Similarly, lower value of total body clearance (Cl_B) was noted for goats 2.34 ± 0.17 ml/kg/min (Agrawal *et al.,*2001) and also in camel as 0.97 ml/kg/min (Wasfi *et al.*,1999) after i.m. administration, in dogs 2.66 ml/kg/min (Baggot *et al.,* 1985) and in cow calves 0.09 ± 0.002 L/kg/h in normal condition which is higher than that of febrile condition 0.05 ± 0.01 L/kg/h after i.v. administration of amikacin (Saini and Shrivastava, 1997).

This difference in the values of Cl_B amongst various species of the animals indicated that difference in their glomerular filteration rates of amikacin which is polar organic base hence weakly bound to serum proteins and is excreted as unchanged into the urine by

glomerural filteration as supported by Carli *et al.* (1990).

The ultimate objective of the study of disposition kinetics is to determine an appropriate dose regimen of drugs. For any antimicrobial agent the dosage regimen is calculated to maintain the minimum therapeutic concentration (MIC) throughout the course of infections. An average plasma concentration of 1.0- 4.0 μg/ml has been reported to be the minimum therapeutic concentration (MIC_{90}) of amikacin against most gram positive, gram negative and atypical bacteria (Leroy *et al.,* 1978, Agrawal *et al.,* 2001). Keeping in view of synergistic effects of the immune system and other *in vivo* factors as well as to cover most of the susceptible organisms, in this discussion, the MIC_{90} of 1.0 μg/ml of amikacin has been taken into consideration. Amikacin possessed excellent antibacterial activity (MIC for 90% of tested strains i.e. $MIC_{90} \leq 2.0$ μg/ml) against most common gram-negative aerobic pathogens, including *E. coli*, *K. pneumoniae*, *Enterobacter spp*, and *Brucella spp* (Shaffer *et al.,* 1953) *Mycobacteria* (Suter, 1952). Thus, in the present study dosage regimen was derived at MIC of 1.0 and 2.0 μg/ml for amikacin at dosage interval of 8 and 12 h.

All calculated data for loading (D*) and maintenance (D_0) doses for different therapeutic levels at different dosage intervals (τ) were noted to be significantly lower except loading (D^*) at dosage interval of 8 h in 5th day of amikacin administration as compared to 1st day of amikacin administration for intravenous route (Table 2) which suggested that amikacin dose has to be reduced for safe and effective for treating systemic microbial infections.

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Research Article

EFFECT OF LICORICE ON PHARMACOKINETICS OF NIMESULIDE IN BIRDS

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ABSTRACT

This investigation was designed to evaluate the influence of Licorice, a CYP2C9 inhibiting flavonoid on the pharmacokinetics of nimesulide in broiler chicken. Birds of 2 kgs b wt, randomly divided into two groups with 8 birds in each. To both the groups (I & II) nimesulide was administered at 2 $mg.kg^{-1}$ b wt as single oral dose, where as group II birds were administered 500 $mg.kg^{-1}$ b wt of licorice extract as pretreatment one hour before the nimesulide administration. Blood samples were collected from tarsal vein at pre-determined time intervals prior to and after administration of nimesulide. Plasma was separated and analyzed for nimesulide by HPLC assay. Based on plasma concentrations of nimesulide, the pharmacokinetic parameters were determined by non-compartmental methods. The pharmacokinetic parameters such as $AUC_{0-\infty}$, Cl_{β}, $t_{1/2}\beta$, Vd_{ss} and MRT were not altered significantly when compared between two groups. There was no significant difference in either plasma concentrations or pharmacokinetic parameters of nimesulide in both the groups studied. We conclude that the licorice, a putative inhibitor of CYP2C9 and CYP3A4 has no effect on the activity of these enzymes in birds.

Keywords: Licorice, Nimesulide, Pharmacokinetic, birds, CYP2C9.

INTRODUCTION

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are often indicated as a supplement to antimicrobial therapy in veterinary medicine to reduce the pain, fever and inflammation (Shukla *et al.*, 2007). NSAID is the most prescribed medications for treating various painful conditions. Among NSAIDs nimesulide uses have been increasingly reported in both human and veterinary practices.

Nimesulide is a non-steroidal anti-inflammatory drug belonging to the sulphanilamide class, and is the first COX-2 preferential inhibitor. It is shown to have anti-inflammatory, analgesic and antipyretic effects in animal experimental models (Rabasseda, 1996). The drug exerts its effects through a variety of mechanisms (Bennett, 1999), but the most important seems to be selective inhibition of COX-2, as demonstrated in man and various animal species (Famaey, 1997; Fabiola *et al.*, 1998). Nimesulide has been reported to cause hepatotoxicity and nephrotoxicity, it should be used vigilantly even in adults (Schattner *et al.*, 2000). According to Vignati *et al.* (2005) nimesulide was reported to be metabolized by CYP2C9 and CYP3A4. But there is no interaction study between a CYP2C9 and CYP3A4 inhibitor and nimesulide. Many clinical reports indicated that the biotransformation of drugs will be compromised in inflammatory responses due to infections that down-regulate cytochrome P450 (Morgan, 1997; Renton, 2001; Slaviero *et al.*, 2003).

Currently, healthcare professionals are deeply concerned about drug-herb interactions and how concurrent administration may interact with pharmacokinetics of drug and how it helps in reduction of side effects of NSAIDs (Kennedy and Seely, 2013).

Glycyrrhiza glabra, also known as licorice, belonging to family Leguminaceae and is a sweetening agent used in foods and beverages. Glycyrrhizin is a well-known putative CYP2C9 enzyme inhibitor (Kent *et al.*, 2002), which metabolizes nimesulide.

So the present study is designed to know the pharmacokinetic interaction of licorice with nimesulide and to study the effect of *Glycyrrhiza glabra* extract, a putative CYP2C9 inhibitor on pharmacokinetics of nimesulide.

MATERIALS AND METHODS

Experimental animals

Adult birds were procured from M/s Srinivasa Hatcheries, Vijayawada, India. They were maintained under standard management and husbandry conditions with free access to feed and water. The experiments were approved by Institutional Animal Ethics Committee (IAEC), N.T.R College of Veterinary Science, Gannavaram, Andhra Pradesh, India.

Drugs and chemicals

Oral bolus formulation of nimesulide (Mensu-OD®) was procured from M/s Horizon Bioceuticals Pvt. Ltd., Himachal Pradesh. Pure technical grade powder of nimesulide which was employed as external standard in HPLC assay was procured from Sigma Aldrich, USA. Heparin 20,000 IU/vial was obtained from M/s Loba Chemie, Mumbai, India. Acetonitrile and other chemicals

of HPLC grade used in the experiment were procured from M/s Merck, Mumbai, India. Water for HPLC was obtained by Millipore water purification system and was filtered through 0.2 µm filter prior to use. All other chemicals used in the study were of analytical grade.

Licorice (*Glycyrrhiza glabra*) roots were obtained commercially from a local crude herbs selling shop. The roots were washed in distilled water, shade dried and made into course powder and stored at room temperature until further use. Licorice extract was prepared by dissolving 200 grams of powder in 2000 ml of 1:1 acetone and distilled water (Tiwari *et al.*, 2011). The conical flask was subjected to constant stirring using an orbital shaker overnight at room temperature with a speed of 100 rpm. The extract prepared was filtered using Whatman's filter paper (640md) and the filtrate was subjected to slow evaporation. The extract was administered to the birds at the rate of 500 mg.Kg^{-1}.

Experimental design

Adult birds were divided into 2 groups consisting eight birds in each group. Nimesulide was administered as single oral bolus dose (2 mg kg-1) in both the groups whereas birds in group II were pretreated with licorice (500mg.Kg^{-1}, orally) 60 min prior to administration of nimesulide. The content of glycerrhizin in licorice varies from 2-25% depending on species and prolonged consumption leads to adverse effects like hypokalemia (Omar *et al.*, 2012). No observed effect level for purified glycyrrhizin was 2mg/kg/day and the oral bioavailability is poor when administered as licorice extract, which has hampered attempts to establish clear dose-effect levels in animals and humans (Isbrucker and Burdock, 2006). In our study licorice extract dose of 500mg.Kg^{-1} was selected to induce maximum effect on pharmacokinetic interaction, if any and no adverse effects were observed at this dose.

Blood samples (0.5 ml) were collected into heparinized tubes by tarsal (either left or right) venipuncture prior to and at 0.166, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h time interval after nimesulide administration. They were centrifuged at 3000 rpm for 5 min and plasma was harvested and stored at -20ºC till analyzed for nimesulide by HPLC.

Assay of nimesulide

Nimesulide concentrations in plasma were determined by high performance liquid chromatography (HPLC) as described by Ptacek *et al.*, (2001) with slight modifications. In brief, acetonitrile was added to plasma sample in the ratio of 1:1 (0.2 mL: 0.2 mL). After vortex mixing at high speed for 20s, the tubes were subjected to centrifugation for 5 min at 8000g. The clear supernatant thus obtained was transferred to a tube. The aliquot was filtered through a 0.22 µm nylon membrane filter and 20 µL of filtrate was injected into the HPLC system.

The HPLC system (Shimadzu Corporation, Kyoto, Japan) comprised of LC-20AD quaternary gradient pump, Rheodyne manual loop injector with a 20µL loop, SPD-20 AV UV-Vis detector, column oven CTO-10ASVP and work station software Labsolutions version 4-0512-039 for data analysis. Separation of nimesulide was performed by using a C18 reverse phase column (4.6 x 250mm, 5µm particle size) as stationary phase and a mixture of 70 parts of acetonitrile and 30 parts of 0.05M ammonium acetate buffer (pH 4.6) as mobile phase at a wavelength of 404 nm. The flow rate was adjusted at 0.8 mL min^{-1}. There were no interfering substances in the plasma at the retention time of nimesulide as evident by the chromatograms obtained for plasma blank and spiked plasma standards. Peak heights were taken for the quantification of nimesulide in plasma from calibration curves obtained on analysis of blank plasma samples spiked with nimesulide (external standards) and analyzed as described for the experimental samples. The limit of quantification for nimesulide was 0.03125µgml^{-1}. The method was found to be linear and reproducible in the concentration range of 0.0625- 2 µg.ml^{-1} nimesulide.

Pharmacokinetic analysis

Plasma concentration versus time data of nimesulide obtained in birds of both groups was utilized for calculating various pharmacokinetic parameters with an interactive least squares linear regression by computer software (PK Solver Version 2.0, 2010 by Zhang Yang). Best-fit model was chosen by using minimal Akaike information criteria estimation (Yamaoka and Nakagawa 1978). Peak plasma concentration (Cmax) and time to reach peak concentration (tmax) were calculated from the actual plasma data of each bird.

Statistical analysis

The plasma concentrations and pharmacokinetic variables of nimesulide are expressed as mean±S.E. Differences in plasma concentrations and pharmacokinetic data between the two groups were analyzed for statistical significance using two samples t-test assuming unequal variances using 'Instat' software. AUC, $t_{1/2}\beta$, C_{max} values were log transformed prior to the analysis. All P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

The plasma levels of nimesulide as a function of time in eight birds after single oral administration of nimesulide (2 mg kg^{-1}) in control group and birds pretreated with licorice group are presented graphically in Fig 1. Various pharmacokinetic parameters estimated by non-compartmental analysis of plasma

concentrations of nimesulide of both groups are summarized in Tables 1.

Nimesulide is an acidic NSAID which showed high anti-inflammatory, antipyretic and analgesic activity and also adverse effects like moderate incidence of gastric side effects, renal and liver toxicity (Biscarini *et al.*, 1988). It undergoes extensive metabolism, primarily by cytochrome P450 isozyme CYP2C9 and CYP3A4 (Vignati *et al.*, 2005). But there is no interaction study between a CYP2C9 and CYP3A4 inhibitor and nimesulide. Many clinical reports indicated that drug biotransformation is compromised in inflammatory responses due to infections that down-regulate cytochrome P450 (Morgan, 1997; Renton, 2001; Slaviero *et al.*, 2003).

Licorice is a sweetening agent used in foods and beverages. A recent study investigated the interaction of licorice with aspirin in animal models and suggested that licorice may reduce stomach irritation as well as the risk of stomach ulcers associated with aspirin (Hawkins and Ehrlich, 2017)

Hence the present study was aimed to investigate the pharmacokinetic interaction of licorice (*Glycyrrhiza glabra* root extract), a CYP2C9 inhibiting flavonoid with nimesulide, a NSAID and CYP2C9 substrate in broiler chicken.

Following oral administration of nimesulide alone (group I) at a dose rate of 2mg.kg^{-1} in birds, initial mean nimesulide plasma concentration of 0.18±0.04 µg.ml^{-1} was observed at 0.166 h. Mean peak plasma levels (C_{max}) of nimesulide observed in this study was 0.75±0.09 µg.ml^{-1} at t_{max} of 1.41±0.16 h after oral administration of nimesulide.

In nimesulide alone treated birds, the elimination rate constant (β) and elimination half life ($t_{1/2}\beta$) observed were 0.16±0.03 h^{-1} and 5.43±1.23 h^{-1} respectively. Villa *et al* (2007) reported almost similar elimination half life ($t_{1/2}\beta$) for nimesulide was reported in horses. However a shorter half life of 4 h in humans (Bernaregigi, 1998) and a longer half-life 20.08±0.79 h in bovine calves (Mahapatra *et al.*, 2009) was reported. The variation in elimination half lives of nimesulide may be due to species difference. So far no pharmacokinetic study of nimesulide in chicken was conducted.

The AUC in pharmacokinetics forms the basis for calculation of some kinetic parameters like MRT, Cl_B, and Vd_{ss}. etc. Mean±SE value of $AUC_{0-\infty}$ obtained in the present study for nimesulide alone group was 3.06±0.59 µg.h.ml^{-1} after single oral administration of nimesulide (2mg.kg^{-1}). Comparatively higher AUC value of 31.75±7.21 µg.h.ml^{-1} for nimesulide was reported in horses when given at a dose rate of 1 mg.kg^{-1} (Villa *et al.*, 2007). In the above study the variation in AUC might be due to species variation and also with lesser dose administration.

Vd_{ss} provides an estimate of drug distribution to various peripheral tissues in the body after absorption and is independent of elimination process. In the present study, the Vd_{ss} obtained for nimesulide group I birds was 2.47±0.38 L.kg^{-1} whereas lower Vd_{ss} of 0.22 L.kg^{-1} was reported in bovine calves (Mahapatra *et al.*, 2009).

Clearance (Cl_B) is a characteristic of the drug and indicates volume of plasma cleared of the drug by various elimination processes per unit time. The clearance obtained in the present study was 0.88±0.19 L.kg^{-1}.h $^{-1}$.

Mean residence time (MRT) is the mean time required for a drug molecule to transverse through the body and thus reflects time associated with absorption, distribution and elimination. In the present study, MRT values obtained for nimesulide in group I was 3.25±0.44 h.

Effect of licorice pre-treatment on the plasma levels and pharmacokinetics of nimesulide was investigated. The initial nimesulide plasma concentration of 0.02±0.02 µg.ml^{-1} was observed at 0.166 h, this value is lesser compared to the initial plasma concentration of 0.18±0.04 µg.ml^{-1} obtained in the group I, however it is not significantly lesser. The peak plasma concentration (Cmax) of nimesulide was 0.75±0.15 µg.ml^{-1} which is almost similar to the value obtained in the nimesulide alone group 0.75±0.09 µg.ml^{-1}.

It appears that licorice pre-treatment has not altered the nimesulide concentration and hence the Cmax for nimesulide alone and nimesulide in licorice pretreated group were almost similar. The dose of licorice may not be sufficient to induce the change. There were

Table 1:
Pharmacokinetic parameters (Mean ±SEM) of nimesulide in different groups of birds (n = 8)

Parameter	Unit	Group I (Nimesulide)	Group II (Nimesulide+ Licorice extract pre-treatment)
β	h^{-1}	0.16±0.03	0.12±0.03
$t_{1/2}\beta$	h	5.43±1.23	7.82±1.69
AUC_{0-t}	µg.h.ml^{-1}	3.06±0.59	3.54±0.80
$AUC_{0-\infty}$	µg.h.ml^{-1}	3.06±0.59	3.78±1.00
AUC_{0-t}/ $AUC_{0-\infty}$	Per cent	100±0.00	97.33±2.67
$AUMC_{0-t}$	µg.h^2.ml^{-1}	11.41±3.43	16.56±6.53
$AUMC_{0-\infty}$	µg.h^2.ml^{-1}	11.41±3.43	27.21±17.02
MRT	h	3.25±0.44	4.09±0.63
Vd_{ss}	L.kg^{-1}	2.47±0.38	2.76±0.51
Cl_B	L.kg^{-1}.h^{-1}	0.88±0.19	0.74±0.14
C_{max}	µg.ml^{-1}	0.75±0.09	0.75±0.15
t_{max}	h	1.41±0.16	3.21±0.34

Values are mean ±SEM (n=8), Unpaired t- test using 'Instat' software. Means without any superscripts in a column do not differ significantly (P>0.05)

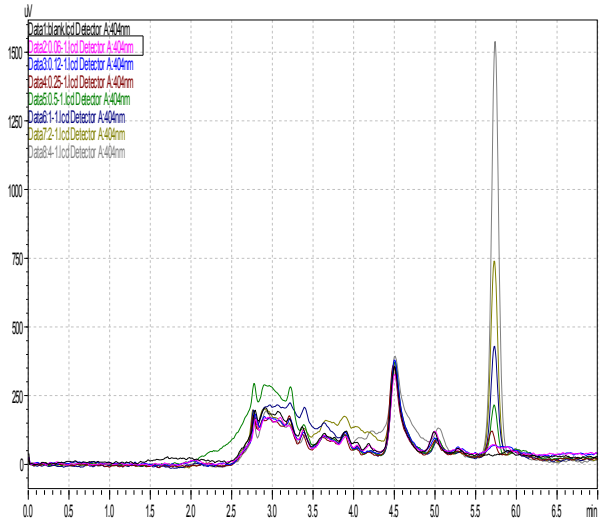

Fig. 1:
Chromatogram of nimesulide standard in plasma.

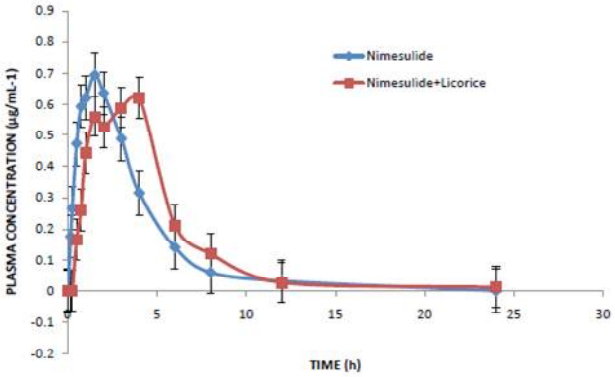

Fig. 2:
Semi logarithmic plot of nimesulide concentration in plasma versus time after single oral administration of nimesulide in different groups of birds (n = 8).

no significant differences in elimination half-lives ($t_{1/2\beta}$), area under curve ($AUC_{0-\infty}$), and mean residence time (MRT) obtained for nimesulide alone and nimesulide in licorice pretreated group. However, morin, a CYP2C9 and CYP3A4 inhibiting polyphenolic compound decreased Cmax of diltiazem when it was co administered with diltiazem at a dose rate of 15mg.kg^{-1} in rats (Chon and Han, 2005).

The results obtained suggest that there were no pharmacokinetic interaction between licorice and nimesulide in chicken when licorice was given 60 min prior to the administration of nimesulide in a single dose. There were no significant differences in volume of distribution at steady state (Vd_{ss}) and the total body clearance (Cl_B) of nimesulide alone and nimesulide in licorice pretreated group.

Licorice, a CYP2C9 and CYP3A4 inhibitor (Kent *et al.*, 2002), did not significantly alter the important pharmacokinetic parameters like elimination half-life and clearance of nimesulide. However there are reports that glycyrrhetinic acid and glycyrrhizin altered the pharmacokinetic parameters of other CYP3A4 substrates like cyclosporine A (Hou *et al.*, 2012; Huang *et al.*, 2008). Probably the experimental design made in the present study had no scope to detect such alterations because of administration of licorice in single dose at 60 min prior to the administration of nimesulide. Although a non-significant increase of C_{max} and AUC of nimesulide alone treatment was observed, present study had no scope to detect alterations in the pharmacokinetic parameters between the groups because of administration of licorice in single dose. It is, thus, concluded that the licorice, a putative inhibitor of CYP2C9 and CYP3A4 has no effect on the activity of these enzymes in birds Hence further studies are required with higher concentrations/prolonged treatment of licorice to study the pharmacokinetic interaction of nimesulide with licorice.

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Research Article

EFFECT OF MULTIPLE ORAL ADMINISTRATIONS OF CIPROFLOXACIN ON HAEMATO-BIOCHEMICAL PARAMETERS IN BROILER CHICKENS

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ABSTRACT

Ciprofloxacin is a clinically popular second generation fluoroquinolone and one of the cheapest prescribed antimicrobial drugs in veterinary and human medicine, having broad spectrum activity with great efficacy against aerobic Gram-negative bacteria especially the *Enterobacteriaceae*. The present study was planned with an aim to evaluate the safety of multiple oral administrations of ciprofloxacin in broiler chickens based evaluation on haematological and biochemical parameters. Ciprofloxacin was repeatedly administered in eight broiler chickens *via* oral route at the dose rate of 10 mg/kg, once in a day, for five days. Blood was collected at 0 day (for control sample before drug administration) and at 6 h, 12 h, 24 h, 48 h, 72 h, 96 h , 120 h, 168 h and 240 h. Hematological parameters including haemoglobin concentration (Hb), packed cell volume (PCV), total leukocyte count (TLC), total erythrocyte count (TEC) and differential leukocyte counts (DLC), and plasma biochemical parameters *viz*. alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), acid phosphatase (ACP), creatine kinase (CK), blood glucose, total bilirubin (T-BIL), creatinine, blood urea nitrogen (BUN), total protein and albumin were estimated before first dosing (0 h; as a control) and at various time points post dosing of drugs. Based on haematological and biochemical evaluations and clinical appreciation, ciprofloxacin was found safe to use at the dose of 10 mg/kg repeated at 24 h for five consecutive days.

Key Words: Ciprofloxacin, oral route, safety assessment, broiler chicken

INTRODUCTION

The fluoroquinolones are a series of synthetic antibacterial agents that are used in the treatment of a variety of bacterial infections. They are the broad spectrum antimicrobial agents having concentration-dependent bactericidal activity with low minimum inhibitory concentrations (MICs) against susceptible micro-organisms, and have pharmacokinetic characteristics like large volume of distribution and low plasma protein binding (Brown, 1996). Ciprofloxacin, a second generation fluoroquinolone has a good oral bioavailability, good to excellent tissue penetration and is considered relatively safe. It rapidly blocks bacterial DNA replication by inhibiting DNA gyrase, an essential prokaryotic enzyme that catalyzes chromosomal DNA supercoiling (Fisher *et al.*, 1989). Fluoroquinolones that are approved for humans and are of potential interest for veterinary medicine include ciprofloxacin (Papich, 2018).

Ciprofloxacin is an active drug against both Gram-negative and Gram-positive bacteria including pseudomonal and staphylococcal species. Among second-generation compounds, ciprofloxacin exhibits the strongest sensitivity against Gram negative bacteria particularly against *Enterobacteriaceae* (Greenwood, 2008). Thus, ciprofloxacin is promising drug in treating mixed infections in broiler chickens having involvement of *Escherichia coli* and *Salmonella* species as secondary infection. The reports on safety of ciprofloxacin in broiler chickens based on haemato-biochemical or histopathological evaluations at therapeutic dosage is not available, instead such reports for other fluoroquinolones (Devada *et al.*, 2012) are available. Safety reports of ciprofloxacin, based on studies on joint cartilages and evaluation of haemato-biochemical parameters, are also reported in cow calves (Bhavsar *et al.*, 2003) and buffalo calves (Sarvaiya *et al.*, 2006). However, such reports are not available for broiler chickens. Thus, the present investigation was carried out to assess the safety of ciprofloxacin in broiler chickens after its multiple oral administrations at the dose rate of 10 mg/Kg body weight once a day, for five consecutive days.

MATERIALS AND METHODS

Experimental birds

The animal experimentation protocol of present study was prior approved by the Institutional Animal Ethics Committee (IAEC) of College of Veterinary Science and Animal Husbandry (CVSc & AH), Sardarkrushinagar Dantiwada Agricultural University (SDAU), Sardarkrushinagar, Gujarat, India. Eight male broiler chickens of Vencobb strain aged from 4 to 6 weeks were procured from Department of Livestock Production and Management (LPM), CVSc & AH, SDAU, Sardarkrushinagar and were kept under observation for 14 days before dosing for experiment. During this acclimatization period, all the experimental birds were kept under constant observation to preclude existence

of any diseases or abnormality. All the standard management procedures were followed as per CPCSEA guidelines for taking care of birds and to avoid any kind of discomfort and stress to them during the experimental period. The birds were kept in labelled individual cages for better identification and easy restraining purposes.

Drug and chemicals

Ciprofloxacin hydrochloride powder of I.P. grade (Indian Pharmacopoeia) was obtained from Shantam Pharmaceuticals Pvt. Ltd., Gandhinagar, Gujarat, as a gift sample. Ready to use *in vitro* diagnostic kits for all biochemical parameters were purchased from Agappe Diagnostic Ltd, Ernakulam, Kerala, India, except acid phosphatase kit which was procured from Crest Biosystems, Goa, India.

Experimental design and sample collection

For oral administrations, fine powder of ciprofloxacin HCl (I.P. grade) was dissolved in sterile water as a vehicle, to the concentration of 20 mg/ml and oral dosing was done using clean and sterile curved gavage feeding needle (14 G). Blood samples from treated birds were collected from wing veins into K_3EDTA (tripotassium ethylene diamine tetraacetic acid) blood collection tubes (2 ml capacity) for haematological analyses and into clean dried heparinized micro-centrifuge tubes (2 ml capacity) for plasma biochemical analysis. Blood was collected at 0 day (for control sample before drug administration) and at 6 h, 12 h, 24 h (day 1), 48 h (day 2), 72 h (day 3), 96 h (day 4), 120 h (day 5), 168 h (day 7) and 240 h (day 10). For haematological parameters, 0.5 to 1.0 ml blood was collected, whereas 1.0 to 1.5 ml blood was collected to harvest plasma needed for analyzing biochemical parameters. Plasma was separated after refrigerated centrifugation of blood samples (4000 RPM, 10 minutes, 4^{o}C). The plasma samples were transferred to cryovials (2 ml capacity) and stored at -20^{o}C and assayed for biochemical parameters on same day.

Haemato-biochemical analysis

All the biochemical parameters (except for acid phosphatase) were estimated from harvested plasma samples by RX Monaco, a fully-automated bench-top clinical chemistry analyser (Randox Laboratories Ltd., United Kingdom). Acid phosphatase was analysed by semi-automatic bio-chemistry analyser (RX-50V, Microlab, India).

Haematological parameters viz. Haemoglobin (Hb), Packed Cell Volume (PCV), Total leukocyte count (TLC), Total erythrocyte count (TEC) and Differential leukocyte count (DLC) for whole blood of poultry were analyzed manually as per standard methods described (Bernard *et al.*, 2000; Samour, 2006).

Statistical analysis

Safety data generated after haematological and plasma biochemical analyses were compared for 'mean values before drug administration' and 'mean values after drug administration' by 'paired sample t-test' using SPSS Statistics version 19 software.

RESULTS

The safety results of repeated oral dosing of ciprofloxacin based on evaluation of hematological and biochemical parameters are summarized in Table 1 and Table 2, respectively.

There were significant differences in the values of hematological parameters like PCV, TLC, TEC and heterophil between pre-dosing (0 day or control) and post-dosing observations from samples collected on different time period. However, other parameters like Hb, lymphocyte, monocyte and eosinophil counts did not show such significant alteration before and after drug administration. (Table 1).

No significant difference was noted in the mean values of all blood biochemical parameters except glucose and albumin. The mean values of glucose on 1st and 2nd day ($p \leq 0.01$) while mean values of albumin on 2nd day ($p \leq 0.01$) and 5th day ($p \leq 0.05$) were significantly differed from their respective mean values observed at 0 day (Table 2).

DISCUSSION

Generally, fluoroquinolones are considered to be relatively safe, compared with other classes of commonly used antimicrobial agents (Andriole, 2005). Most reactions are considered to be minor and reversible upon discontinuing treatment. Fluoroquinolone toxicities are, for the most part, dose and animal species dependent (Bertino and Fish, 2000). In veterinary species, reported toxicities include gastrointestinal disturbances (such as nausea, vomiting and diarrhoea), arthropathies in young animals (especially dogs), and ocular toxicities including retinal degeneration in cats by enrofloxacin (Martinez *et al.*, 2006).

In the present study, significant differences in the values of PCV, TLC, TEC and heterophil count was observed. Heterophil count showed only transient change at 24 h. The values of PCV, TLC and TEC were significantly increased at many time points but returned to normal range. The values of PCV (on 2nd and 3rd day at $p \leq 0.05$; and on 4th, 5th, 7th and 10th day at $p \leq 0.01$), TLC (on 4th day at $p \leq 0.05$; and on 5th, 7th and 10th day at $p \leq 0.01$), TEC (on 12 h, 7th and 10th day at $p \leq 0.05$; and on 2nd and 5th day at $p \leq 0.01$), and heterophil count (on 1st day at $p \leq 0.05$) were found to be significantly different from pre-treatment values of respective parameters observed on 0 day (Table 1). The pattern of increase in TLC and TEC is probably due to advancing age (Samour, 2006). Other parameters like Hb, lymphocyte, monocyte

and eosinophil counts did not show such significant changes.

No significant difference was noted in the mean values of all blood biochemical parameters except glucose and albumin estimated before and after repeated oral administration of ciprofloxacin. Decrease in blood glucose content was observed on 1st and 2nd day but degree of hypoglycemia was mild and transient in nature as levels returns to statistically insignificant values at 3rd day and onwards. Drug induced hypoglycemia is an established fact associated with the use of fluoroquinolones in human beings, but in comparison to newer members of fluoroquinolones, ciprofloxacin causes hypoglycemia of very low severity and in low frequency (Murad *et al.*, 2009). In present study, pre-dose normal value of glucose ranged from 237 to 275 mg/dl. Thus, the fluctuated blood glucose values were well within the normal range. Decrease in albumin was temporary and disappeared once drug was withheld after course of treatment.

Enrofloxacin when administered in healthy broiler chickens at the recommended therapeutic dose 10 mg/kg body weight via drinking water for five successive days, caused a significant increase ($p<0.05$) in gamma glutamyl transferase, uric acid and creatinine levels but during the withdrawal period, the elevated levels declined gradually and differences became statistically insignificant (Sureshkumar *et al.*, 2013). Thus, fluoroquinolones impact on biochemical parameters showed reversible trend once

Table 1
Effect of multiple dose oral administrations of ciprofloxacin, repeated every 24 h for five days at the dose of 10 mg/kg body weight, on hematological parameters (mean±SE) in broiler chickens (n=8)

Parameters	Unit	Time of Collection (Values of parameters are represented as Mean±SE)								
		0 h	6 h	12 h	24 h	48 h	72 h	96 h	120 h	168 h	240 h
Hb	g/dl	11.76±0.26	11.61±0.27	11.76±0.32	11.59±0.33	11.58±0.33	11.85±0.30	12.00±0.31	11.93±0.30	11.81±0.26	11.89±0.30
PCV	%	34.98±0.96	35.15±0.83	35.76±0.85	35.60±0.96	35.95±0.67*	35.98±0.64*	36.64±0.90**	36.24±0.76**	36.51±0.70**	36.24±0.84**
TLC	10^3/ cu mm	23.93±0.60	23.91±0.50	24.33±0.40	24.15±0.29	24.36±0.39	24.40±0.42	24.74±0.42*	25.11±0.36**	25.25±0.49**	25.30±0.46**
TEC	10^6/ cu mm	2.47±0.06	2.54±0.07	2.60±0.07*	2.60±0.08	2.69±0.03**	2.70±0.11	2.66±0.08	2.78±0.09**	2.80±0.10*	2.85±0.07*
Heterophils	%	25.88±0.79	26.50±0.60	26.50±0.96	26.63±1.12*	24.75±0.49	25.38±1.22	26.38±1.24	25.13±0.64	26.13±0.95	25.50±0.94
Lymphocytes	%	65.88±1.33	65.88±0.69	64.88±1.46	65.75±1.67	67.50±0.96	67.88±1.74	66.38±1.46	67.25±1.25	65.25±1.44	66.25±1.56
Monocytes	%	5.38±0.92	5.13±0.74	5.63±0.96	5.00±0.57	4.63±0.60	4.25±0.62	4.75±0.77	4.13±0.58	5.13±0.77	5.13±0.81
Eosinophils	%	2.88±0.40	2.50±0.60	3.00±0.27	2.63±0.26	3.13±0.40	2.50±0.42	2.50±0.46	3.50±0.42	3.50±0.53	3.13±0.30

*Values having * and ** superscript in same raw indicate significant difference at $p \leq 0.05$ and $p \leq 0.01$, respectively*

Table 2
Effect of multiple dose oral administrations of ciprofloxacin, repeated every 24 h for five days at the dose of 10 mg/kg body weight, on plasma biochemical parameters (mean±SE) in broiler chickens (n=8)

Parameter	Unit	Time of Collection (Values of parameters are represented as Mean±SE)									
		0 h	6 h	12 h	24 h	48 h	72 h	96 h	120 h	168 h	240 h
ALT	U/L	7.25±0.36	6.78±0.31	6.99±0.35	7.55±0.55	7.20±0.36	6.90±0.18	7.21±0.31	7.90±0.47	7.36±0.32	7.48±0.37
AST	U/L	192.61±4.31	197.23±4.91	194.81±3.68	197.46±4.13	194.03±4.80	192.81±2.67	196.30±4.52	189.91±4.21	191.09±2.43	190.56±3.53
ALP	U/L	694.74±34.34	696.35±31.58	694.48±33.12	699.61±30.85	701.69±32.76	694.84±35.76	696.90±32.45	696.61±32.86	700.03±34.14	694.53±32.52
ACP	U/L	3.68±0.12	3.68±0.15	3.69±0.16	3.64±0.15	3.65±0.15	3.73±0.14	3.71±0.15	3.69±0.14	3.74±0.11	3.70±0.12
CK	U/L	1214.30±39.96	1212.01±42.37	1216.56±44.67	1212.73±39.49	1221.39±38.90	1219.54±43.52	1218.16±40.76	1216.99±43.93	1214.45±44.14	1215.61±44.86
Blood glucose	mg/dl	261.25±4.29	253.75±6.55	253.00±4.46	252.13±5.38**	247.88±3.32**	255.00±5.80	251.00±5.01	254.00±4.41	258.38±4.75	261.13±5.02
Total Bilirubin	mg/dl	0.12±0.01	0.11±0.00	0.11±0.00	0.11±0.01	0.12±0.01	0.12±0.01	0.12±0.01	0.12±0.01	0.11±0.01	0.11±0.01
Creatinine	mg/dl	0.16±0.01	0.17±0.01	0.16±0.01	0.15±0.01	0.16±0.01	0.16±0.01	0.16±0.01	0.17±0.01	0.15±0.01	0.16±0.01
BUN	mg/dl	1.55±0.15	1.54±0.16	1.53±0.16	1.54±0.14	1.53±0.16	1.53±0.18	1.51±0.17	1.48±0.13	1.49±0.15	1.56±0.13
Total Protein	g/dl	3.85±0.09	3.83±0.09	3.81±0.12	3.90±0.08	3.89±0.06	3.91±0.07	3.85±0.11	3.79±0.09	3.91±0.08	3.93±0.08
Albumin	g/dl	1.44±0.04	1.39±0.04	1.40±0.04	1.41±0.04	1.35±0.03**	1.44±0.03	1.40±0.04	1.38±0.04*	1.41±0.02	1.41±0.04

*Values having * and ** superscript in same raw indicate significant difference at $p \leq 0.05$ and $p \leq 0.01$, respectively*

the drug is withdrawn after treatment done within therapeutic regimens.

Gatifloxacin with same dosage regimen in broiler chickens was also found safe based on hematological, biochemical and histopathological evaluations (Devada *et al.*, 2012).

Ciprofloxacin was found safe in cow calves following multiple dose intravenous administration at the therapeutic dose of 5 mg/kg body weight, as no alterations were found in joint cartilage (Bhavsar *et al.*, 2003). Sarvaiya *et al.* (2006) also found the ciprofloxacin to be safe in buffalo calves at same therapeutic dosage regimen based on evaluation of haemato-biochemical parameters. Arthropathy of weight bearing joints is a common toxic effect of long term fluoroquinolone therapy in some animal species particularly in young ones. Histopathological study of joint cartilage is not attempted in the present study as the therapeutic dosage regimen was followed for only five days, similar to that mostly observed in field conditions.

Results of the present study showed that none of the broiler birds under safety assessment had any gross deformity or abnormalities in the gait or pain at joints throughout the period of experiment. The change in the values of some haematological and biochemical parameters, if any in the present safety study, were not beyond normal ranges after multiple dosing of ciprofloxacin in broiler chickens. The health status of ciprofloxacin treated broiler chickens was found to be good enough, when adjudged by normal behaviour, growth rate and absence of any clinical signs and symptoms. Thus, based on haematological, biochemical and clinical appreciation, it is safe to use ciprofloxacin at the dose of 10 mg/kg, once in a day, for five consecutive days to treat susceptible bacterial infections in broiler chickens.

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Research Article

TOXICODYNAMIC INTERACTIONS OF IMIDACLOPRID WITH ANTIEPILEPTICS IN MICE – AN INSIGHT INTO ITS MECHANISM OF ACTION

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ASTRACT

The toxicodynamic interactions of imidacloprid with some antiepileptic agents were studiedto investigate about its possible mechanism of action in mice. Imidacloprid prolonged the sleeping time induced by pentobarbitone and hexabarbitone, whichindicates the inhibition of hepatic metabolising enzymes involved in their metabolism by imidacloprid.Prior administration of phenytoin reduced restlessness, diazepam reduced restlessness and tremors induced by imidacloprid. Carbamazepine administered prior to imidacloprid antagonised all the characteristic behavioural symptoms induced by imidacloprid. These results suggested possibility of involvement of GABAergic system and a direct excitatory action at level of neuromuscular junction by imidacloprid.

Key words: Imidacloprid;Toxicodynamic interactions; Experimentally induced convulsions.

INTRODUCTION

Pesticides are chemical compounds including different insecticides, fungicides, rodenticides, herbicides etc. Excessive and wide spread use of pesticides cause environmental pollution (Koller, 1979) and potential health hazards. So all living beings are directly/indirectly exposed to pesticide residues found in animal or plant products, environment etc. (Dahiya and Chauhan, 1982, Agnihotri *et al.*, 1987, Ram *et al.*, 1987, Kaushik*et al.*, 1991, Kannan *et al.*, 1992). Being toxic for pests, pesticides have an inherent degree of toxicity for other living organisms also because of similar physiological and biochemical reactions. So their excess or continuous use even at recommended level has led to health problems in animals and human beings.

The course for development of pesticides showing selective toxicity against pests and little/no effects on animal and human beings has led, especially in last 2-3 decades, to the discovery of a new group of nitroguanidine insecticides having broad spectrum of activity. Imidacloprid, a neonicotinoid from this group is one such compound which is experimentally used and tried extensively mainly for sucking insects viz. aphids, leaf hoppers, leaf miner, beetles etc. (Elbert *et al.*, 1990, Schmeer*et al.*, 1990, Abbink, 1991, Altmann, 1991, Pfluger and Schmuck, 1991).The compound has found its application against termites, soil insects and some biting insects like Colorado beetle, Weevil and also it is being used for seed dressing, soil treatment and foliar treatment. It also has topical application to treat adults and larvae of fleas in canines and felines in some countries like USA. The compound is available in many trade names like Admire, Confidor, Merit, Premier, Premise, Provado etc.

The present investigation was, therefore, undertaken to study the toxicodynamic interactions of imidacloprid with various anti-epileptic agents. Such studies will help in better understanding of toxic mechanisms, developing remedial measures against toxicity and assessing safety evaluation for wide spread use of imidacloprid.

MATERIALS AND METHODS

Experimental animals

Swiss albino male mice weighing 20-25g were procured from Disease Free Small Animal Houseof the University and housed in the Departmental Small Animal House in polyacrylic cages kept at room temperature with a natural light-dark cycle. The animals were acclimatized to laboratory conditions for 3-4 days before the experiments were conducted. The animals were provided feed and water *ad libitum.*The feed but not water was withdrawn at scheduled time before and during experiments unless it was otherwise specifically stated for a particular parameter. All experiments were conducted between 9:00 a.m. and 5:00 p.m. in noise free laboratory conditions. The prior approval of Institutional Animal Ethics Committee for the protocol of this study was obtained and formulation of imidacloprid (Confidor, 200 SL) was used for this study.

For each experiment, mice were randomly divided in three groups of 5 animals each. The control group was administered 10ml/kg of gum acacia solution (2%) and treatment groups received 22mg/kg or 44mg/kg ($2/5^{th}$ or $4/5^{th}$ of MTD) of imidacloprid intraperitoneally. Assessment of interactions of imidacloprid with

antiepileptic agents

Pentobarbitone or hexabarbitone induced hypnosis

Hypnosis was induced by pentobarbitone (40 mg/kg, i.p.) or hexabarbitone (180 mg/kg, i.p.) in mice. The criterion for hypnosis was the loss of righting reflex i.e. when animal could not correct its posture at least three times within 15 seconds when placed on its back. Animals treated with pentobarbitone or hexabarbitone were placed on their backs as soon as they lost the righting reflex. Time was noted when they spontaneously corrected their posture. Criterion for awakening i.e. regaining of righting reflex was the correction of posture at least three times within fifteen seconds, when they were placed on their backs. The interval between the loss of righting reflex and the awakening of animal was taken as sleeping time (duration of sleep) which was recorded in min. Treatment of animals in each group was done 30minutes before administration of pentobarbitone or hexabarbitone. Time taken for induction and duration of sleep was noted. Mortality, if any, was recorded.

Interaction with fluoxetine

Mice were kept off feed for 3h before experimentation. The animals in three groups were exposed to 22mg/kg or 44mg/kg (2/5th or 4/5th of MTD) or 55mg/kg (MTD) of imidacloprid by intraperitoneal route 7h after administering fluoxetine (15mg/kg, p.o.). Animals were observed for some characteristic behavioural symptoms induced by imidacloprid treatment (these symptoms included tremors, respiratory rate, body posture and limb position, restlessness and flexion ofhead). Scoring of these symptoms was done as per Irwin schedule (as described by Turner, 1965) and these characteristic symptoms were noted at interval of 15, 30 and 45 min after administration of imidacloprid.

Interaction with diazepam

Mice were kept off feed for 12h before study. The animals in three groups received 22mg/kg or 44mg/kg (2/5th or 4/5th of MTD) or 55mg/kg (MTD) of imidacloprid by intraperitoneal route given ½h later to administration of diazepam (2.5mg/kg, i.p.). Scoring of some characteristic behavioural symptoms induced by imidacloprid treatment in these animals (as mentioned earlier) was done as per Irwin schedule (as described by Turner, 1965) at 15, 30 and 45 min after imidacloprid administration.

Interaction with carbamazepine

The feed was withdrawn from animals 3h before conducting the study. All the animals were administered carbamazepine at the rate of 50 mg/kg, p.o. Four hours later these three groups received 22mg/kg or 44mg/kg (2/5th or 4/5th of MTD) or 55mg/kg (MTD) of imidacloprid intraperitoneally. Some characteristic behavioural symptoms induced by imidacloprid treatment in these animals (as mentioned earlier) were noted at interval of 15, 30, and 45 min after administration of these doses of imidacloprid. Scores were assigned to these symptoms as per Irwin schedule (as described by Turner, 1965).

Interaction with phenytoin

Animals were kept off-feed for 12h before exposure to test drug. One hour after administration of phenytoin (10mg/kg, i.p.) animals in these groups received 22mg/kg or 44mg/kg (2/5th or 4/5th of MTD) or 55mg/kg (MTD) of imidacloprid by intraperitoneal route. All the animals were observed for some characteristic behavioural symptoms induced by direct imidacloprid treatment (as mentioned earlier).These symptoms were noted at 15, 30 and 45 min after administration of imidacloprid. Scores were assigned to different symptoms as per Irwin schedule (as described by Turner, 1965).

Statistical analysis

Data was analysed by one wayANOVA followedby Dunnett'smultiple comparison test.P values<0.05 were considered statistically significant.

RESULTS

Assessment of interactions of imidacloprid with antiepileptic agents

Assessment of effect on pentobarbitoneor hexabarbitoneinduced hypnosis

Imidacloprid significantly enhanced the duration of hypnosis induced by pentobarbitone (Table 1). At higher dose there was 20% mortality. The mortality occurred during hypnosis.Early onset of hypnosis induced byhexabarbitoneand increase in sleeping time was seen and at higher dose 33% mortality was recorded (Table 2).

Assessment of interaction with fluoxetine

Characteristic behavioural symptoms except head flexion did not seem to be affected by fluoxetine. The number of animals showing flexion of head at 44 mg/kg and 55 mg/kg of imidacloprid were reduced to almost half by prior administration of fluoxetine (Table 3).

Assessment of interaction with diazepam and carbamazepine

Diazepam antagonized the restlessness and tremors induced by imidacloprid at 44 mg/kg and 55 mg/kg dose levels(Table 4). Carbamazepine showed antagonistic effect on all the behavioural profiles produced by imidacloprid at different dose levels (Table 5).

Assessment of interaction with phenytoin

Phenytoin significantly reduced the restlessness and head flexion induced by imidacloprid at 44 and 55 mg/kg (Table 6). Phenytoin did not show any effect on the respiratory rate, body posture and limb position and tremors induced by imidacloprid.

DISCUSSION

Seizure is characteristic feature associated with disordered and rhythmic high frequency discharge of impulse by a group of neurons in brain and status epilepticus is characterized by repeated episodes of epilepsy without the patient having recovered from previous attack.

Effect on interactions of imidacloprid with antiepileptic agents

Imidacloprid increased the duration of pentobarbitone and hexabarbitone induced hypnosis at both the dose levels and produced mortality at higher dose only. Similar increase in pentobarbitone induced sleeping time by imidacloprid and isoproturon has beenreported by Premlata (2001) and Sarkar (1990) respectively. Prolonged barbiturate induced sleeping time can be due to effect on brain permeability (Paul *et al.*, 1957) or inhibition of barbiturate metabolizing enzymes (Murphy and Dubois, 1956). Moreover Stevens *et al.* (1972) suggested that impaired metabolism of hexabarbital and not the altered sensitivity of brain to barbiturateis the cause for increased hexabarbital sleeping time. Since imidacloprid prolongs the sleeping time induced by both the barbiturates, it indicates the inhibition of hepatic metabolising enzymes by imidacloprid.

Fluoxetine did not alter the imidacloprid induced behaviour in mice. This suggests that probably there is no involvement of serotonergic mechanism in imidacloprid induced behaviour.Phenytoin antagonized restlessness, diazepam antagonised restlessness and tremors induced by imidacloprid, while carbamazepine antagonized all the imidacloprid induced characteristic behavioural symptoms.

Phenytoin produces its action by stabilization of neuronal membrane, thus prolonging recovery of inactivated Na^+ channels. Thus it limits the repetitive firing of action potentials evoked by sustained depolarization (McLean and McDonald, 1983). Carbamazepine also acts in similar manner. However at higher concentration phenytoin enhances responses to GABA also (McLean and McDonald, 1986). This study showed that carbamazepine antagonized the effects of imidacloprid, whereas phenytoin showed no effect on tremors, body posture and limb position, respiratory rate, and head flexion. Hence effect of imidacloprid on neuronal membrane can be ruled out. However the possibility of involvement of GABA receptors cannot be ruled out. Diazepam has the ability to enhance GABA mediated synaptic inhibition (Picrotoxin acts reverse to it and it decreases GABA mediated synaptic inhibition). Diazepam antagonized imidacloprid induced tremors and

Table 1:
Effect of imidacloprid on pentobarbitone (40 mg/kg, i.p.) induced hypnosis in mice

Dose (mg/kg, i.p.)	Time of onset of sleep (min)	Duration of sleep (min)	% mortality
Control	4.8±0.37	26.0±3.66^A	0
22	4.8±0.37	42.6±1.36^B	0
44	5.2±1.01	49.8±11.20^B	20

Values are mean ± SEM of five observations. Means bearing different superscripts differ significantly (P<0.05).

Table 2:
Effect of imidacloprid on hexabarbitone (180 mg/kg, i.p.) induced hypnosis in mice

Dose (mg/kg, i.p.)	Time of onset of sleep (min)	Duration of sleep (min)	% Mortality
Control	39.5±6.46	85.1±14.12	0
22	31.2±2.28	116.7±17.90	0
44	24.7±4.00	119.4±31.60	33

Values are mean ± SEM of five observations. Means bearing different superscripts differ significantly (P<0.05).

Table 3:
Effect of fluoxetine (15 mg/kg, p.o.) on some characteristic behavioral symptoms induced by imidacloprid in mice

Dose(mg/kg, i.p.)	Score for severity of symptoms at different time intervals (min)														
	Restlessness (0)			Respiratory rate (4)			Body posture and limb position (4)			Tremors (0)			Presence of flexion of head(+)		
	15	30	45	15	30	45	15	30	45	15	30	45	15	30	45
Imidacloprid 22	0.2 ±0.25	0 ±0.00	0 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	3.7 ±0.25	4 ±0.00	4 ±0.00	0 ±0.00	0 ±0.00	0 ±0.00	-	-	-
Fluoxetine +imidacloprid 22	0 ±0.00	0 ±0.00	0 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	0 ±0.00	0 ±0.00	0 ±0.00	-	-	-
Imidacloprid 44	1.4 ±0.40	1.8 ±0.48	1.8 ±0.48	3.4 ±0.24	3.6 ±0.4	3.4 ±0.24	3.2 ±0.20	3 ±0.31	3.2 ±0.48	0.4 ±0.40	0.8 ±0.73	1.2 ±0.00	5	5	4
Fluoxetine +imidacloprid 44	2 ±0.00	1.2 ±0.48	0.6 ±0.40	3.4 ±0.24	3.2 ±0.20	3.8 ±0.20	3 ±0.00	3 ±0.00	3.4 ±0.40	1.6 ±0.40	1.2 ±0.20	0.8 ±0.00	2	2	2
Imidacloprid 55	2 ±0.00	2.6 ±0.24^A	2.8 ±0.20	3.4 ±0.40	4.2 ±0.48	5 ±0.00^A	2.8 ±0.20	2 ±0.00	2 ±0.00	1.2 ±0.20	1.6 ±0.00	2 ±0.00	5	5	5
Fluoxetine +imidacloprid 55	2 ±0.00	0.7 ±0.25^B	2.3 ±0.33	3.4 ±0.24	2.7 ±0.47	3.3 ±0.33^B	3 ±0.30	2.7 ±0.47	2.6 ±0.33	1.4 ±0.40	1.5 ±0.00	1 ±0.00	3	3	3

Values are mean ±SEM of observations of five animals. Means bearing different superscripts differ significantly (P<0.05).

Table 4:
Effect of diazepam (2.5mg/kg, i.p.) on some characteristic behavioral symptoms induced by imidacloprid in mice

Dose(mg/kg, i.p.)	Score for severity of symptoms at different time intervals (min)														
	Restlessness (0)			Respiratory rate (4)			Body posture and limb position (4)			Tremors (0)			Presence of flexion of head(+)		
	15	30	45	15	30	45	15	30	45	15	30	45	15	30	45
Imidacloprid 22	0.2 ±0.25	0 ±0.00	0 ±0.00	4 ±0.00^{A}	4 ±0.00	4 ±0.00	3.7 ±0.25^{A}	4 ±0.00	4 ±0.00	0 ±0.00	0 ±0.00	0 ±0.00	-	-	-
Diazepam+ 22 imidacloprid	0.2 ±0.20	0 ±0.00	0 ±0.00	3.2 ±0.20^{B}	3.8 ±0.20	3.6 ±0.24	2.8 ±0.20^{B}	3.4 ±0.40	4 ±0.00	0 ±0.00	0 ±0.00	0 ±0.00	4	1	1
Imidacloprid 44	1.4 ±0.40	1.8 ±0.48	1.8 ±0.48^{A}	3.4 ±0.24	3.6 ±0.40^{A}	3.4 ±0.24	3.2 ±0.20	3 ±0.31	3.2 ±0.48	0.4 ±0.4	0.8 ±0.48	1.2 ±0.73	5	5	4
Diazepam+ imidacloprid 44	1.2 ±0.37	0.6 ±0.24	0 ±0.00^{B}	3 ±0.31	2.2 ±0.20^{B}	2.8 ±0.37	3 ±0.00	2.4 ±0.40	1.2 ±0.73	0.2 ±0.2	0.8 ±2.0	0 ±0.00	3	5	3
Imidacloprid 55	2 ±0.00	2.6 ±0.24^{A}	2.8 ±0.20^{A}	3.4 ±0.40	4.2 ±0.48^{A}	5 ±0.00	2.8 ±0.20	2 ±0.00^{A}	2 ±0.00^{A}	1.2 ±0.20	1.6 ±0.24	2 ±0.00^{A}	5	5	5
Diazepam+ imidacloprid 55	1 ±0.44	0.8 ±0.20^{B}	0.6 ±0.24 ^{B}	3.6 ±0.40	2.6 ±0.24^{B}	2.8 ±0.86	2.8 ±0.48	3.2 ±0.20^{B}	3 ±0.31^{B}	0.6 ±0.24	1 ±0.44	0.2 ±0.20^{B}	5	5	3

Values are mean ±SEM of five observations. Means bearing different superscripts differ significantly (P<0.05).

Table 5:
Effect of carbamazepine (50mg/kg, p.o.) on some characteristic behavioral symptoms induced by imidacloprid in mice

Dose(mg/kg, i.p.)	Score for severity of symptoms at different time intervals (min)														
	Restlessness (0)			Respiratory rate (4)			Body posture and limb position (4)			Tremors (0)			Presence of flexion of head(+)		
	15	30	45	15	30	45	15	30	45	15	30	45	15	30	45
Imidacloprid 22	0.2 ±0.25	0 ±0.00	0 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	3.7 ±0.25	4 ±0.00	4 ±0.00	0.0± 0	0.0± 0	0.0± 0	-	-	-
carbamazepine+ imidacloprid 22	0.2 ±0.20	0.4 ±0.40	0.2 ±0.20	4.4 ±0.24	3.6 ±0.24	3.8 ±0.20	3.8 ±0.20	3.8 ±0.20	3.8 ±0.00	0.0± 0	0.0± 0	0.0± 0	1	1	2
Imidacloprid 44	1.4 ±0.40	1.8 ±0.48 ^{A}	1.8 ±0.48	3.4 ±0.24^{A}	3.6 ±0.40	3.4 ±0.24	3.2 ±0.20	3 ±0.31	3.2 ±0.40	0.4± 0.4	0.8± 0.48	1.2± 0.73	5	5	4
carbamazepine+ imidacloprid 44	1.2 ±0.37	0.4 ±0.24^{B}	0.6 ±0.40	4.8 ±0.20^{B}	4.4 ±0.24	3.8 ±0.37	3 ±0.31	3.6 ±0.24	3.2 ±0.20	0.2± 0.2±	0 0.00	0.2± 0.2	4	3	3
Imidacloprid 55	2 ±0.00^{A}	2.6 ±0.24^{A}	2.8 ±0.20^{A}	3.4 ±0.40^{A}	4.2 ±0.48	5 ±0.00	2.8 ±0.20^{A}	2 ±0.00^{A}	2 ±0.20^{A}	1.2± 0.20^{A}	1.6± 0.24^{A}	2.0± 0	5	5	5
carbamazepine+ imidacloprid 55	0.4 ±0.20 ^{B}	0.6 ±0.24 ^{B}	0.6 ±0.33 ^{B}	4.8 ±0.20 ^{B}	4.8 ±0.20	5 ±0.00	3.6 ±0.24^{B}	3 ±0.31^{B}	3.6 ±0.00^{B}	0.0± 0.00 ^{B}	0.6± 0.24^{B}	1.0± 0.44	1	2	3

Values are mean ±SEM of five observations. Means bearing different superscripts differ significantly (p<0.05).

Table 6:
Effect of phenytoin (10 mg/kg,i.p.) on some characteristic behavioral symptoms induced by imidacloprid in mice

Dose(mg/kg, i.p.)	Score for severity of symptoms at different time intervals (min)														
	Restlessness (0)			Respiratory rate (4)			Body posture and limb position (4)			Tremors (0)			Presence of flexion of head(+)		
	15	30	45	15	30	45	15	30	45	15	30	45	15	30	45
Imidacloprid 22	0.2 ±0.25	0 ±0.00	0 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	3.7 ±0.25	4 ±0.00	4 ±0.00	0 ±0.00	0 ±0.00	0 ±0.00	-	-	-
Phenytoin+ imidacloprid 22	0 ±0.00	0 0±0.00	0 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	4 ±0.00	0 ±0.00	0 ±0.00	0 ±0.00	-	-	-
Imidacloprid 44	1.4 ±0.40	1.8 ±0.48^{A}	1.8 ±0.48 ^{A}	3.4 ±0.24	3.6 ±0.40	3.4 ±0.24	3.2 ±0.20	3 ±0.31	3.2 ±0.48	0.4 ±0.40	0.8 ±0.48	1.2 ±0.73	5	5	4
Phenytoin+ imidacloprid 44	0.8 ±0.20	0.5 ±0.28^{B}	0.2 ±0.25^{B}	3.4 ±0.24	3.5 ±0.28	3.7 ±0.25	3.2 ±0.20	3.2 ±0.25	3.5 ±0.28	1 ±0.44	0.7 ±0.25	0.5 ±0.28	1	2	-
Imidacloprid 55	2 ±0.00^{A}	2.6 ±0.24^{A}	2.8 ±0.20^{A}	3.4 ±0.40	4.2 ±0.48	5 ±0.00	2.8 ±0.21	2 ±0.00^{A}	2 ±0.00	1.2 ±0.20	1.8 ±0.24	2 ±0.00	5	5	5
Phenytoin + imidacloprid 55	0.8 ±0.37^{B}	1 ±0.40^{B}	0.6 ±0.33^{B}	3 ±0.00	3.5 ±0.28	3.6 ±0.33	2.8 ±0.20	3.7 ±0.25^{B}	3.3 ±0.33	0.8 ±0.20	1.8 ±0.20	3.3 ±0.33	-	1	-

Values are mean ±SEM of five observations. Means bearing different superscripts differ significantly (P<0.05).

imidacloprid potentiated the picrotoxin induced convulsions. It shows a possibility of involvement of GABAergic system in effects/actions of imidacloprid. Leela *et al.* (2003) has also demonstrated that

carbamazepine has direct neuromuscular blocking action. Antagonism of imidacloprid action by carbamazepine may be at the level of neuromuscular junction suggesting a direct excitatory action of imidacloprid at neuromuscular junction in higher doses i.e. 44 and 55 mg/kg intraperitoneally.These results suggested possibility of involvement of GABAergic system and a direct excitatory action at level of neuromuscular junction by imidacloprid.

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Research Article

PHARMACOKINETICS OF IVERMECTIN FOLLOWING SINGLE DOSE SUBCUTANEOUS ADMINISTRATION IN CATTLE CALVES

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ABSTRACT

The present study was undertaken to evaluate the pharmacokinetics of ivermectin 0.2 mg/kg b.wt in cattle calves. The study was conducted in four cross-bred male cattle calves (1.0-1.5 yrs in age, weighing 110±5 kg). The plasma concentration of ivermectin was determined by HPLC. The decay in plasma concentration of drug was biexponential in cattle calves. The C_{max} value of 32.31 was obtained at T_{max} of 4.4 days in cattle calves, following SC administration of ivermectin. The elimination half life (β_HL), volume of distribution (V1_F) and AUC were calculated as 24.59 days, 2.28 $L.kg^{-1}$, 393.9 day.ng/mL in cattle calves, following SC administration of ivermectin. A dosage regimen of 0.2 mg/kg at 14 days interval is recommended in cattle.

Keywords: Cattle calf, ivermectin, pharmacokinetics, subcutaneous

INTRODUCTION

Ivermectin used to treat billions of livestock and pets around the world. Ivermectin was the world's first endectocide, forerunner of a completely new class of antiparasitic agents, potently active against a wide range of internal and external nematodes and arthropods. It is a semisynthetic derivative of avermectin B1and consists of an 80:20 mixture of the equipotent homologous 22, 23 dehydro B1a and B1b. This antiparasitic agent, developed by Merck & Co., is frequently used in veterinary medicine, due to its broad spectrum of activity, high efficacy and wide margin of safety (Fisher *et al.*, 1989). It is a highly lipophilic substance that dissolves in most Organic solvents, but is practically insoluble in water (0.0004% m/v). Ivermectin was first marketed in 1981 by Merck Sharp and Dohme as an antiparasitic agent (Steel, 1993), and it remains the leading worldwide antiparasitic agent for livestock.

Topical ivermectin 1% cream was found to be both, anti-inflammatory and antiparasitic against papulopustular rosacea caused by Demodex mites (Schaller *et al.*, 2017). Ivermectin exhibited antiviral activity against porcine circovirus 2, by preventing the entry of viral cap protein in to the nucleus both in vitro and vivo (Wang *et al.*, 2019)

It has exceptional potency against endo- and ectoparasites at extremely low doses (doses recommended are expressed as µg/kg); this accounts for its large margin of safety. Toxicity to ivermectin is rare across animal species. The signs of toxicosis are mydriasis and depression, followed by ataxia, recumbency, and death. It has no adverse effects on breeding performance. Many rumino-reticular delivery systems, as well as oral, topical, and injectable formulations of ivermectin, are currently available at the dosage recommended by manufacturers, namely, 200 µg/kg in ruminants (500 µg/kg for topical application) and equines, 300 µg/kg in pigs, and 6 µg/kg in dogs. Its use has revolutionized the treatment of nematode and arthropod parasites in animals and has provided hope for the control or even eradication of filariases in humans (Aranzazu *et al.*, 2009). All important gastrointesti-nal and lung nematodes are susceptible to the drug, includ-ing sensitive mites (Baggot, 1988), ticks (Marriner *et al.*, 1987), biting flies, and parasitic dipteran larvae (Timothy, 2005).

The pharmacokinetic parameters of Ivermectin vary extensively and in accordance with many factors that can all influence the drug's plasma concentration. These factors, which include the species, route of administration, vehicle used in the commercial formulation, bodyweight, body condition, physiological status, and amount and type of nutrition, create difficulties when extrapolating data from one species to another and should be considered in clinical practice in order to achieve effective levels that will last as long as possible.. The purpose of the present study was to determine the pharmacokinetics and dosage regimen of ivermectin following single dose subcutaneous (SC) administration in cattle calves.

MATERIALS AND METHODS

Experimental animals

The present study was conducted in four cross-bred male cattle calves (1.0-1.5 yrs in age, weighing 110±5 kg). Cross-bred male cattle calves for this study were procured from Instructional Dairy Farm (IDF), of college of veterinary and animal sciences, Pantnagar. All these animals were housed in animal house of department of Veterinary Pharmacology and

Toxicology and kept on pre-experimental period for one month before the commencement of experiment to acclimatize them to new environment. Physical and clinical examination was done before the start of experiment. The animals were reared under uniform management and husbandry conditions, maintained on standard ration and water provided *ad libitum*. The animals were kept under constant observation before the commencement of the experiment. Institutional Animal ethics committee principles were followed strictly throughout the course of this study. Animals were handled gently and carefully. Deworming was done one month before the start of experimentation with the help of fenbendazole which was given at the rate 5mg/kg body weight. Pure technical standard ivermectin (Sigma Aldrich Ltd) and other HPLC grade chemicals were used in this study.

Estimation of ivermectin

Injectable formulation (20µl)of ivermectin (Noromectin® ; M/s Norbrook Laboratories Ltd.) was used in the study. Pharmacokinetic study of ivermectin was conducted following a single dose (0.2 mg kg^{-1}) Subcutaneous (SC) injection in neck region of cattle. The blood samples were collected from jugular vein of calves in heparinized microcentrifuge tubes by disposable plastic syringes at time interval of 0 min, 15 min, 30 min, 1h, 3 h, 6 h, 12 h, 1 day, 3 day, 6 day, 9 day and up to 42 days. The blood samples collected in heparinized tubes following administration of Ivermectin were centrifuged at 5000 rpm (15 min) for separation of plasma. The plasma thus obtained was collected in micro centrifuge tubes and stored at -20°C till further analysis. An intervening wash out period of one month was given to all the animals before commencement of new experiment.

Extraction and derivatization of ivermectin from plasma samples

Extraction of plasma samples was carried out as per the method described by Perez *et al.* (2007) and Na-Bangchang *et al.* (2006) with slight modifications**.** 1 ml of acetonitrile and 0.25 ml of deionised water was added to 1 ml of plasma sample, vortex mixed for 20-30 seconds and centrifuged at 12,000g for 12 minutes (4°c). The supernatant was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 30-40°c. The residue was subjected to derivatization according to the method of De Montigny *et al.* (1990) . The residue was dissolved in 100 µL of 1-methylimidazole solution in acetonitrile (1:2 v/v). To initiate the derivatization, 150 µL of trifluoroacetic anhydride solution in acetonitrile (1:2 v/v) was added. After completion of the reaction (< 30 s), an aliquot (20 µL) of this solution was injected directly in to HPLC (Shimadzu Corporation, Kyoto, Japan, Model RF-10A_{XL}, LC10AT) and analysed using. The isocratic mobile phase consists of acetic acid (0.2% in water), methanol, and acetonitrile (4:32:64, v/v/v). The flow rate was kept at 0.7 $ml.min^{-1}$ at a temperature of 30°C with fluorescence detection at an excitation wavelength of 365 nm and an emission wavelength of 475 nm using C_{18} reverse phase column (125 mm x 4 mm Lichrospher 100 RP-18 5µm) with a guard column (Lichrospher 100 RP-18e, 5µm, Merck Kga A, 64271 Darmstadt, Germany) and syringe (Manufactured by Hamilton,Co., RE No. Nevada, USA). Ivermectin was quantified from its respective retention time.

Preparation of standard curve

The standards for ivermectin were made by dissolving 1 mg of pure ivermectin in 1 ml of methanol from which concentrations of 100, 50, 25, 10, 5, 1 $ng.ml^{-1}$ were made in methanol. 20 µl of these concentrations was injected into HPLC system and quantified under the HPLC conditions mentioned above. The standard calibration curve for ivermectin was obtained by plotting concentrations *versus* mean of the peak areas obtained for their respective standards. The limit of quantification (LOQ) for ivermectin was 1$ng.ml^{-1}$. The method for ivermectin was found to be linear and reproducible in the concentrations ranging 100 to 1 $ng.ml^{1}$. A retention time of 24 .1 min for ivermectin was observed**.**

The concentrations of the ivermectin standard were made in drug free plasma as 100, 50, 25, 10, 5, 1 $ng.ml^{-1}$ applying serial ten times dilution (100 µl standard + 900 µl drug free plasma) of 1000, 500, 250, 100, 50, 10 $ng.ml^{-1}$ of standard in methanol, in equal volumes of drug free plasma, each time. The extraction from plasma was done by the same procedure as mentioned earlier. The areas obtained by chromatography were plotted against concentration in order to get a standard calibration curve. Recovery of the drug was done by deproteinizing the plasma having above mentioned drug concentration. Recovery percent of ivermectin from plasma was 83.2.

Pharmacokinetic analysis of data

The plasma concentrations and pharmaco-kinetic variables of Ivermectin were expressed as mean ± S.E. The pharmacokinetic analysis of the plasma concentration obtained following SC administration of Ivermectin in this study was done by pharmacokinetic software “Phasight WinNonlin” version 5.3.

RESULTS

The plasma concentration-time profile following single dose (0.2 $mg.kg^{-1}$) subcutaneous administration of Ivermectin (Noromectin®) in male cattle calves depicted in Figure 1.

The plasma samples were collected up to 42 days. The concentration of ivermectin could be detected

only up to 30 days. The mean peak plasma concentration was 32.315±0.64 ng.ml^{-1} attained at 4.44 days post administration which decreased slowly to a minimum of 2.165 ± 0.02 ng.ml^{-1} at 30th day. The pharmacokinetic parameters describing the disposition kinetics of ivermectin following single dose (0.2 mg.kg^{-1}) subcutaneous administration are presented in Table 1. A two-compartment model adequately (r= 0.89) described the plasma concentration-time profile of ivermectin in male cattle calves following single dose subcutaneous administration.

The mean values of zero_time intercept of distribution phase (A) and elimination phase (B) in the present study were calculated to be 3641.42±991 ng.mL^{-1} and 0.165±0.03 ng.ml^{-1}, respectively. The elimination rate constant of first phase (K_{10}) and second phase (β) were 0.222±0.001 and 0.029±0.003 day^{-1}, respectively, with an elimination half-life of first phase (K_{10}_HL) and second phase (Beta_HL) calculated as 3.114±0.02 and 24.59±3.37 day, respectively. The transfer rate constant from central to peripheral compartment (K_{12}) and from peripheral to central compartment (K_{21}) were 0.001±0.0006 and 0.029±0.003 day^{-1}, respectively. The volume of distribution of central compartment (V1_F; when fraction of drug absorption is not known), and volume of distribution of peripheral compartment (V2_F; when fraction of drug absorption is not known) were 2283.8±43.4 and 204.781±78.3 ml.kg^{-1}, respectively. The clearance from central compartment (CL_F; when fraction of drug absorption is not known) and clearance from peripheral compartment (CLD2_F; when fraction of drug absorption is not known) were estimated as 508.265±9.78 and 5.331±1.49 ml.kg^{-1}.day^{-1} respectively. The rate constant of distribution phase (α) was 0.225±0.001day^{-1} with distribution half-life (Alpha_HL) of 3.078±0.024 day. The rate constant of absorption phase (K01) was 0.225±0.002 day^{-1} with absorption half-life (K01_HL) of 3.071±0.03 day. The mean area under curve (AUC) was 393.937±7.67 ng.ml^{-1} day.

DISCUSSION

A two-compartment model adequately described the plasma concentration-time profile of ivermectin in cattle calves following single dose (0.2 mg.kg^{-1}) SC administration in the present study. The values of C_{max} in the present study were 32.31 ng.ml^{-1} in cattle calves

Table 1:
Pharmacokinetic parameters of ivermectin in plasma following its single dose (0.2 mg.kg^{-1}) subcutaneous administration in cattle calves (n=4)

Parameters		Units		Calf Number		Mean±SE
		I	II	III	IV	
V1_F	ml/kg	2389.182	2198.939	2319.253	2228.196	2283.8±43.4
K01	1/day	0.23	0.227	0.221	0.222	0.225±0.002
K10	1/day	0.219	0.220	0.226	0.224	0.222±0.001
K12	1/day	0.003	0.001	0.003	0.0007	0.001±0.0006
K21	1/day	0.027	0.036	0.020	0.034	0.029±0.003
AUC	day.ng/ml	381.970	413.199	381.175	399.406	393.937±7.67
K01_HL	Day	3.0004	3.041	3.131	3.114	3.071±0.03
K10_HL	Day	3.162	3.148	3.063	3.084	3.114±0.02
α	1/day	0.222	0.223	0.230	0.225	0.225±0.001
β	1/day	0.027	0.036	0.020	0.034	0.029±0.003
α _HL	Day	3.114	3.117	3.011	3.071	3.078±0.024
β_HL	Day	25.287	18.980	33.860	20.242	24.59±3.37
A	ng/ml	2289.299	3708.035	2147.346	6421.028	3641.42±991
B	ng/ml	0.209053	0.213508	0.162020	0.078790	0.165±0.03
CL_F	ml/day/kg	523.597	484.028	524.692	500.743	508.265±9.78
V2_F	ml/kg	257.895	109.780	400.201	51.251	204.781±78.3
CLD2_F	ml/day/kg	7.179	4.049	8.335	1.762	5.331±1.49
Tmax	Day	4.417	4.448	4.436	4.464	4.441±0.009
Cmax	ng/ml	31.410	33.912	31.136	32.805	32.315±0.64

Abbreviations: V1_F= Volume of distribution of central compartment when fraction of drug absorption is not known; K01=Rate constant of absorbtion phase; K10=Elimination rate constant of first phase; K12= Transfer rate constant from central to peripheral compartment; K21=Transfer rate constant from peripheral to central compartment; AUC= Total area under the curve (from time zero to infinity); K01_HL= Absorption half –life; K10_HL=Elimination half life of first phase; α=First order rate constant; Regression coefficient for the distribution phase of the disposition curve; β=Regression coefficient for the elimination phase); α_HL= Distribution half-life; β_HL= Elimination half-life of second phase; A= Zero time intercept of plasma concentration of distribution phase; B =Zero-time intercept of plasma concentration of elimination phase; CL_F =The clearance from central compartment when fraction of drug absorption is not known; V2_F =Volume of distribution of peripheral compartment when fraction of drug absorption is not known ; Tmax =Time to reach peak plasma concentration ; Cmax =Peak plasma concentration.

following SC administration of ivermectin. These findings could be well corroborated with C_{max} (33.1 ng/mL) in cattle (Echeverria *et al.*,1997), 32.7 ng/mL in cattle (propylene glycol: glycerol-formal vehicle 60:40 v/v) following SC route of administration. (Lifschitz *et al.*, 2004; Alvinerie *et al.*, 1998) have also reported C_{max} of 28.5 ng/mL in cattle by intraruminal route of administration. The $C_{max\,in}$ the present study could also be compared with other species viz sheep (32.2 and 30 ng/ml; (Bogan, 1988) and (McKellar, 1991), respectively) and pigs (28.4 ng/mL; (Scott and McKellar, 1992)

A lower peak plasma concentration (C_{max}) as compared to the present study has been observed by other workers in cattle using different formulations (22.6, 12.2 and 16 ng/mL; (Lifschitz *et al.*, 1999b), (Gayrard *et al.,* 1999) and (Laffont *et al.,* 2001), respectively). Sheep (24.1, 25.8 and 12.5 ng/mL; (Echeverrý´a *et al,* 2002), (Barber *et al.,* 2003) and (Chiu *et al.,* 1990 a) respectively). Goats (21.8 and 9.3 ng/mL; (Gonzalez *et al.,* 2006) and (Escudero *et al.,* 1997), respectively).However, higher peak plasma concentration (C_{max}) level compared to present study has been reported in cattle (42.8, 133.2, 40 and 39 ng/mL; (Lanusse *et al.,* 1997), (Chiu *et al.*, 1990 a), (Lifschitz *et al.*, 2000) and (Laffont *et al.*, 2001), respectively). Pigs (39.6 ng/mL; (Lifschitz *et al.,* 1999a), horses (51.3 ng/mL; (Perez *et al.*, 2002) and dogs (44.3 ng/mL; (Daurio *et al.,* 1992). The higher peak plasma concentration (C_{max}) in the present study may be attributed to the formulation (propylene glycol: glycerol-formal 60:40 v/v) as a vehicle in the injectable product (Noromectin®). Injectable product has the advantage that higher maximum plasma concentration are achieved and, thus presumably (by gradient diffusion) greater skin penetration and ectoparasiticidal activity, whereas the oral product is more easily administered and may have greater activity against some intestinal nematodes.

The value of T_{max} in the present study was 4.44 days in cattle calves following SC administration of ivermectin. These findings could be well corroborated with T_{max} (4 days) in cattle (Lanusse *et al.,* 1997). A lower value of T_{max} compared to the present study has been reported in cattle (2.25 and 2.32 days; (Lifschitz *et al.,* 1999b) and (Echeverrý´a *et al.,* 1997), respectively), sheep (1.7 and 1.24 days; (Cerkvenik *et al.,* 2002) and (Barber *et al.,* 2003), respectively) and goats (3 and 2.85 days; (Gonzalez *et al.,* 2006) and (Alvinerie *et al.,* 1993), respectively). However, higher level of Tmax (15.1 day) compared to present study has been reported in cattle by sustained release bolus through intraruminal route (De montigny *et al.,* 1990).

The mean elimination half-lives in the present study were 24.59 days in cattle calves following SC route of administration of ivermectin. These findings could be well corroborated with mean elimination half-life of 17.2 days in cattle by subcutaneous route (Lanusse *et al.*, 1997). However, lower mean elimination half-lives compared to present study has been reported in sheep (9.6 days; (Gonzalez *et al.*, 2007), goats (7.4 days; (Gonzalez *et al.*, 2006) and pigs (1.18 days)(Craven *et al.*, 2001). The higher mean elimination half_life in the present study could be due to low water solubility of Ivermectin and its pre-cipitation in SC tissues favour slow absorption from the injection site, resulting in a prolonged

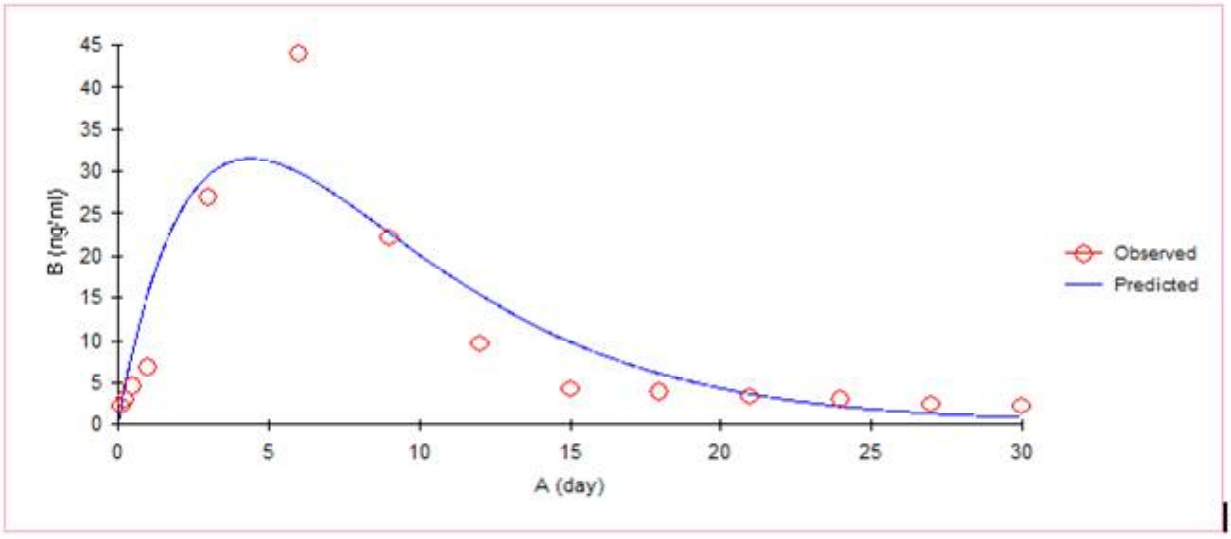

Figure 1 :
Plasma concentration-time plot of observed concentration (mean) Vs predicted profile of Ivermectin (Noromectin®) following single dose (0.2 $mg.kg^{-1}$) subcutaneous administration in cattle calves (n=4)

presence in the bloodstream. Retention in the body is also increased due to slow absorption from the injection site.

Volume of distribution is a measure of extravascular distribution of a drug and higher values would always be advantageous for therapeutic purposes indicating excellent tissue penetration. In the present study, the volume of distribution (V1_F) was 2.28 $L.kg^{-1}$ in cattle calves following SC administration of ivermectin. These findings could be quite similar with volume of distribution (2.7 $L.kg^{-1}$) in cattle (Bousquet-Me´lou *et al.*, 2004), goats (2.8 $L.kg^{-1}$; (Gonzalez *et al.*, 2006) and pigs (2.7, $L.kg^{-1}$(Craven *et al.*, 2001). Due to its high lipophilic nature, Ivermectin is exten-sively distributed with wide volume of distribution (Vd) in all species. Inter-individual variation can also be attributed to differences in body con-dition, age, sex, and physiological status (Cerkvenik *et al.,* 2002). A lower Volume of distribution (1.2 $L.kg^{-1}$) compared to present study has been reported in cattle (Echeverrý´a *et al.,* 1997). However, higher volume of distribution (3.4 $L.kg^{-1}$) compared to present study has been reported in cattle (Lanusse *et al.,* 1997), sheep (5.3, 3 and 12.8 $L.kg^{-1}$; (Prichard *et al.*, 1985), (Gonzalez *et al.*, 2007) and (Cerkvenik *et al.,* 2002), respectively.

The AUC is the parameter that integrates both time and intensity of drug concentration. The area under the concentration time curve characterizes the relative availability of drug in the body (Dudley, 1991). The area under curve (AUC) in the present study was 393.93 $ng.ml^{-1}$day in cattle calves following SC administration of Noromectin® respectively. These findings could be well corroborated with AUC (328.8 and 381.1 $ng.ml^{-1}$day; (Echeverrý´a *et al.*, 1997) and (Laffont *et al.,* 2001] respectively) in cattle. However, higher AUC compared to present study has been reported in cattle (459 and 595.1$ng.ml^{-1}$ day; (Lanusse *et al.,* 1997) and (Laffont *et al.,* 2001) respectively), sheep (440 $ng.ml^{-1}$ day; (Prichard *et al.,* 1985), horse (550.4 $ng.ml^{-1}$ day; (Marriner *et al.,* 1987). A lower area under curve (AUC) compared to present study has been reported in cattle (189 and 278 $ng.ml^{-1}$day; (Lifschitz *et al.,* 1999b) and (Lifschitz *et al.,* 2000**),** respectively).

Plasma clearance of drug is the volume of the blood or plasma cleared of drug by metabolism and excretion per unit of time. It is a better index of efficiency of drug elimination than half-life as it gives the clearance of drug from blood per unit of time (Baggot, 1988). The value of clearance in this study was 0.5 $L.kg^{-1}$ day^{-1} in cattle calves following SC administration of ivermectin. These findings could be well corroborated with plasma clearance (0.48 $L.kg^{-1}.day^{-1}$) in cattle (Lanusse *et al.*, 1997) and in sheep (0.56 $L.kg^{-1}.day^{-1}$; (Prichard *et al.*, 1985). However, higher plasma clearance compared to present study has been reported in sheep (1.11 and 3.24 $L.kg^{-1}.day^{-1}$ (Gonzalez *et al.*, 2007) and (Cerkvenik *et al.,* 2002), respectively), goats (1.56 $L.kg^{-1}.day^{-1}$; (Echeverrý´a *et al.*, 2002) and pigs (4.15 $L.kg^{-1}.day^{-1}$; Craven *et al.*, 2001). A lower plasma clearance compared to present study has been reported in cattle (0.27 and 0.35 $L.kg^{-1}.day^{-1}$(Laffont *et al.,* 2001) and (Bousquet-Me´lou *et al.,* 2004), respectively.

Ivermectin persists in the body for a prolonged period, not only due to low plasma clearance but also due to the accumulation in fat tissue. Plasma clearance appears to be greater in pigs than in (goats > sheep > cattle) polygastric species (Ara´nzazu *et al.*, 2009)

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Research Article

IN VITRO AND *IN VIVO* ANTIINFLAMMATORY ACTIVITY OF HYDROETHANOLIC EXTRACT OF *RHODODENDRON ARBOREUM*

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ABSTRACT

Evaluation of anti-inflammatory potential of hydroethanolic extract of *Rhododendron arboretum* (RAHE) using *in vitro* and *in vivo* methods was undertaken in this study. *In vitro* methods included inhibition of albumin denaturation test and of estimation anti-proteinase activity whereas in vivo methods included formalin and Freund's ,adjuvant induced rat paw edema model for assessment of anti-inflammatory potential of RAHE. Results of the study revealed 67.50% inhibition of albumin denaturation at 500μg/ml concentration whereas diclofenac showed 52% inhibition. RAHE showed 51.28% anti-proteinase activity at concentration of 500μg/ml whereas diclofenac showed a response of 41.20% and significant ($P<0.05$) reduction in paw edema was observed following oral administration of RAHE which indicated potent anti-inflammatory activity of the RAHE. It is concluded from this study that hydroethanolic extract of *Rhododendron arboretum* (RAHE) following *in vitro* and *in vivo* assessment revealed potent anti-inflammatory potential.

Keywords: Anti-inflammatory potential, *hydroethanolic extract, in vitro* and *in vivo*, paw edema, *Rhododendron arboretum, rats.*

INTRODUCTION

For ages nature has provide a vast source of resources to mankind for development of a large number of drugs for the treatment of diseases of man and animals. More than 35,000 plant species have been characterized for medicinal purposes. India is known for its ancient tradition of herbal medicines and is home of thousands of important medicinal plant species (David *et al.*, 2015). *Rhododendron arboreum* belonging to the family Ericacaeae, known locally as as *Burans,* was originally highlighted in the areas of North Central India, Himalayas, areas of Kashmir, in Assam and Manipur and specifically and grown only in high altitude regions ranging from 600 mts. above sea level or above. The tree is the state tree of Uttarakhand and the national flower of Nepal. The flowers blossom biannually whereas green leaves are available on the tree throughout the year (Srivastava, 2012). The plant is traditionally used for the treatment of various health conditions like heart diseases, diarrhea, blood dysentery, fever, inflammation etc. The leaves are used in the preparation of corolla which is sweet in taste and used when fish bones gets stuck in gullet. Young leaves are used to alleviate headache (Chauhan *et al.*, 2016). The medicinal and important pharmacological properties of the plant include hepatoprotective, antioxidant, immunomodulatory, antiinflammatory, antidiabetic, anticancer, antinociceptive, adaptogenic, synthetase inhibitory and central nervous system depressant activity (Gill *et al.*, 2015).

Acute inflammation is classically characterized by signs like erythema, edema, pain, heat and loss of functions. Chronic inflammation leads to progressive shift in type of cells present at the site of inflammation which leads to healing of the injured tissue from the incidence of inflammation. Acute models are designed in order to modulate erythema and changes in vascular permeability, for the measurement of local pain and rat paw edema (Pinheiro *et al.*, 2002) whereas chronic models are designed to produce response of drugs which may modulate the disease process as in the case of drug induced hepatotoxicity, nephrotoxicity, immunosuppression etc (Chfeng *et al.,* 2005). Thus, in view of the above facts, this study was designed to evaluate extracts of *Rhododendron arboreum* for in vitro and in vivo anti-inflammatory potential.

MATERIALS AND METHODS

Preparation of hydroethanolic extract of Rhododendron arboreum (RAHE) leaves

Leaves of *Rhododendron arboreum* were separated and shade dried in laboratory for 22 days followed by further drying in a fan incubator at 37^{0}C for 6-7 days to remove the extra moisture present in the leaves. The dried leaves were further grinded in a mixer to obtain a fine homogenous powder of light brown color. Dried powder of the leaves was soaked in water and ethanol in 60:40 ratio and adding extract in 1:10 ratio and allowed to stand for 24 hours. The filtrate obtained on the next day was filtered using muslin cloth and filtered again with the help of Whatman filter paper no. 42. The obtained filtrate was subjected to rotatory vacuum evaporator at 40^o-50^o C and further dried in incubator at 37^{o}C-39^{o}C for 48 hrs to get dried extract

which was further used for *in vivo* and *in vitro* evaluation of anti-inflammatory activity.

In vitro inhibition of albumin denaturation test

Anti-inflammatory activity of *Rhododeron arboreum* was evaluated by using inhibition of albumin denaturation technique according to the methods of Govindappa *et al.* (2011). The extract solution was prepared in varying concentrations from 100µg/ml to 500µg/ml and 1% aqueous solution of bovine serum albumin was added to it. The pH was balanced using 1N HCl. The mixture was incubated at 37^{0}C for 20 minutes and then heated at 51^{0}C for 20 minutes. After cooling of the samples, the supernatant was measured spectrophotemetrically at 660 nm. The experiment was performed in triplets and reading were taken after every 60 seconds. The percent inhibition of albumin denaturation was calculated by the following formula:

$$\text{Percent inhibition} = \frac{\text{Absorbance control - Absorbance sample}}{\text{Absorbance control}} \times 100$$

In vitro antiproteinase activity test

Antiproteinase activity was determined by the method of Kulkarni *et al.* (2015). RAHE was prepared in varying concentrations form 100µg/ml to 500µg/ml. To 1 ml of the sample in a test tube 0.06 mg trypsin and 1ml of 20mM Tris HCL Buffer (pH7.4) were added and this mixture was incubated at 37^{0}C for 5 minutes and then 1 ml of 0.8% (w/v) casein was added to the mixture. The mixture was then incubated at 37^{0}C for 20 minutes. 2ml of 70% perchloric acid was added in order to terminate the reaction. The suspension was centrifuged and absorbance of supernatant was measured at 210 nm against buffer as blank. The experiment was conducted in triplets and the percent inhibition of proteinase inhibitory activity was calculated by the following formula:

$$\text{Percent inhibition} = \frac{\text{Absorbance control-Absorbance sample}}{\text{Absorbance control}} \times 100$$

In vivo anti-inflammatory potential of RAHE

Freund's adjuvant induced paw edema

Eighteen rats were taken and divided into 3 groups with 6 animals each. 0.1 ml of 500 µg of Freund's adjuvant suspension containing heat killed *Mycobacterium tuberculosis* cells was injected in the right hind paw of each rat in all groups. Group 1 was taken as control and did not receive any treatment following administration of CFA on day 1 of the trial. Rats of group 2 were given diclofenac @ 0.2 ml/animal (10 mg/kg b wt, IM) at alternate days on day 2 and day 4; group 3 received RAHE at 100 mg/kg b wt, orally daily for 12 days. The swelling was recorded daily for 12 days using digital caliper (Fehrenbacher *et al.,* 2012).

Formalin induced paw edema test in rat

The study was conducted in rats following the methods of Arzi *et al.* (2015). Eighteen rats were taken and divided into 3 groups with 6 animals each. 0.2 ml of 2% formalin (standard formalin was diluted with normal saline to obtain the final conentration) was injected in the right hind paw of each rat in all groups. Rats of group 1 were injected with formalin on day 1 and did not receive any treatment during the course of the trial. Group 2 received formalin and day 1 and therapeutic treatment was started from day 2 by injecting 0.2 ml diclofenac/ animal, IM on day 2 and 4 of the study and group 3 received RAHE at 100 mg/kg b wt, orally daily for 9 days. The swelling was recorded daily for 9 days using digital calliper.

RESULTS

The ability of the plant extract to inhibit albumin denaturation was examined as (%) 30, 45, 55, 62.5 and 67.5 at 100, 200, 300, 400 and 500 concentrations (µg/ml), respectively. The highest inhibition of 67.50% was observed at 500µg/ml concentration of RAHE. Diclofenac, the standard anti-inflammatory drug used in this study, displayed a inhibition of 52% at a concentration of 50 µg/ml, thus, indicating the significant anti-inflammatory potential of the plant extract in this experiment (Figure 1).

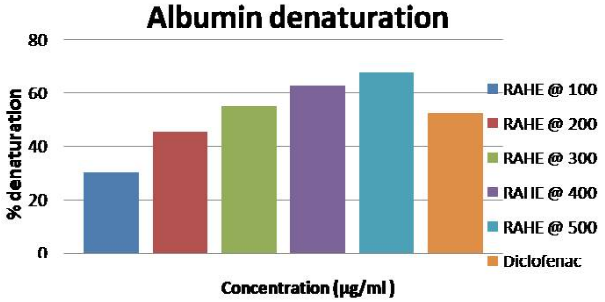

Figure 1:
Effect of hydroethanolic extract of (RAHE) on inhibition of albumin denaturation activity

Antiproteinase activity test

This study revealed that maximum inhibition of 51.28% was observed at 500 µg/ml concentration of RAHE. Diclofenac, the standard drug displayed a response of 41.2% at a concentration of 50 µg/ml, thus indicating the significant anti-inflammatory potential of the plant extract (Figure 2).

Freund's adjuvant induced paw edema test

A significant ($P<0.05$) reduction in paw edema following administration of diclofenac in group 2 was observed from 4th day post administration of complete Freund's adjuvant whereas a significant ($P<0.05$) reduction in paw edema was recorded following 5th day of administration of RAHE daily for 12 days in rats. It

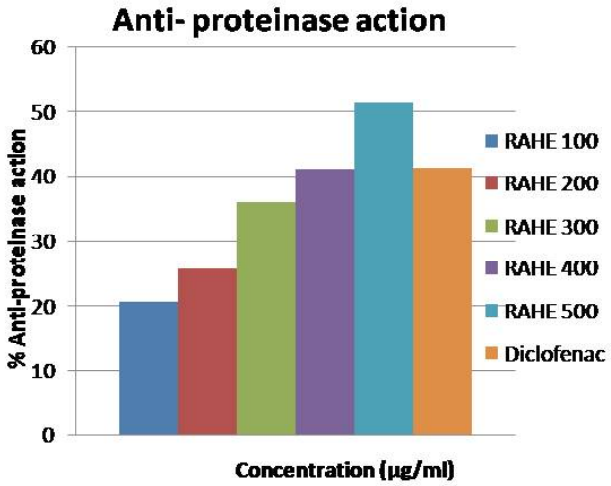

Figure 2:
Effect of hydroethanolic extract of RAHE on anti-proteinase action test.

can be interpreted from the study that RAHE caused a reduction in paw edema in Freund's adjuvant treated animals following oral administration for 12 days in rats. Diclofenac the standard drug also displayed a similar reduction in paw edema at effects equivalent to RAHE after 5th day of the study (Figure 3).

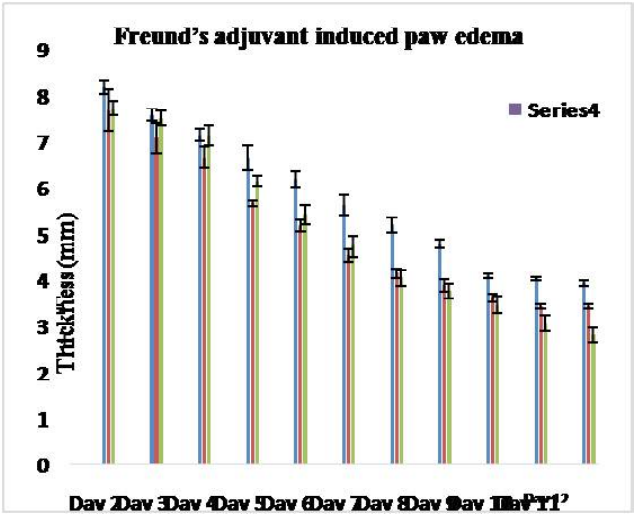

Figure 3:
Effect on Freund's adjuvant induced paw edema following oral administration of RAHE for 12 days in rats. (n=6)

Formalin induced paw edema test

The study was conducted in rats following the methods of Arzi *et al.* (2015). Eighteen rats were taken and divided into 3 groups with 6 animals each. 0.2 ml of 2% formalin (standard formalin was diluted with normal saline to obtain the final concentration) was injected in the right hind paw of each rat in all groups. Rats of group 2 were injected with formalin on day 1 and did not received any treatment during the course of the trial. Group 2 received formalin and day 1 and therapeutic treatment was started from day 2 by injecting 0.2 ml diclofenac/animal, IM on day 2 and 4 of the study and group 3 received RAHE at 100 mg/kg b wt, orally daily for 9 days. The swelling was recorded daily for 9 days using digital calliper.

There was a significant decrease in paw edema following administration of diclofenac in rats from day 3rd of the treatment whereas a significant ($P<0.05$) reduction in paw edema was observed in group 3 from day 5th following oral administration of RAHE for days in rats. Thus, from the findings of the study, it can be interpreted that RAHE caused a significant reduction in paw edema from 5th day onwards in comparison to standard drug diclofenac which caused reduction in paw edema from 3rd day itself, so RAHE produced a significant ($P<0.05$) decline in paw edema equivalent to the extent of standard drug diclofenac (Figure 4).

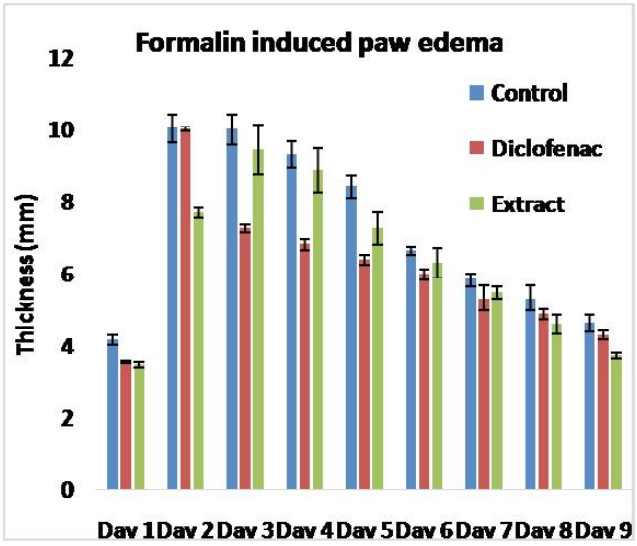

Figure 4 :
Effect on Formalin induced paw edema following oral administration of RAHE for 9 days in rats (n=6)

DISCUSSION

In vitro in and *in vivo* anti-inflammatory study of RAHE exhibited 67.50% inhibition of albumin denaturation at 500µg/ml concentration whereas diclofenac showed 52% inhibition. RAHE showed 51.28% antiproteinase activity at concentration of 500µg/ml whereas diclofenac showed a response of 41.20%. Leukocyte proteinase plays an important role in elaborating the cellular and tissue damage during the development of any kind of inflammatory reaction in the body. This process is inhibited by proteinase inhibitors which directly inhibit the proteinase to prevent development of inflammatory reaction. The proteinase

are mainly found in lysosomes and are the source for serine proteinase (Bermudez *et al.,* 2015).

A significant (P<0.05) reduction in paw edema was observed following oral administration of RAHE indicative of potent anti-inflammatory activity of the extract. Inflammation is characterized by the 5 cardinal signs viz hypersensitivity, redness, swelling, heat and loss of function (Medzhitov, 2010). Subcutaneous injection of inflammatory agents such as Freund's adjuvant leads to development of edema, hyperalgesia or increased activity to abnoxious stimulus are basic signs which appears upon inflammation with Freund's adjuvant. The study of these cardinal signs is used to measure the inflammatory response of the body and various compounds such as NSAID'S can be developed in order to prevent the condition, Freund's adjuvant is reported to have more inflammatory, pain inducing and tissue necrosis properties than commonly used inflammatory agents (Stils, 2005). The findings of the study reveals the loss of inflammatory signs with treatment groups thus indicating the antiinflammatory effect of RAHE in rats as compared to the standard drug diclofenac and control. The findings of the study can be correlated to the findings of Verma *et al.* (2011) who recorded protection in paw edema upon administration of *Rhododendron arboreum* etanolic extract@ 100 mg/kg b wt, and 200 mg/kg b wt, supporting the anti- inflammatory effect of the extract.

Similar activity in studies conducted by Raghu and Agrawal(2016) was recorded in ethanolic extract of *Rhododendron* showing an increase in antiinflammatory activity of the extract due to the presence of in-vitro lipoxygenase inhibitory property in the extract.

The anti-inflammatory properties of *Rhododendron arboreum* has also been reported earlier (Gill *et al.*, 2015). Ethyl extract fraction of *Rhododendron arboreum* at 100, 200 and 400 mg/kg b wt, showed dose dependent and a significantly increased anti-inflammatory activity in arachidonic induced paw edema, cotton pellet granuloma and Freund's adjuvant induced paw arthritis. The activity might be due to presence of high amount of flavonoids in the extract (Verma *et al.,* 2012). Hydroethanolic extract of *Rhododendron arboreum* has been shown to have potent antioxidant potential due to presence of flavonoids (Swaroop *etal*., 2005) which could be attributed to its anti-inflammatory activity. Finding of the study ,thus , *in vitro* and *in vivo* studies revealed potent anti-inflammatory activity of hydroethanolic extract of *Rhododendron arboretum.*

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Research Article

SAFETY EVALUATION OF REPEATED ORAL ADMINISTRATION OF GEMIFLOXACIN AND PIPERINE IN LAYER BIRDS

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ABSTRACT

The present study was designed to investigate safety of gemifloxacin and piperine @ 10 mg/kg/day orally for 5 days in layer birds. It was carried out by monitoring clinical observations, hematological (TEC, HB, PCV, MCV, MCH, MCHC, TLC, DLC and platelet count) and blood biochemical parameters (blood glucose, ALT, AST, ALP, LDH, serum creatine kinase, serum total bilirubin, serum total protein, serum albumin, serum uric acid and serum creatinine). There were no clinical signs of adverse reactions and all the hemato-biochemical parameters were found to fluctuate within normal range during treatment period and the values were not significantly different from corresponding control day (0 day) values. It suggests that repeated oral administration of gemifloxacin or piperine alone daily once for five days is safe in layer birds.

Key words: Gemifloxacin, Piperine, Safety, biochemical parameters, layer birds

INTRODUCTION

Antibacterial are frequently used in animal production all over the world for disease treatment and prevention, as well as for growth promotion or performance enhancement. In poultry, the fluoroquinolones are used extensively for disease prevention and treatment. Fluoroquinolones are class of synthetic bactericidal agents widely used in treatment. Gemifloxacin is a potent, novel broad spectrum antibacterial compound. It belongs to fourth generation fluoroquinolones having enhanced affinity for bacterial topoisomerase IV. It is useful for the treatment of respiratory and urinary tract infections. It is also active against other major pathogens involved in respiratory tract infections, including *Haemophilus influenza, Moraxella catarrhalis*, *Legionella pneumophila*, *Chlamydia spp.* and *Mycoplasma spp*. (Hannan and Woodnutt, 2000). The safety of gemifloxacin has been evaluated in humans (Amitabh *et al.*, 2012; Visoriya *et al.*, 2014) and broiler birds (Gohel *et al.*, 2018). Piperine, the major plant alkaloid present in black pepper (*Piper nigrum*) and long pepper (*Piper longum*), is reported to have bioavailability-enhancing activity for nutritional substances and drugs (Patil *et al.,* 2011). It enhances gastrointestinal as well as local absorption and inhibits various metabolizing enzymes. The safety of piperine has been evaluated in rats (Bhat and Chandrasekhar, 1986). Despite the great potential for clinical use of gemifloxacin and piperine, the data on its safety profile in layer birds are scarce. Looking to this fact, the present study was therefore, undertaken to determine the safety of gemifloxacin and piperine in layer birds.

MATERIALS AND METHODS

Experimental Animals

The study was conducted on 12 layer birds (synthetic white leghorn) of 53-56 weeks weighing between 1.2 and 2.0 kg. The layer birds were obtained from and maintained at the Poultry Research Station (PRS), College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand. The study was undertaken during the months of November and December when the ambient temperature was recorded between 12 to 30 ºC. Birds were kept under observation for three weeks prior to commencement of experiment. The layer birds were kept in clean cages and were provided standard layer ration. Water was provided *ad libitum*. The experimental protocol for general procedure and use of layer birds for conducting the present study has been reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the college (IAEC/GVC/VPT/249/2016). Layer birds were rehabilitated after completion of study. All necessary managemental procedures were adopted to keep the layer birds free from stress.

Drugs and chemicals

Technical grade powder of gemifloxacin mesylate (GEM0360816) was received on gratis from Hetero Drugs Limited, Hyderabad. Piperine (P49007-5G0000005526) with purity e" 97 % was purchased from Sigma-Aldrich Chemicals Private Limited, Bangaluru. Standard assay kits for estimation of biochemical parameters were purchased from Coral Clinical Systems,

Goa, India.

Experimental design

Safety of repeated oral administration of gemifloxacin and piperine @ 10 mg/kg body weight once daily for 5 consecutive days was assessed by daily estimating haematology and serum biochemistry parameters. All layer birds were observed twice a day throughout the experiment period. Changes in posture, laying performance and response to handling as well as the abnormal behaviour, if any, were recorded.

Collection of blood

Blood samples from all twelve birds were withdrawn from wing vein into sterile heparinized and non-heparinized test tubes at 0 day (before drug administration) and on 1st, 2nd, 3rd, 4th and 5th day for haematological and serum biochemical analysis. Blood smears for determination of differential leukocyte count (DLC) were prepared from fresh blood at the time of blood collection (Schalm, 1967). Blood samples (0.5 mL) collected in tubes with K_3EDTA were utilized for hematological evaluation, whereas blood samples (2 mL) collected in centrifuge tubes without K_3EDTA were allowed to clot at room temperature. Serum was harvested by centrifugation at 5000 rpm for 10 minutes at 10 ºC (Eppendorf 5804 R, Germany) and stored at – 65 ºC for biochemical analysis.

Hematological parameters

Haematological parameters like total erythrocyte count (TEC), hemoglobin (HB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocyte count (TLC), platelet count were estimated using automated hematology analyzer (BC-2800Vet, Shenzhen Mindray, China). Whereas, DLC was carried out as per method described by Schalm, (1967).

Biochemical parameters

Various serum biochemical parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), total bilirubin (TB), total protein (TP), albumin (ALB), uric acid (UA) and creatinine (CR) (except blood glucose) were estimated using standard assay kits with the help of Clinical Biochemistry Analyzer (BS-120, Shenzhen Mindray, China) as per manufacturer's protocol/method described in assay kit of respective biochemical parameters. Blood glucose level was estimated using

Table 1:

Effect of daily oral administration of gemifloxacin or piperine alone (10 mg/kg body weight) for 5 days on hematological parameters in layer birds (Mean±SE, n=6)

Group	Day	TEC	HB	PCV	MCV	MCH	MCHC	TLC	L	H	M	E	PLTC
		10^6/µL	g/dL	%	fL	pg	g/dL	10^3/µL	%	%	%	%	10^3/µL
Gemifloxacin	0	2.71± 0.08	12.58 ±0.21	34.13 ±0.59	126.5 ±1.75	46.62 ±1.46	36.9 ±0.97	23.57 ±0.34	58.67 ±2.04	31.5 ±1.82	7.17 ±0.60	2.67 ±0.42	32.17 ±1.47
	1	2.71± 0.07	12.52 ±0.38	34.25 ±0.72	126.73 ±0.97	46.2 ±0.67	36.48 ±0.66	23.67 ±0.69	61 ±1.03	29.33 ±1.09	7.33 ±0.71	2.33 ±0.42	32.83 ±0.65
	2	2.70± 0.05	12.47 ±0.22	34.1 ±0.57	126.83 ±1.21	46.25 ±0.53	36.5 ±0.24	23.78 ±0.24	58.5 ±1.41	30.83 ±1.33	7.5 ±0.67	3.17 ±0.48	32.33 ±0.56
	3	2.68± 0.07	12.67 ±0.29	34.22 ±0.93	128.12 ±1.13	47.33 ±0.44	36.98 ±0.40	23.55 ±0.41	58.17 ±2.02	30.33 ±1.58	8.5 ±0.43	3 ±0.37	32.83 ±0.60
	4	2.69± 0.04	12.55 ±0.20	34.07 ±0.65	126.93 ±1.41	46.62 ±0.51	36.82 ±0.35	23.47 ±0.33	60.83 ±2.02	28.83 ±1.45	7.5 ±0.56	2.83 ±0.31	33 ±1.57
	5	2.68± 0.05	12.6 ±0.18	34.37 ±0.70	128.4 ±0.97	46.97 ±0.46	36.67 ±0.44	23.72 ±0.26	58.83 ±2.68	30 ±2.32	8.17 ±0.54	3 ±0.37	32.17 ±0.60
Piperine	0	2.62± 0.04	12.53 ±0.23	34.42 ±0.68	131.48 ±2.67	46.45 ±0.74	36.4 ±0.66	23.31 ±0.19	60.83 ±1.14	28.67 ±1.45	6.83 ±0.54	3.67 ±0.49	32.33 ±0.61
	1	2.76± 0.1	12.72 ±0.30	34.27 ±0.74	124.88 ±2.26	46.3 ±1.34	37.07 ±0.50	23.82 ±0.47	58.5 ±1.41	30.83 ±1.33	7.5 ±0.67	3.17 ±0.48	32.5 ±0.76
	2	2.75± 0.08	12.68 ±0.21	34.4 ±0.52	125.77 ±2.17	46.27 ±1.12	36.82 ±0.33	23.69 ±0.34	58.83 ±1.62	30.33 ±1.58	7.67 ±0.61	3.17 ±0.40	32.17 ±0.60
	3	2.68± 0.08	12.82 ±0.14	34.7 ±0.60	130.05 ±1.83	48.02 ±1.18	36.93 ±0.45	23.05 ±0.32	60 ±2.03	29.33 ±2.19	7.83 ±0.54	2.83 ±0.17	31.67 ±1.15
	4	2.69± 0.09	12.63 ±0.23	34.65 ±0.79	129.13 ±1.72	47.02 ±1.12	36.43 ±0.42	23.06 ±0.33	60.83 ±1.08	29.5 ±1.06	6.5 ±0.43	3.17 ±0.48	33.67 ±2.06
	5	2.68± 0.07	12.48 ±0.14	34.17 ±0.38	128.15 ±3.25	46.73 ±1.36	36.48 ±0.43	23.15 ±0.25	58.5 ±0.85	31 ±0.89	7.5 ±0.72	3 ±0.52	32.5 ±0.85

All values in treatment groups are not significantly different ($p < 0.05$) when compared to control (0 Day).

Table 2:
Effect of daily oral administration of gemifloxacin or piperine alone (10 mg/kg body weight) for 5 days on blood biochemical parameters in layer birds (Mean±SEM, n=6)

Group	Day	BG	ALT	AST	ALP	LDH	CK	TB	TP	ALB	UA	CR
		mg/dL	U/L	U/L	U/L	U/L	U/L	mg/dL	g/dL	g/dL	mg/dL	mg/dL
Gemifloxacin	0	225.5 ±4.31	13.13 ±0.14	198.25 ±12.31	93.42 ±6.42	643.86 ±6.60	336.54 ±4.85	0.15± 0.01	3.93 ±0.14	1.32 ±0.02	5.72± 0.12	0.31± 0.01
	1	226.67 ±3.08	13.09 ±0.17	199.61 ±10.36	96.99 ±6.65	645.04 ±7.90	334.27 ±7.89	0.16± 0.02	3.91 ±0.05	1.31 ±0.02	5.77± 0.08	0.31± 0.02
	2	227.33 ±6.26	13.11 ±0.17	201.9 ±16.46	91.69 ±6.59	643.04 ±4.49	341 ±5.96	0.15± 0.01	3.93 ±0.11	1.31 ±0.01	5.76± 0.21	0.32± 0.01
	3	227.33 ±5.20	13.08 ±0.07	202.65 ±10.39	92.18 ±5.44	644.91 ±6.25	338.78 ±5.61	0.15± 0.01	3.98 ±0.16	1.32 ±0.01	5.85± 0.13	0.31± 0.01
	4	226.5 ±6.74	13.06 ±0.05	202.65 ±12.48	91.32 ±7.03	641.1 ±5.98	337.75 ±4.79	0.14± 0.01	3.93 ±0.15	1.32 ±0.02	5.75± 0.13	0.32± 0.01
	5	226.17 ±3.56	13.17 ±0.15	198.52 ±13.84	88.81 ±6.37	643.97 ±6.00	341.55 ±7.24	0.16± 0.01	4 ±0.13	1.32 ±0.01	5.73± 0.15	0.31± 0.01
Piperine	0	227.67 ±5.77	13.1 ±0.08	202.5 ±14.90	91.92 ±4.75	644.36 ±6.31	337.16 ±5.38	0.16± 0.01	3.86 ±0.16	1.31 ±0.01	5.73± 0.15	0.32± 0.01
	1	221.83 ±4.57	13.06 ±0.14	205.07 ±9.46	90.77 ±6.23	645.32 ±5.59	341.91 ±5.70	0.16± 0.01	3.93 ±0.11	1.32 ±0.02	5.84± 0.16	0.31± 0.01
	2	222.83 ±4.85	13.14 ±0.15	201.75 ±6.99	97.08 ±7.74	643.69 ±3.23	342.36 ±6.93	0.15± 0.01	3.91 ±0.22	1.3 ±0.02	5.86± 0.1	0.32± 0.02
	3	228.33 ±4.06	13.13 ±0.09	203.68 ±14.20	88.6 ±4.89	643.2 ±6.19	339.57 ±6.12	0.16± 0.02	3.96 ±0.21	1.32 ±0.01	5.70± 0.09	0.32± 0.01
	4	223.83 ±4.60	13.1 ±0.13	209.69 ±12.77	87.07 ±5.77	651.04 ±5.89	340.69 ±3.92	0.15± 0.01	3.93 ±0.15	1.31 ±0.01	5.69± 0.06	0.30± 0.01
	5	225.5 ±5.17	13.01 ±0.18	203.27 ±9.19	91.53 ±5.86	648.17 ±3.09	342.94 ±6.65	0.16± 0.01	3.84 ±0.12	1.3 ±0.02	5.77± 0.09	0.31± 0.01

All values in treatment groups are not significantly different ($p < 0.05$) when compared to control (0 Day).

Glucose Monitor (Morepen Laboratories Limited, Delhi).

Statistical analysis

All the data have been presented as mean ± SE. Statistical comparison of the mean values in different groups was made using one-way analysis of variance (ANOVA), using software SPSS (Version 25). Significant differences ($p<0.05$) between different experimental groups were determined by Duncan's test.

RESULTS AND DISCUSSION

Clinical observation

All layer birds appeared active, clinically healthy and did not reveal any remarkable clinical signs during the entire experimental period. Besides there was no morbidity and mortality at all tested treatment levels/ groups throughout the period of experiment.

Hematological parameters

The mean values of total erythrocyte count (TEC), hemoglobin (HB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocyte count (TLC), differential leukocyte count (lymphocytes (L), heterophils (H), monocytes (M), eosinophils (E) and platelet count (PLTC) following oral administration of gemifloxacin and piperine at dose of 10 mg/kg/day for 5 days in layer birds are presented in Table 1. The average values of haematological parameters observed for consecutive five days in gemifloxacin or piperine treated layer birds did not differ significantly ($p<0.05$) from the corresponding values observed on day zero.

Biochemical parameters

The mean values of blood glucose (BG), serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), serum alkaline phosphatase (ALP), serum lactate dehydrogenase (LDH), serum creatine kinase (CK), serum total bilirubin (TB), serum total protein (TP), serum albumin (ALB), serum uric acid (UA) and serum creatinine (CR) following oral administration of gemifloxacin or piperine in layer birds are presented in Table 2. No significant alterations ($p < 0.05$) in biochemical parameters have been observed following oral administration of gemifloxacin or piperine at dose of 10 mg/kg/day for 5 days in layer birds compared to control (0 day).

The results of present studies are well supported by previous reports of non-significant change in hemato-biochemical parameters with no adverse effects or

clinical observation following repeated administration of gemifloxacin (320 mg, PO, once-daily for 5 or 7 days) in human (Lowe and Lamb, 2000; Allen *et al.*, 2001; Ball *et al.,* 2001, 2004; Tillotson *et al.,* 2004; Amitabh *et al.,* 2012; Visoriya *et al.,* 2014), gemifloxacin (10 mg/kg/day, PO, for 5 days) in broiler birds (Gohel *et al.*, 2018), levofloxacin (10 mg/kg, PO, at 12 h interval for 14 days) in layer birds (Patel *et al.*, 2009), gatifloxacin (10 mg/kg, PO, at 12 h interval for 14 days) in layer birds (Patel, 2010), piperine (20 mg/kg/day, PO, for 8 weeks) in rats (Bhat and Chandrasekhara, 1986[a]), Trikatu (piperine 3.4 % w/w) formulation (2000 mg/kg, PO, dose and at 5 mg/kg/day, PO, for 28 days) in Charles Foster rats (Chanda *et al.*, 2009), Benjakul (piperine, the major active component) formulation (100 mg and 200 mg tablets three times daily after meal for 14 days) in healthy Thai subjects (Amorndoljai *et al.*, 2011; Jumpa-ngern *et al.*, 2013) and marbofloxacin (5 mg/kg/day, PO, IM, for 5 days) alone and marbofloxacin (5 mg/kg/day, PO, IM, for 5 days) with piperine pretreatment (10 mg/kg/day, PO for 5 days) in rats (Chauhan, 2017).

The results of non-significant haemato-biochemical alterations following repeated oral administration of gemifloxacin or piperine at the dose rate of 10 mg/kg/day for five days is safe and well tolerated in layer birds.

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Short communication

AMELIORATION OF ARSENIC INDUCED REPRODUCTIVE TOXIC EFFECTS BY *ECLIPTA ALBA*

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ABSTRACT

The present study was designed to assess the ameliorating potential of dried powder of *Eclipta alba* plant (DPEA) @ 1000 and 2000 ppm concentration feed on reproductive toxicity parameters against arsenic induced toxicity in white leghorn cockerels. Various reproductive parameters including mass sperm motility and sperm count were examined after 90 day exposure of arsenic in cockerels. The mass motility of the sperms reduced significantly ($P \leq 0.05$) in groups II, IV and V as compared to control. There was a significant ($P \leq 0.05$) increase in the percentage of dead sperms in group II as compared to control or group I. There was a significant ($P \leq 0.05$) decline in sperm concentration of cockerels as compared to control in all the groups. DPEA at higher dose revealed improvement in altered reproductive indices. There was a significant ($P \leq 0.05$) decrease in the weight of the testes as compared to control group.

Key words: Arsenic, cockerels, semen quality, chronic, reproductive toxicity.

Arsenic causes several toxic effects on hepatic, cardio-pulmonary, renal, immunological and reproductive systems. *Eclipta alba,* also known as Bhringaraj, is widely used in India as a chologuague, in hepatic enlargement for jaundice and other ailments of the liver and gall bladder. The present study was undertaken to investigate the phytotherapeutic role of *Eclipta alba* in arsenic intoxicated poultry birds with the following objectives. Thirty five, four to six weeks old male white leghorn chicks, were randomly divided into five groups of seven birds each. The birds in poultry shed were kept in battery cage system under standard managemental conditions. After two weeks of adaptation period, 90 days feeding trial was started as per Table 2. The research project was approved by IAEC for conducting this investigation. After 90 days feeding trial, cockerels were humanely sacrificed, the abdomen was cut open. The right and left vas deferens were surgically excised from their point of entry at the cloaca to the inferior edge of kidneys. The contents of the vas deferens was collected in a test tube .During collection, the semen tube was maintained at 38-40°C with an insulated jacket. A water bath and a slide warmer were used to maintain the samples at this temperature until sperm motility was assessed. Sperm mass motility in neat semen was examined as per the guidelines given in Table 1.

The semen sperm concentration ($x10^6/mm^3$) was measured by using haemocytometer by the method of Allen and Champion (1955).

$C = 50,000 \times N \times D$,

Where

C = Concentration of sperms per mm^3 of diluted semen, N = Number of spermatozoa counted, D = Dilution rate.

The percentage of dead spermatozoa was done by differential staining (vital staining) technique. Stock solution of stain was prepared by dissolving 1gm eosin powder and 5 gmnigrosin powder in 100 ml of 2.9% aqueous solution of sodium citrate dehydrate. The semen sample was warmed up to 30º C and a drop was put on prewarmed slide. Then 2-3 drops of the stain was put and mixed gently. This mixture was kept at room temperature for 5 -10 minutes. Then it was spread

Table 1:
Grading of neat semen

S.No.	Type of Wave motion	Eddies/ 15 sec	Approx. initial motility (%)	Grade
1.	Less motility	0	5-10	Dead, 000
2.	Slight motility, no wave motion	0	10-20	Very poor, 00
3.	Not clear waves/eddies. Some throbbing movements.	<1	20-40	Poor, 0
4.	Not clear waves/eddies. Oscillating and circulating spermatozoa in the field.	1-2	50-60	Fair, +
5.	Slow moving waves/eddies appeared scattered in field	2-3	60-70	Better, ++
6.	Not so rapid waves/eddies. Swirls moving towards periphery.	3-4	70-80	Good, +++
7.	Extremely rapid waves/eddies. Origin and disappearance of waves is unclear.	4-5	80 and above	Very good, ++++

Table 2:
Effect on reproductive parameters following oral administration of different dietary levels of arsenic and DPEA in feed for 90 days in WLH cockerels (Mean ±SE, n = 7)

Groups Parameters	I	II	III	IV	V
Mass motility	3.60±0.24^{a}	2.20±0.20^{b}	3.40±0.24^{a}	2.60±0.24^{c}	2.80±0.20^{d}
Dead, %	23.80±1.56^{a}	39.00±3.19^{b}	25.40±3.33^{a}	27.00±0.89^{a}	24.20±0.58^{a}
Sperms conc (x10^{6}/mm^{3})	2.10±0.09^{a}	1.48±0.10^{b}	2.02±0.05^{c}	1.93±0.09^{d}	2.03±0.07^{ce}

Means bearing different superscripts a, b, c, d and e differ significantly (P≤0.05) with columns in a row. Groups I = control with normal feed, II = arsenic@100ppm, III = arsenic@ 100ppm + silymarin, IV = arsenic@100ppm+ *Eclipta alba*@1000ppm and and V = arsenic@100ppm+*Eclipta alba* @ 2000ppm.

into a thin smear and was allowed to dry in the air.

The smear was examined under oil immersion. The head of the dead spermatozoa appeared pink against the blue-blackish background while live appeared colourless. 200 sperms in different fields in random zigzag manner were counted by the following formula. The data was statistically analysed by using ANOVA technique for significant difference in the values of different groups as 5% level of significance.

$$\% \text{ of dead sperms} = \frac{\text{No. of dead spersm counted}}{\text{Total number of dead spers}} \times 100$$

The mass motility of the sperms reduced significantly (P< 0.05) in groups II, IV and V as compared to control. Group III showed no significant (P<0.05) changes as compared to control group. There was a significant (P < 0.05) increase in the percentage of dead sperms in group II as compared to control or group I. Group III, IV and V showed no significant (P<0.05) change as compared to control group (Table 2).

There was a significant (P≤0.05) decline in sperm concentration of cockerels as compared to control in all the groups. The sperm concentration in the II group showed the highest decline. The decrease in group II and IV was more as compared to group III and V, group III, IV and V were significant (P≤0.05) when compared to group II (Table 2). There was a significant (P≤0.05) decrease in the weight of the testes as compared to control group. Group II showed the highest decline in the weight of the testis.

Arsenic is known to have detrimental effects on reproductive system, it selectively accumulates in the testes and epididymis (Kim and Kim, 2015).

There was a significant (P≤0.05) increase in dead sperm percentage and decrease in sperm concentration in the cockerels of group II as compared to control at the end of trial. The mass motility of the sperms in the II group decreased significantly (P≤0.05).The parameters reflecting male infertility include low sperm count and motility. Low sperm count has been associated with decreased production in the testis, their retention in the seminiferous tubules and phagocytosis or blockage in the excurrent ducts (Mathur *et al.*, 2010). Significant decrease in the sperm count of the cockerels could be the result of depletion of germ cell lineage supported by the observed vacuolization in seminiferous tubules or due to failure in the release of differentiating spermatids. Our findings are in agreement with the findings of Pant *et al.* (2004), who also reported the decreased sperm count, motility and increase in abnormal sperm concentration in adult mice following arsenic exposure.

The DPEA treated groups showed results reaching normalcy. These groups showed non-significant changes in the dead sperm per cent and sperm concentration as compared to control which supports our findings of DPEA treated groups reaching normalcy. Arsenic induced oxidative stress created cellular toxicity and increased vulnerability of spermatozoa to oxidative stress due to low activity of antioxidant enzymes and high PUFA content. This effect of arsenic was counteracted by *Eclipta alba* known to have antioxidant potential (Zhen *et al.,* 2007). It is concluded from this study that arsenic @ 100 ppm in diet produced reproductive toxic effects which were ameliorated by feeding of powdered *Eclipta alba* @ 1000 and 2000ppm in a 90 days study in cockerels.

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