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Review Article

LIPOSOMES: A NOVEL TRANSPORTER FOR VETERINARY PHARMACEUTICAL AGENTS

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ABSTRACT

Liposomes are nanosized phospholipid vesicles that can serve as delivery platforms for a wide range of therapeutic substances. Liposomes are easily formulated, highly modifiable and easily administered delivery platforms. Liposomes are biodegradable and nontoxic and have long *in vivo* circulation time. Targeting of liposome with various receptor help in site specific distribution of drug at active site. Liposomal drugs exhibit reduced toxicities and retain enhanced efficacy compared with free drugs. Incorporating agents that have intrinsic imaging properties into liposomes can create platforms that provide concomitant therapeutic and diagnostic functions.

Key Words: Liposomes, novel transporter, liposome drug delivery system

INTRODUCTION

About 90% of potential therapeutic agents have low bioavailability and poor pharmacokinetics. In order to provide better therapeutic efficacy, the pharmacological agents can be incorporated into novel drug delivery systems. These nanodrug delivery systems include polymeric nanoparticles, magnetic nanoparticles, nanocrystals, nanoemulsions and liposomes. These nanodrug delivery systems are known to enhance the therapeutic indices of the incorporated drugs through a number of ways. These delivery systems protect the drug from the internal body environment, improve the bioavailability and pharmacokinetics by improving site specific delivery (Irache *et al.,* 2011). Among various drug delivery systems, several liposome based therapeutic agents in animals have been evaluated during the past decade and have been demonstrated to be highly versatile and easy to modify and are relatively simple to formulate (Torchilin, 2005).

LIPOSOME: STRUCTURE, COMPOSITION AND TYPES

The name liposome is derived from two Greek words: 'Lipos' meaning fat and 'Soma' meaning body. Liposome is defined as "microscopic spherical vesicles that form when phospholipids are hydrated or exposed to an aqueous environment" (Bangham *et al.,* 1965). Liposome as a microstructure consisting of one or more concentric spheres of lipid bilayer separated by water or aqueous buffer compartments (Weiner *et al.*, 1989). Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural non-toxic phospholipids. The typical characteristic of bilayer forming lipids is their amphiphilic nature: a polar head group covalently attached to one or two hydrophobic hydrocarbon tails. When these lipids, e.g., phosphatidylcholine or phosphatidyl glycerol are exposed to an aqueous environment, interactions between themselves lead to spontaneous formation of closed bilayers (Frezard, 1999). The incorporation of cholesterol into the lipid bilayer membrane improves the stability of liposomes in serum (Senior and Gregoriadis, 1982).

There are mainly three types of liposome. These are multilamellar vesicle, large unilamellar vesicle and small unilamellar vesicle. Multilamellar vesicles consist of several concentric bilayers and have range in size from 500 to 5000 nm. They are ideal for trapping hydrophobic drugs in additional lamellae. Large unilamellar vesicles consist of one concentric lipid bilayer surrounding a large inner aqueous environment and have range in size from 200 to 800 nm. They are ideal for trapping large amounts of hydrophilic drugs. Small unilamellar vesicles consist of one concentric bilayer and small size in the range of 100 nm. They are ideal for long-term circulation (Torchilin, 2005).

ADVANTAGES OF LIPOSOME DRUG DELIVERY SYSTEM

Liposomes increase efficacy and therapeutic index of drug and stability of entrapped drug via encapsulation. They are non-toxic, flexible, biocompatible, completely biodegradable and non immunogenic for systemic and non-systemic administrations and reduce the toxicity of the encapsulated agent and reduce the exposure of sensitive tissues to toxic drugs production. They have flexibility to couple with site-specific ligands to achieve active targeting (Himanshu *et al.,* 2011).

AN EFFECTIVE DELIVERY SYSTEM

Liposomes serve as effective delivery platforms due to several favourable characteristics. They can encapsulate both hydrophobic and hydrophilic compounds and can be used for intracellular drug delivery (Rose *et al.*, 2005). Moreover, the vesicle size, surface charge, and surface properties can be easily modified using different

compounds and preparation parameters (Rose *et al.*, 2005). For example, adding polymers such as poly(ethylene) glycol (PEG) to the liposomal surface (PEGylation) can create long-circulating liposomes that can evade capture from the reticuloendothelial system (RES), stay in the body longer and demonstrate extended-release of the encapsulated drug over time (Lukyanov *et al.,* 2004).

Moreover, attaching antibodies and other markers to liposome surfaces can allow for diagnostic imaging and targeted therapy (Torchilin, 2005). When liposomal based drug products circulate in body the release of drug done by external stimuli such as temperature and pH (Kono, 2001).

pH - sensitive liposome

pH-sensitive liposomes are constructed from pH-sensitive components. After being endocytosed in the intact form, due to lower pH inside the endosome, pH sensitive liposome fuse with endovascular membrane and release their content into cytoplasm. Serum-stable, long-circulating PEGylated pH-sensitive liposomes were prepared using a combination of PEG and a pH-sensitive, terminally alkylated co-polymer of N-isopropylacryl amide and methacrylic acid (Roux *et al.,* 2004).

PHARMACOKINETIC CONSIDERATIONS

Through encapsulation of drugs in liposomes, the volume of distribution is significantly reduced and the concentration of drug at the desired site of action increased. Liposomal drug delivery increases the amount of drug that can be effectively delivered to tumor sites in anticancer therapy (Martin, 1998). Liposomes given intravenously usually interact with plasma high density lipoproteins and the opsonins, which bind to the surface of vesicles and mediate their endocytosis by macrophages. The rate of liposome clearance from blood circulation will, therefore, depend on the ability of opsonins to bind to the liposome surface. The rate can be manipulated through appropriate selection of liposome characteristics (Gregoriadis, 1990). For instance, "fluid" vesicles are removed more rapidly from blood circulation than "rigid" ones. Clearance from the blood stream is also influenced by vesicle size and surface charges (Liu and Liu, 1996). The longest half-life is obtained when liposomes are relatively small (diameter < 0.05μm) and carry no net surface charge (Frezard, 1999). The pharmacokinetic behavior of liposomes depends on the route of injection such as intraperitoneal, subcutaneous or intramuscular route (Qussoren *et al.*, 1997).

BIOMEDICAL APPLICATION OF LIPOSOME

The characteristics of liposomes demonstrate their potential in several areas of veterinary medicine. In particular, liposomes can serve as potent delivery platforms for transport of cancer therapeutics, vaccine, analgesic drugs, antimicrobials and in diagnostic imaging procedure (Table 1).

1. Cancer chemotherapeutics

Modern cancer therapy involves the use of several antineoplastic chemotherapeutic drugs. These drugs are potent at eliminating cancer cells *in vitro* but are observed to have significant barriers to *in vivo* efficacy (Egusquiaguirre *et al.,* 2012). These barriers include a lack of selectivity for cancer cells, low bioavailability at tumor sites, larger volumes of distribution, and toxicity to normal tissues (Bae *et al.,* 2011). Nanotechnology-based drug delivery systems, such as liposomes, can overcome these barriers through a variety of mechanisms. Due to their small size (10–100nm), they are ideal for intracellular uptake, have high encapsulation capacities and can be designed for specific targeting of tumor cells (Bae *et al.,* 2011; Egusquiaguirre *et al.,* 2012). Furthermore, leaky microvasculature and highly impaired lymphatic drainage of tumor can allow the accumulation of liposome within the tumor (Egusquiaguirre *et al.,* 2012). Liposomes have demonstrated a promising potential for delivery of anticancer drugs in animals.

Study conducted in dogs having sarcoma and carcinoma on various part of body, treated with doxorubicin, encapsulated LTSL (Hauck *et al.,* 2006). Another study conducted in 10 cats with advanced feline soft tissue sarcomas. Cats were treated with doxorubicin encapsulated liposomes concomitantly with daily palliative radiotherapy. Result showed that out of 10 cats 7 cats achieving partial (=5) or complete (=2) response with a median response duration of 237 days (Kleiter *et al.,* 2010).

Table 1:
Liposome based drugs used in various clinical conditions in animals and birds

Species	Agent	Disease	References
Dogs	Doxorubicin	Spontaneous canine tumors	(Hauck *et al.,* 2006)
Cats	Doxorubicin in conjunction with radiotherapy	Soft-tissue sarcoma	(Kleiter *et al.,* 2010)
Dogs	HSA cell lysates	Canine hemangiosarcoma	(U'Ren *et al.,* 2007)
Chickens	*Salmonella fimbriae* proteins	*Salmonella enterica* vaccine	(Li *et al.,* 2004)
Dogs	Endostatin DNA	Soft-tissue sarcoma	(Kamstock *et al.,* 2006)
Green-cheeked conures	Butorphanol tartrate	Experimentally induced arthritic pain	(Paul-Murphy *et al.,* 2009)

Hafeman *et al.* (2010) studied in 5 dogs suffering from malignant histiocytosis. When given liposomal clodronate (0.5 ml/kg over a 60-min period) for three treatment at 2 weeks interval, there was significant reduction in tumor volume in dogs.

There are certain new ligands which were found in laboratory animal study for targeting tumor cells are antibodies, folate, transferring, vasoactive intestinal peptide and chondroitin sulphate. Internalizing antibodies are required to achieve a much-improved therapeutic efficacy with antibody-targeted liposomal drugs.The antibody CC52 is directed against rat colon adenocarcinoma CC531 lines. When, these antibodies are attached to PEGylated liposomes, there was specific accumulation of liposomes in a rat model of metastatic CC531 (Kamps *et al.*, 2000). Targeting tumors with folate-modified liposomes represents a popular approach, because folate receptors (FR) are frequently over expressed in a range of tumor cells.The folate modified doxorubicin loaded liposomes are successfully used in the treatment of acute myelogenous leukaemia (Pan *et al.,* 2002). Transferrin (Tf) receptors (TfR) are over expressed on the surface of many tumor cells and so antibodies against TfR, as well as Tf itself, are popular ligands for liposome targeting to tumors and inside tumor cells (Hatakeyama *et al.,* 2004). Tf-coupled doxorubicin-loaded liposomes demonstrate increased binding and toxicity against C6 glioma cells of rats (Eavarone *et al.,* 2000).Long circulating PEGylated liposomes are used for intracellular delivery of cisplatin into gastric cancer (Iinuma *et al.,* 2002). Vasoactive intestinal peptide (VIP) has been used to target PEG liposomes with radionuclides to VIP receptors on the surface of tumor cells and resulted in enhanced breast cancer inhibition in rats (Dagar *et al.,* 2003). Cisplatin-loaded liposomes that specifically bind to chondroitin sulphate, which is over expressed in many tumor cells, have been used for the successful suppression of tumor growth and metastases *in vivo* (Lee *et al.,* 2002).

2. Delivery of vaccine

There has been considerable research on the use of nanosized based delivery systems such as liposomes for delivering adjuvants that can enhance the immunogenicity of novel vaccines. Virosomes are the cornerstone for the development of new vaccines. In these applications, the liposome surface was modified with fusogenic viral envelope proteins (Kaneda, 2000).These systems can potentially enhance immunogenicity through a number of ways. First, many nanoparticles can mimic pathogen-associated molecular patterns, activating innate immune response through pattern recognition receptors. Second, nanoparticles such as liposomes are taken up preferentially by antigen presenting cells resulting in an enhanced T-cell activation.In particular, cationic liposomes serve as potent vaccine design platforms due to their ability to bind with DNA and elicit an immune response. Furthermore, some nanoparticles can be constructed with virus like particles on their surface thereby providing the necessary immune stimulation without the actual virus DNA that can cause infection. Finally, delivery systems such as liposomes can act as targetable depot formulations that provide extended delivery of antigen to a specific location for a designated amount of time. Due to the potentially favourable characteristics of liposomes for vaccinations against a range of veterinary pathogens, liposome-based vaccination in food animals has generated much research interest in the past decade. In humans, Studies showed that virosomes containing spike proteins of influenza virus on their surface elicits high titres of influenza specific antibodies (Huckriede *et al.,* 2003). Clinical trials of virosome influenza vaccine in children showed that it is highly immunogenic and well tolerated (Herzog *et al.,* 2002). A similar approach was used to prepare virosomal hepatitis A vaccine that elicited high antibody titres after primary and booster vaccination of infants and young children (Usonis *et al.,* 2003). In a study in 2009, with non parenteral administration (eye drop) of liposome encapsulated inactivated APEC (avian pathogenic *E. coli*) as a vaccine inoculated in 120 chickens showed that IgA and IgG antibodies in their oral mucus. When subsequently challenged with a live strain of APEC showed good immunity against infection (Yaguchi *et al.,* 2009). Comparative study done between Lasota vaccine and liposome encapsulated NDV vaccine in chicken. Result showed that the antibody production and cell counts were significantly higher in the birds vaccinated with the liposomal ND vaccine (Onuigbo *et al.,* 2012). Sawicka *et al.* (2012) used liposomes encoded with MIC3 protein which is active against *T. gondii* infection in ewe. This vaccine when given to ewes, elicited strong immune response. This warrants further study for vaccine design in livestock.

3. Transport of analgesic drugs

Liposomes have been demonstrated to act as depot formulations for pain medication. Technological advances in recent years have made it possible for the incorporation of many different types of analgesics into liposomes. Opioids remain the most widely studied analgesic drugs for liposomal delivery. A rodent model of monoarthritis administered epidural LE-hydromorphone compared with plain hydromorphone. Result showed that thermal pain threshold increased after 2 hours in plain hydromorphone however, it increased after 72 hours in case of LE-hydromorphone. This shows that LE-hydromorphone prolongs the duration of analgesia compared with free drug (Schmidt *et al.,* 2011).

The ability of liposomes to target antibiotics to the site of a facultative intracellular bacterial infection has been examined in several *in vitro* cultures and experimental animal models. Liposomal formulations have been

compared with free drug formulations against various bacteria. Rifampin delivered weekly for two weeks in tuftsin-bearing liposomes was at least 2,000 times more effective than the free drug in lowering the load of lung bacilli in infected mice (Agarwal *et al.*, 1994). Liposome encapsulated clarithromycin is more effective than the free form against *Mycobacterium avium* infections in a model of intramacrophage derived from human blood (Onyeji *et al.,* 1994).

4. Antifungal therapy

Despite the advent of the newer, less toxic azole antifungal drugs, amphotericin B (AmB) remains the preferred drug for the treatment of most serious systemic fungal infections. This is, in part, due to the increase in fungal diseases world-wide such as candidiasis, aspergillosis, cryptococcosis and histoplasmosis. C clinicians prefer the use of other antifungal drugs such as ketoconazole, itraconazole or fluconazole, alone or in combination with AmB in order to reduce its dose, hence, reducing possibilities of nephrotoxicity.Thus, the toxicity of AmB, either acute or chronic, has limited its clinical use. It has been established that liposomal AmB (LAmB) is more effective and less toxic than conventional preparation for the treatment of critically ill patients. Most of the increase in therapeutic effect is thought to be the result of a decrease in toxicity, which allows larger dosages of AmB to be used safely. High doses of LAmB to treat dogs with blastomycosis with different degrees of severity (localized and disseminated) achieving successful outcomes and none of the patients developed renal failure (Krawiec *et al.,* 1996). The inhalation of aerosolized LAmB, for localized fungal pneumonias, AmB was not detected in the serum and other organs such as liver, kidneys and brain. This indicates that the toxic side-effects in other tissues are minimal (Lambros *et al.,* 1997).

5. Antiparasitic therapy

The persistence of Praziquintal (PZQ) concentrations in the liver after the administration of liposomal PZQ compared with free PZQ (10 days and 1 h, respectively) suggest the use of LPZQ as a chemoprophylactic treatment of schistosomiasis (Akbarieh *et al.,* 1992). Liposomal mebendazole has also shown increased activity and reduced side effects in the treatment of echinococcosis (Croft, 1986).

6. Liposome in diagnostic imaging procedure

Liposomal contrast agents have been used for experimental diagnostic imaging of liver, spleen, brain, cardiovascular system, tumors, inflammation and infections (Torchilin, 1996).Computerized tomograhphy contrast agents (primarily heavily iodinated organic compounds) can be included in the inner water compartment of liposomes or incorporated into the liposome membrane. For example, iopromide has been incorporated into plain and PEGylated liposomes and demonstrated favourable biodistribution and imaging potential in rats and rabbits (Sachse *et al.*, 1993; Sachse *et al.*, 1997). Computed tomography images of peptide-targeted 64Cu-liposome distribution in a mouse xenograft have been shown by Jensen (2014).

Liposomes for sonography are prepared by incorporating gas bubbles (which are efficient reflectors of sound) into the liposome or by forming the bubble directly inside the liposome as a result of a chemical reaction, such as bicarbonate hydrolysis yielding carbon dioxide. Gas bubbles stabilized inside the phospholipid membrane demonstrate good performance and low toxicity in rabbit and porcine models.

STORAGE OF LIPOSOMAL DRUG PRODUCTS

Liposomal products are potentially prone to hydrolytic degradation and leakage. Hence, it is desirable to freeze-dry the suspension to a powder and store in dried form (Kirby and Gregoriadis, 1984). The powder can be reconstituted to an aqueous suspension immediately before use. By doing so, small unilamellar vesicles (SUVs) may be converted to multilamellar vesicles (MLVs) dispersion upon rehydration. Addition of a carbohydrate (trehalose) during freeze-drying prevents fusion and leakage of the vesicles (Crowe *et al.,* 1986). Lyophilization increases the shelf life of liposomal product at 4°C for 6 months (Ghanbarzadeh *et al.,* 2013).

DISADVANTAGES OF LIPOSOME DRUG DELIVERY SYSTEM

Phospholipids used in preparation of liposomes undergo oxidation and hydrolysis when there is temperature or pH variation (Himanshu *et al.,* 2011).Leakage and fusion of encapsulated drug (Himanshu *et al.,* 2011). Production cost for preparation of liposomal drug formulation is high. Product required freeze drying. Despite the fact that liposomes are nontoxic, liposomes, lipid micelles and solid-lipid nanoparticles are known to cause acute hypersensitivity reactions. These reactions are putatively caused by the activation of the complement by the surface of the lipid particles and can be studied in animal sensitivity models. In a pig sensitivity model, the most commonly observed adverse effects were shown to be anaphylactoid shock, characterized by pulmonary hypotension and cardiac arrhythmias (Szebeni *et al.,* 2007).

FUTURE DIRECTIONS

Further trials in large-scale animal studies are required before several liposome-based therapeutics being translated for widespread clinical and commercial use. Liposomes can be engineered to form hybrids with semiconducting nanocrystals called quantumdots that have novel magnetic and imaging properties and also be loaded with a chemotherapeutic to easily target various organs and to kill cancer cell *iv-vivo.* In addition to curative therapies, liposomes may also be used for dietary

supplementation in animals. There is a possibility in the future of liposomes being used to supplement a broad range of trace minerals and vitamins to prevent morbidity in companion and farm animals.

CONCLUSIONS

Finally, liposomes are easily formulated, highly modifiable and easily administered delivery platforms for veterinary pharmaceutical agents. Experimental studies revealed that liposome based therapeutic agents are highly versatile, easy to modify and simple to formulate. They are promising agents in intracellular delivery of cancer chemotherapeutic agents, analgesic agents, antimicrobial agents, vaccine and diagnostic imaging properties.

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Research Article

A STUDY ON DETERMINATION OF MTD AND EFFECT OF SUBACUTE EXPOSURE OF THIACLOPRID AND ITS AMELIORATION BY RESVERATROL IN MALE RATS

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ABSTRACT

The aim of present study was to ascertain the maximum tolerated dose (MTD) and to investigate the effects of subacute toxicity of thiacloprid on b wt gain and relative organ weight and its amelioration by resveratrol in adult male Wistar rats. The MTD of thiacloprid was determined to be 345 mg/kg orally in male rats in a pilot dose range finding study. Animals were observed for toxic signs and symptoms after oral administration of thiacloprid in single dose. Rats were randomly allocated into six groups (n = 6 rats /group). Thiacloprid was administered orally to two group of rats by gavage once daily for 28 days at doses of 10% MTD and 5% MTD. Resveratrol co-treatment was also given in thiacloprid administered groups. B wt of each rat was recorded on day 0 and at an interval of two days till the completion of experiment and changes in weight of various body organs (liver, kidney, brain, and spleen) were recorded after sacrifice. The sign and symptoms of toxicity were piloerection, decreased motility and reactivity, convulsions, tremors, tachypnea, dyspnea, labored breathing and red incrusted snout. Thiacloprid treatment resulted in decreased b wt gain as compared to the naïve. A significant change was also observed in relative organ weight in thiacloprid administered groups as compared to naïve which was restored by resveratrol co-treatment. The study revealed a mild to moderate toxic effect of thiacloprid on b wt gain and relative organs weight.

Keywords: Thiacloprid, resveratrol, MTD, b wt gain, relative organ weight

INTRODUCTION

Thiacloprid [3-(6-chloro-3-pyridylmethyl)-1, 3-thiazolidin-2-ylidenecyanamide (IUPAC)], is an insecticide of the neonicotinoid class. It is effective against sucking insects on plants and companion animals, against turf insects and some beetles. Due to its systemic activity, thiacloprid is extensively used for soil application, seed and foliar treatment. Like other neonicotinoids, thiacloprid shares structural similarity and a common mode of action with the tobacco toxin, nicotine (EPA, 2005). It acts by disrupting the insect's nervous system through acting as a nicotinic acetylcholine receptor inhibitor (Osterauer and Kohler, 2008). Thiacloprid has genotoxic and cytotoxic effects; it significantly increases the micronucleus in rat bone marrow cells (Sekeroglu *et al.,* 2011) and in human peripheral blood lymphocytes (Kocaman *et al.*, 2012). Thiacloprid intoxication induces endocrine disturbance which may lead to tumor and hypertrophy of glands (Sekeroglu *et al.*, 2014). Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin produced naturally by several plants in response to injury or when the plant is under attack by pathogens such as bacteria or fungi (Fremont, 2014). The major dietary sources of stilbenes include grapes, wine, soy, peanuts, and peanut products (Cassidy *et al.,* 2000).

The determination of maximum tolerated dose (MTD) is imperative for toxicity study of insecticides. The information is lacking on MTD of thiacloprid by oral route and ameliorating effect of resveratrol on b wt gain and relative organ weight parameters following its exposure in adult male Wistar rats. Therefore, the present study was undertaken to determine MTD of thiacloprid orally and assess the effect of sub acute toxicity of thiacloprid on b wt gain and relative weight changes of liver, kidney, brain and spleen and its amelioration by resveratrol in male rats following oral administration.

MATERIALS AND METHODS

Thiacloprid formulation (Alanto 240 SC, Thiacloprid 21.7%) was commercially obtained from authorized dealer of Bayer Cropscience Limited, Sabarkantha, Gujarat and resveratrol from Sigma-Aldrich Company. Adult male Wistar rats weighing between 120 to 140 g were procured from Disease Free Small Animal House, LUVAS, Hisar. The animals were acclimatized to laboratory conditions for 7 days before start of experiment. Animal house temperature varied between 22 to 27º C throughout the study. The prior approval of institutional animal ethics committee was obtained for the use of laboratory animals.

Determination of MTD:

MTD of thiacloprid was determined in male rats by oral route following the method as described by Moser and Padilla (1997). The pilot dose range finding studies were conducted in small groups of rats (n=3) using several

doses including few lethal doses. Single dose of thiacloprid was administered in a group of 3 animals and observations were made at various time intervals. Thereafter, several iterations were conducted to determine MTD. Out of these doses, a maximum dose was selected that produced clear signs of toxicity but not resulting in lethality i.e. maximum tolerated dose (MTD).

Effect on b wt gain and relative organ weight

Rats were divided in 6 groups, each comprising of 18 rats.

Group 1: Naïve control: 3% gum acacia suspension was given once daily orally for 28 days.

Group 2: Thiacloprid (10% THIA): Thiacloprid suspension (MTD/10) in 3% gum acacia was administered once daily orally for 28 days.

Group 3: Thiacloprid (5% THIA): Thiacloprid suspension (MTD/20) in 3% gum acacia was administered once daily orally for 28 days.

Group 4: Resveratrol (2 mg/kg): Resveratrol (2 mg/kg) in 3% gum acacia was administered orally in a single dose for 28 days.

Group 5: Thiacloprid (10% THIA) + Resveratrol (2 mg/kg): Resveratrol (2 mg/kg) and thiacloprid suspension (MTD/10) in 3% gum acacia were administered separately once daily orally for 28 days.

Group 6: Thiacloprid (5% THIA) + Resveratrol (2 mg/kg): Resveratrol (2 mg/kg) and thiacloprid suspension (MTD/20) in 3% gum acacia were administered separately once daily orally for 28 days. A gap of 12 hours was maintained between resveratrol and thiacloprid administration.

The relative organ weights were calculated by using formula: organ weight / b wt X 100.

Statistical analysis

Data were expressed as Mean ± SE. One way ANOVA along with Duncan's multiple comparison tests as post-hoc test using SPSS 16.0 (SPSS Inc., 233 South Wacker Drive, 11th Floor, Chicago) for a computer programme. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Following the pilot dose range finding experiment, MTD of thiacloprid in adult male Wistar rats was found to be 345 mg/kg b.wt. by oral route (Table 1). Effect of various doses used for determination of MTD on gross observable behavior was noted, in which toxic symptoms started in 40-50 min after thiacloprid administration and were found to be dose dependent on onset and severity of effect. The main clinical signs were piloerection, decreased motility and reactivity, poor reflexes, spastic gait, spasmodic state, convulsions, tremors, tachypnea, diarrhea, narrowed palpebral fissure, closed eyelids and red incrusted snout. Dyspnea and labored breathing, were observed just before death.

Following administration of thiacloprid and resveratrol, body weights of rats were recorded on alternate day till completion of experiment and are presented in Table 2. Thiacloprid treatment alone at both the dose levels (10% THIA and 5% THIA) significantly ($p<0.05$) reduced the b wt gain in dose dependent manner as compared to naïve group animals in 28 days trial. Resveratrol co-treatment in thiacloprid administered animals significantly restored ($p<0.05$) b wt gain in 10% THIA + RT group as compared to 10% THIA group of rats while resveratrol co-treatment restored the weight gain non significantly ($p<0.05$) in 5% THIA + RT as compared to 5% THIA group of rats in 28 days exposure.

The weight gain in animals serves as index of growth rate (Palani *et al.,* 1999). A significant reduction in b wt gain (g) was observed in present study in groups treated with thiacloprid while non-significant decrease in weight gain was observed in group treated with resveratrol alone in 28 days exposure. Resveratrol co-treatment in thiacloprid treated group significantly increased the weight gain in 28 days trial. This may be because of the hepato-protective effect of resveratrol or by reduction in protein catabolism caused by thiacloprid.

Our results are in agreement with Bhardwaj *et al.* (2010), where similar results of loss in b wt gain in an oral toxicity study of imidacloprid in female rats with doses of 0, 5, 10, 20 mg/kg b.wt./day for 90 days were observed. Decrease in b wt gain was observed at 20 mg/kg b.wt./day and no mortality occurred during treatment period, while food intake was reduced at high dose level.

In present study, reduced b wt gain in resveratrol administered groups may be because of anti-obesity effects of resveratrol on adipocytes in body storage tissues. Resveratrol also decreases adipogenesis and viability in maturing preadipocytes, mediated through down-regulating adipocyte specific transcription factors and enzymes and also by genes that modulate mitochondrial function. Resveratrol also increased lipolysis and reduced lipogenesis in mature adipocytes (Baile *et al.,* 2011).

The relative organ weight of vital organs of male rats in all treatment groups is expressed as g/100 g b. wt. and presented in Table 3 respectively. A statistically significant increase ($p<0.05$) was observed in liver weight in both 10% THIA and 5% THIA groups as compared to naïve group rats in 28 days exposure. Resveratrol co-treatment significantly decreased ($p<0.05$) the liver weight in 10% THIA + RT and 5% THIA + RT group rats as compared to 10% THIA and 5% THIA treatment group rats in 28 days trial. A statistically significant increase ($p<0.05$) was observed in kidney weight in 10% THIA treatment group rats as compared to naïve group of rats while no significant change in weight of kidney was observed in 5% THIA group as compared to naïve group rats in 28

Table 1.
Maximum tolerated dose (MTD) of thiacloprid administered orally in adult male Wistar rats

Dose (mg/kg)	Number of rats died/number of rats administered	Percent mortality
400	3/3	100
380	2/3	66.66
360	1/3	33.33
350	1/3	33.33
345	0/3	00
340	0/3	00
335	0/3	00
330	0/3	00

MTD of thiacloprid: 345 mg/kg b.wt. Orally

Table 2.
Effect of thiacloprid on absolute b wt gain (g) and its amelioration by resveratrol in adult male rats in 28 days study

Treatment	28 days
Naïve	$48.83^{a} \pm 2.57$
RT	$45.17^{ab} \pm 2.39$
10% THIA	$30.33^{d} \pm 1.83$
5% THIA	$36.83^{c} \pm 1.30$
10% THIA+ RT	$38.83^{c} \pm 1.47$
5%THIA + RT	$41.5^{bc} \pm 2.68$

Values are Mean ± S.E; n=6; Values bearing common superscripts within a column do not differ significantly ($p<0.05$)

in weight of liver in 10% THIA and 5% THIA group rats. Our results are in accordance with Bhardwaj *et al.* (2010) where a significant increase in relative liver weight at 20 mg/kg b.wt./day dose was reported. Liver is the main organ of thiacloprid metabolism and thus, physiologically the liver will be affected directly by thiacloprid during the period of exposure. In present study, resveratrol co-treatment significantly reduced the increased relative liver weight by thiacloprid exposure. Resveratrol resulted in reduced weight gain by decreasing the levels of hepatic carnitine palmitoyl transferase and acyl-coenzyme A oxidase (Gómez-Zorita *et al.,* 2012).

Thiacloprid treatment significantly increased the relative kidney weight in comparison to naïve. Resveratrol co-treatment decreased the relative kidney weight in the present study. Our results are in accordance with Palsamy *et al.* (2011) where resveratrol treatment revealed improvement in kidney weight showing its renoprotective nature. This is possibly through the anti-oxidant potential of resveratrol.

No significant changes were observed in weight of heart. It indicates thiacloprid and resveratrol have no significant effect on brain. Spleen weight was reduced in thiacloprid treatment groups as compared to naïve. Our results are in agreement with the study of Vohra *et al.*

Table 3.
Effect of thiacloprid on relative organ weights (g/100 g b.wt.) and its amelioration by resveratrol in adult male rats in 28 days study

Treatment	Organs			
	Liver	Kidney	Brain	Spleen
Naïve	$3.75^{a} \pm 0.19$	$0.59^{a} \pm 0.01$	$0.95^{a} \pm 0.03$	$0.36^{a} \pm 0.03$
RT	$3.24^{a} \pm 0.15$	$0.60^{a} \pm 0.02$	$0.93^{a} \pm 0.03$	$0.35^{a} \pm 0.03$
10% THIA	$4.97^{d} \pm 0.16$	$0.81^{b} \pm 0.05$	$0.99^{a} \pm 0.01$	$0.23^{c} \pm 0.02$
5% THIA	$4.59^{c} \pm 0.17$	$0.68^{a} \pm 0.02$	$0.94^{a} \pm 0.03$	$0.27^{bc} \pm 0.02$
10% THIA+ RT	$4.21^{c} \pm 0.31$	$0.68^{a} \pm 0.01$	$0.97^{a} \pm 0.04$	$0.30^{bc} \pm 0.01$
5%THIA + RT	$4.19^{b} \pm 0.25$	$0.63^{a} \pm 0.03$	$0.91^{a} \pm 0.02$	$0.33^{ab} \pm 0.03$

Values are Mean ± S.E; n=6; Values bearing common superscripts within a column do not differ significantly ($p<0.05$)

days study. Resveratrol co-treatment significantly decreased ($p<0.05$) the kidney weight in 10% THIA + RT as compared to 10% THIA treatment group. No significant changes were observed in weight of brain in all groups. Thiacloprid treatment alone at both the dose levels significantly reduced ($p<0.05$) the spleen weight in dose dependent manner as compared to naïve group animals in 28 days trial. Resveratrol co-treatment restored the spleen weight non significantly ($p<0.05$) in 10% THIA + RT and 5% THIA + RT group rats as compared to 10% THIA and 5% THIA treatment group rats.

In toxicological studies, relative organ weights are important criteria for evaluation of organ toxicity (Crissman *et al.,* 2004). Generally in sub-acute toxicity studies relative weight of affected organ increases.

A statistically significant increase was observed

(2014) where similar results were observed in Swiss albino mice after sub-acute exposure to imidacloprid. This decline in spleen weight in animals exposed to imidacloprid may be attributed to damage of spleen cells which is reflected by decreased weight of the organ (Israa and Layla, 2015). Our results are in agreement with study of Gatne *et al.* (2006), where it was observed that the spleen of mice exposed to imidacloprid are indicative of tissue destruction and injury reflecting imidacloprid induced death of lymphocytes and also showed disintegration of white pulp in the spleen of imidacloprid treated rats at a dose of 160 mg/kg b.wt. In our study, resveratrol co-treatment in imidacloprid treated groups restored the spleen weight. It may be attributed to the protective effect of resveratrol on spleen by reducing oxidative stress (Karabulut *et al.,* 2006).

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Research Article

ANTI-DIARRHEAL ACTIVITY OF STEM BARK OF *FICUS RELIGIOSA* IN WISTAR RATS

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ABSTRACT

The present investigation was aimed to study the anti-diarrheal potential of ethyl alcohol extract of *Ficus religiosa* stem bark in wistar rats. The stem bark of locally collected *F. religiosa* was subjected to ethanolic extract and preliminary phytochemical analysis was carried out. The anti-diarrheal activity of extract at 200 and 400 mg/kg b wt dose was evaluated using castor oil induced diarrhea in wistar rats. Along with castor oil induced diarrhea, the activity was assessed in castor oil induced enteropooling and gastrointestinal motility test (Charcoal meal test). The preliminary phytochemical analysis revealed the presence of alkaloids, sterols, tannin, flavonoids, saponin and glycosides in the ethanolic extract. The extract at 200 and 400 mg/kg significantly inhibited the castor oil induced diarrheal feces by 64.17% and 67.01%, respectively. In castor oil induced enteropooling test the inhibition observed in weight of intestinal contents was 62.20% and 69.23%; and volume of intestinal content was 60.23% and 70.60%. Similarly the extract also reduced the gastrointestinal motility by 47.36% and 63.21% in charcoal meal test. All the results were found statistically significant ($P<0.01$).The extract at both the dose rates significantly inhibited the castor oil induced diarrhea in terms of reduced the frequency of defecation, fecal volume and gastrointestinal motility. The results obtained were dose dependant.

Key words: Castor oil induced diarrhea, *Ficus religiosa,* enteropooling, motility test

INTRODUCTION

Diarrheal diseases are a major problem in Third World countries and are responsible for the death of millions of people each year. *Ficus religiosa,* commonly known as "Peepal" is the most popular member of the genus *Ficus* and it is known by more than 150 names. *Ficus religiosa* (Peepal) belongs to family the Moraceae. It is native from India to South East Asia. Stem bark is used for the treatment of diabetes, diarrhoea, leucorrhoea, menorrhagia, nervous disorders, ulcers, paralysis, astringent, asthma, parasympatholytic, viral infection, bacterial infection, protozoan infections, relaxant possess spasmolytic effects on smooth muscles, skin diseases and for vaginal and other urinogenital disorders. The fruit extract have antitumor activity and is used as purgative and aphrodisiac (Nadkarni, 1954).

The stem bark of *F. religiosa* shown presence of the tannins, saponins, flavonoids, steroids, terpenoids, cardiac glycosides, bergapten, bergaptol, lanosterol, β-sitosterol, stigmasterol, β-sitosterol-d-glucoside (phytosterolin), vitamin K_1, n-octacosanol, methyl oleanolate, lupen-3-one etc. and GC-MS analysis identified thirteen chemical constituents of which the major are 1,2-Benzenediol (9.85%), Caffeine (4.20%) and Stigmasterol, 22,23-dihydro (1.81%) (Manorenjitha *et al.*, 2013). The present study is aimed to explore the anti-diarrheal activity of alcoholic extract of stem bark of *Ficus religiosa* in wistar rats.

MATERIALS AND METHODS

The experimental protocol was duly approved by the Institutional Animal Ethical Committee (IAEC). Research work was carried out in rats of Wistar strain, procured from the CPCSEA recognized Laboratory Animal Breeding Centre. Rats weighing around 150-180 g were used for the present study.

Ficus religiosa bark was collected from the trees inside the campus of Nagpur Veterinary College, Nagpur. The plant material was duly authenticated from Department of Botany, Institute of Science, RTM Nagpur University, Nagpur. The collected bark was dried in shade under room temperature and was subjected to extraction in Soxhlet's extraction apparatus, using ethyl alcohol as solvent.

Anti-diarrheal activity

Anti-diarrheal activity of *Ficus religiosa* bark extract was assessed by using battery of following experiments.

Castor oil-induced diarrhea

Twenty four healthy rats were selected for the study and kept in polypropylene rat cages with raised grid at bottom. Rats were divided into four groups with six animals each and fasted for 24 hours with free access to water prior to the treatments. Control received 2ml/kg, p.o. saline, standard control was treated with diphenoxylate @ 5 mg/kg p. o. The other two groups were treated with *F. religiosa* alcoholic extract @ 200 and 400mg/kg, p. o. by gavage. After 1 hour of treatments, castor oil @ 2ml/rat was administered orally to induce diarrhea (Mandal *et al.*, 2010). Each rat was placed in a separate cage and observed for total feces and diarrheal feces for 6 hr. The observations of diarrheal feces from control group were considered as 100% score.

Castor oil-induced enteropooling

24 wistar rats were divided in to four groups and fasted for 24 hours with free access to water. First group received 2ml/kg saline, second group was administered orally with diphenoxylate @5mg/kg and rest two groups were orally administered with *F. religiosa* extract @200 and 400 mg/kg b wt. After 2 hours of induction of diarrhea in each rat by administration of 2 ml of castor oil, all rats were sacrificed. The small intestine of each rat was removed from pyloric part of stomach to cecum after tying the ends with thread and weighed. The intestinal contents were collected by milking into a graduated tube and their volume was measured. The empty intestine was weighed and the difference between full and empty intestine was calculated for statistical analysis (Verma *et al.*, 2011).

Gastrointestinal motility test (Charcoal meal test)

Twenty four healthy rats divided into four groups were used for this test. Castor oil @ 2 ml/rat was administered orally to all and after one hour rats were orally treated as follows- control 2% gum acacia, standard control loperamide 5mg/kg p.o. and rest two groups alcoholic extracts @ 200 and 400 mg/kg. One hour later, all the rats were orally administered with 1 ml of charcoal meal (10% charcoal suspension in 5% gum acacia). All rats were sacrificed after 1hr and the distance traveled by the charcoal meal from pylorus to caecum was measured and expressed as a percentage of the total distance of the intestine (Pazhani *et al.*, 2001)

RESULTS

The crude ethanolic extraction of stem bark of *Ficus religiosa* yielded reddish brown semisolid extract with an extractability of 38 percent. The preliminary phytochemical screening of the extract revealed the presence of alkaloids, sterols, tannin, flavonoids, saponin and glycosides.

The *F. religiosa* bark extract significantly produced anti-diarrheal effect in castor oil induced diarrhea test (Table 1). It reduced the frequency of wet diarrheal feces at both the doses of 200 mg/kg (6.33±0.272, 64.17%) and 400 mg/kg (5.83±0.251, 67.01%) in comparison with the control (17.67±0.272). The standard drug diphenoxylate at 5mg/kg was observed to be most significant (4.0±0.210, 77.36%) in producing anti-diarrheal effect. The results were statistically significant at 1% level of significance.

In castor oil induced enteropooling method, the mean values recorded for weight of intestinal contents (gm) and volume (ml) of fecal matter was recorded in different groups. The weights of intestinal contents and their percent inhibition were 2.99 ± 0.126 in control, 0.58 ± 0.044

Table 1.
Effect of stem bark of *Ficus religiosa* aqueous extract on castor oil induced diarrhea in rats.

Treatment	Total number of feces	Number of diarrhea feces	% inhibition of diarrhea
N. S. (2ml/kg) + Castor oil (2ml)	20.5±0.349 [a]	17.67±0.272 [a]	-
Diphenoxylate (5mg/kg) + Castor oil (2ml)	10.33±0.344 [c]	4.0±0.210 [c]	77.36%
Extract (200mg/kg) + Castor oil (2ml)	13.0±0.365 [b]	6.33±0.272 [b]	64.17%
Extract (400mg/kg) + Castor oil (2ml)	11.83±0.250 [b]	5.83±0.251[b]	67.01%

Values expressed as mean ±SEM, P<0.01

Table 2.
Effect of stem bark of *Ficus religiosa* aqueous extract on castor oil induced enteropooling in rats.

Treatment	Weight of intestinal contents	Percent inhibition	Volume of intestinal fluid	Percent inhibition
N. S. (2ml/kg) + C. O. (2ml)	2.99±0.126[a]	-	3.47±0.262[a]	-
Diphenoxylate(5mg/kg)+C.O. (2ml)	0.58±0.044[c]	80.60%	0.73±0.061[c]	78.96%
Extract (200mg/kg) + C. O. (2ml)	1.13±0.116[b]	62.20%	1.38±0.064[b]	60.23%
Extract (400mg/kg) + C. O. (2ml)	0.92±0.057[b]	69.23%	1.02±0.132[bc]	70.60%

C. O.- Castor oil, Values expressed as mean ±SEM, P<0.01

Table 3
Effect of stem bark of *Ficus religiosa* aqueous extract on charcoal meal test in rats.

Treatment	Volume of intestinal fluid	Percent inhibition
5% Gum acacia (1ml) + Castor oil (2ml)	78.6±3.938[a]	-
Loperamide (5mg/kg) + Castor oil (2ml)	21.0±0.806[d]	73.28%
Extract (200mg/kg) + Castor oil (2ml)	41.37±1.422[b]	47.36%
Extract (400mg/kg) + Castor oil (2ml)	28.91±1.183[c]	63.21%

Values expressed as mean ±SEM, P<0.01

(80.60%) in treatment control, 1.13 ± 0.116 (62.20%) in 200mg/kg extract and 0.92 ± 0.057 (69.23%) in 400mg/kg of extract. The extracts at 200 and 400 mg/kg significantly inhibited the volume of intestinal fluid which was 1.38±0.064 (60.23%) and 1.02±0.132 (70.60%), respectively, as compared to control 3.47±0.262. Both weight and volume were observed statistically significant at 1% of significance.

In charcoal meal test, the gastrointestinal motility in castor oil induced diarrhea and its inhibition by different treatments was assessed through percentage of distance (cm) traveled by charcoal meal in intestine (from pylorus part of stomach to caecum). It was 78.6 ± 3.938, 21.0 ± 0.806 (73.28%), 41.37 ± 1.422 (47.36%) and 28.91 ± 1.183 (63.21%) in control, standard and extract at 200 and 400 mg/kg, respectively and found significant at 1% level of significance.

DISCUSSION

Castor oil-induced diarrhea in rats is described to be an appropriate model of the complex, prolonged processes of hypersecretion and accelerated transit that characterize secretory diarrhea. The induction of diarrhea with castor oil results from the action of ricinoleic acid which produces changes in the transport of water and electrolytes, resulting in a hypersecretory response. The usual reaction of the intestine to excessive fluid load is increased peristalsis. The reactions of the gut to ricinoleic acid can be considered basic mechanisms of the intestinal response to a wide variety of pathological stimuli (Niemegeers *et al.*, 1984) and also reduce active Na+ and K+ absorption and decrease Na+, K+ ATPase activity in the small intestine and colon (Gaginella and Philips, 1975). Thus the antidiarrheal activity observed was may be due to antisecretory mechanism, as the plant extract significantly reduced the accumulation of fluid in the intestine in terms of weight and volume.

Action of castor oil stimulates the release of endogenous prostaglandins (Méité *et al.*, 2010). Inhibitors of prostaglandin synthesis are known to delay diarrhoea induced with castor oil (Teke *et al.*, 2007). The extract may also have inhibited prostaglandins and produced antidiarrheal effect. Of the numerous phytochemicals (such as alkaloids, tannins, flavonoids and terpenes) present in active extracts, tannins and flavonoids are thought to be responsible for antidiarrhoeal activity by increasing colonic water and electrolyte reabsorption (Enzo, 2006). Flavonoids present in the plant extracts are reported to inhibit release of autacoids and prostaglandins, thereby may inhibit motility and secretion induced by castor oil. Tannins and tannic acid denature proteins in the intestinal mucosa by forming protein tannates which make the intestinal mucosa more resistant to chemical alteration and reduce secretion (Singh *et al.*, 2013). The presence of tannins and flavonoids in the bark extract might be the elemental factors for the antidiarrheal activity of the plant. The results of present study are indicative of antidiarrheal activity of *F. religeosa* stem bark which significantly reduced the frequency of defecation, fecal volume and gastrointestinal motility. The findings may justify the usefulness of *F. religiosa* bark in the management of diarrhea.

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Research Article

ACUTE TOXICITY OF ENROFLOXACIN FORMULATIONS IN RATS

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ABSTRACT

The acute toxicity study of two different formulations of enrofloxacin, tablet and injection was conducted in rats. Enrofloxacin was given as single intraperitoneal injection at the dose rates of 300, 500, 1000 and 2000 mg/kg b wt in test groups I, II, III and IV, respectively. Second formulation was administered as single oral dose of enrofloxacin tablet @ 1000, 2000, 3000, 4000 mg/kg b wt in test groups I, II, III and IV, respectively. Toxicity was assessed by estimation of haematological (Hb, PCV, TEC, TLC), biochemical (total protein, albumin, globulin, creatinine, ALT, AST) and antioxidant (LPO, GSH) parameters. Acute toxicity study of the two formulations of enrofloxacin conducted in albino rats showed mild to moderate haemotoxic and hepatotoxic effects at the dose level @ 1000 mg/kg b wt, and mortality at 2000 mg/kg b wt, intraperitoneally and @ 2000 mg/kg b wt, orally. Enhanced level of GSH in vital organs indicated its anti-oxidative potential in rats.

Key words: Acute toxicity, antioxidents, haematobiochemical, enrofloxacin, rat.

INTRODUCTION

Enrofloxacin, a fluoroquinolone has been developed exclusively for use in veterinary medicine. It has a strong, broad spectrum bactericidal effect indicated in single or mixed bacterial infections and especially in infections caused by anaerobic bacteria related to the deactivation of the enzyme DNA gyrase – a type II topoisomerase (Shen *et al.*, 1989). With the exception of potential cartilage abnormalities in young animals, fluoroquinolones are generally well tolerated with most side effects being mild and serious adverse effects being rare (Owens and Ambrose, 2005).The present investigation was carried out for observing acute toxicity of enrofloxacin in rats.

MATERIALS AND METHODS

The experiment was designed to evaluate the acute toxic effects of two different formulations of enrofloxacin (injectable and tablet) at increasing dose rates in rats. Albino rats (Sprague Dawley) of 2 to 2.5 months of age, weighing between 150 to 200 gm, were procured from Laboratory Animal Resource Centre, IVRI, Izatnagar. The animals were acclimatized for two weeks in the experimental laboratory animal house of the Department under standard managemental conditions. Standard rat feed and water were provided *ad libitum* throughout the experimental period. The studies were performed in accordance with the guidelines on regulation of scientific experiments as approved by the IAEC.

For assessment of acute toxicity of enrofloxacin 10% w/v injection, the rats were randomly divided into five groups (four test groups and one control group) with 3 rats in each group. Since, the intraperitoneal LD_{50} for enrofloxacin could not be found in literatures, the drug was administered at the dose rates of 300, 500, 1000 and 2000 mg/kg b wt intraperitoneally as single dose in group II, III and IV, respectively. Rats were kept under constant observation for consecutive 14 days and examined regularly for appearance of any clinical signs as well as mortality due to toxicity. Mortality seen at 2000 mg/kg b wt. On 15^{th} day all the rats were sacrificed, viscera and internal organs were examined for gross lesions and the blood, liver, kidney and brain samples were collected to evaluate haematological, biochemical and antioxidative parameters.

The acute toxicity of enrofloxacin was done by randomly dividing the rats into five groups (four test groups and one control group) with 3 rats in each group. The rats were kept off fed for 24 h before starting the experiment for oral administration of drug (enrofloxacin tablets, 50 mg), so as to have ease in gavaging as well as to avoid interference of food in gastric absorption of the drug. Experiment was designed as per OECD guidelines and 1/5^{th} dose (1000 mg/kg b.w.) of oral LD_{50} of enrofloxacin (>5000 $mg.kg^{-1}$), (Schmidt, 1985) was administered in the first group as single oral dose in the form of gastric gavage. Before administration, the tablets were powdered and dissolved in distilled water to make a solution. The dose was increased to 2000, 3000 and 4000 mg/kg b wt in the subsequent groups for study of acute toxicity at higher dose rates. Rats were kept under constant watch for consecutive 14 days and observed for appearance of specific clinical signs as well as mortality due to drug toxicity. On 15^{th} day, all the rats were sacrificed, viscera and internal organs were examined for gross lesions and the blood, liver, kidney and brain samples were collected to evaluate haematological, biochemical and antioxidative

parameters.

The acute toxicity study of enrofloxacin injection, 10 % w/v and enrofloxacin tablets, 50 mg was assessed by estimating haematological and biochemical parameters by standard methods using diagonistic kits.

Estimation of oxidative stress related biochemical parameters viz., Lipid peroxidation product, Malondialdehyde (LPO) (Rehman, 1984) Reduced Glutathione (GSH) (Prins and Loos, 1969) in erythrocytes, liver, kidney and brain was carried out as per standard methods.

RESULTS AND DISCUSSION

The acute toxicity study of two different dosage forms of enrofloxacin 10% w/v injection and enrofloxacin tablets 50 mg was carried out in albino rats to examine the safety of the two formulations.

Enrofloxacin 10% w/v injection

No significant changes were observed in values of PCV, hemoglobin, TEC and TLC as compared to control in test groups I and II. The values of PCV, Hb decreased significantly (P<0.05) and TLC depicted highly significant (P<0.01) increase in comparison to the control group in rats of test group III as shown in Table 1.

Test group II and III showed a significant (P<0.001) increase in level of serum creatinine and AST than those of control group (Table 1). The increase in the value of creatinine gave an indication of probable kidney damage at higher dose.

A significant increase in the activity of serum AST in treated groups might be due to hepatic damage caused by enrofloxacin in this study. The rise in serum levels of ALT and AST activity has been attributed to the loss of structural integrity of hepatocytes as these enzymes are located in the cytoplasm and are released into circulation after cellular damage (Ahmed and Khater, 2001). The rise in the activity of these enzymes in this study, thus, might have occurred due to hepatic damage at reasonably higher doses of enrofloxacin in rats.

As depicted in Table 1, there was a significant reduction of LPO levels in treated groups II and III in comparison to control in RBCs which suggested that enrofloxacin did not produce oxidative stress in erythrocytes of enrofloxacin treated rats. A significant increase was however, observed in liver of test groups II and III indicating the production of tissue oxidative stress. The reported value of GSH in RBCs of rats was significantly increased in test groups I and II in comparison to control. The elevation of GSH level in RBCs might have occurred due to enhanced cellular response to overcome the oxidative stress imposed by the enrofloxacin during the study period in rats. A significant decrease was observed in liver GSH profile in rats of test group I, whereas in test groups II and III non-significant decrease in liver GSH level was observed which suggested that enrofloxacin promoted the synthesis of oxidative radicals in liver of enrofloxacin treated rats. No significant changes were observed in GSH profile of other tissues tested.

Enrofloxacin, 50mg tablet

No significant changes were observed in haematological profile of treated rats in either of the groups except the significant (P<0.05) decrease in TEC and highly significant (P<0.01) increase in TLC value in rats of test group III as shown in Table 1. Haemolysis and shrinkage in RBC might be the reason for decline in cell count in treated rats of group III (Singh *et al.*, 2004).

A significant (P<0.001) increase in activities of serum ALT was found in rats of test group III and IV which is known to be the indicator of hepatic damage (Cornelius, 1989).

A significant (P<0.001) increase in serum cholesterol level was recorded in test groups III and IV (Table 1). This alteration in cholesterol levels might be attributed to altered equilibrium between the activities of hydroxyl methyl glutaryl CoA (HMG-CoA) reductase and that of acyl CoA, cholesterol acyl transferase which led to disturbed cholesterol metabolism due to hepatic damage done by the drug (Hochgraf *et al.*, 2000).

The serum concentrations of creatinine in treated group I, II, III and IV, were significantly higher (P<0.001) as compared to rats of control group (Table 1). Higher levels of creatinine is suggestive of nephrotoxic effect (Cornelius, 1989).

As depicted in Table 1, there was a significant reduction of LPO levels in treated groups II, III and IV in comparison to control in RBCs which suggested that enrofloxacin did not produce oxidative stress in erythrocytes of enrofloxacin treated rats. Significantly higher levels of LPO were also observed in the liver in rats of test group II and in kidneys in rats of test groups III and IV indicating the increased production of malondialdehyde (MDA), thereby suggesting the oxidative damage due to higher dosage of enrofloxacin in tissues. The elevation of GSH level in organs like liver, brain and kidney of treated rats in this study might have occurred due to enhanced cellular response to overcome the oxidative stress imposed by the enrofloxacin during the study period in rats.

Thus, enrofloxacin produced mild haemotoxic and moderate hepatotoxic effect at the dose higher than 2000 $mg.kg^{-1}$ b wt and moderate haemotoxic and hepatotoxic effect at the dose i.p. as high as 500 and 1000 $mg.kg^{-1}$ b wt in rats.

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Table 1.
Effect of enrofloxacin on hematobiochemical and antioxident profile after 14 days of single oral and i.p. doses in rats

Parameter	Control		Test Group I		Test Group II		Test Group III		Test Group IV	
	10%w/v injection	50 mg tablets	10% w/v injection	50 mg tablets	10%w/v injection	50 mg tablets	10% w/v injection	50 mg tablets	10% w/v injection*	50 mg tablets
Haematological										
PCV %	54 ± 2.65	54 ± 2.65	47.33±2.33	50 ± 0.82	45.67 ± 1.45	48 ±2.45	42 ± 1.73[a]	45 ± 6.00	-	43 ± 4.36
Hb %	13 ± 0.42	13 ± 0.42	11.67±0.27	12.27 ±0.24	11.73 ± 0.44	12.47±0.24	10.80 ± 0.42[a]	12.1 ± 0.17	-	11.9 ± 0.38
TEC (10^6/µl)	8.13 ± 0.18	8.13 ±0.18	8.28 ± 1.10	7.97 ± 0.19	7.90 ± 0.07	7.81 ± 0.23	6.41± 0.50	6.93 ± 0.49	-	5.94 ± 0.24[a]
TLC (10^3/µl)	10.47± 0.48	10.47±0.48	11.47±0.43	11.13 ±0.18	12.07 ± 0.18	12.13±0.12	14.00 ± 0.53[b]	13.57 ± 0.50[a]	-	16.20 ± 0.81[b]
Biochemcial										
Total Protein (g/dL)	8.19 ± 0.20	8.19 ±0.20	6.81 ± 0.22	8.26 ±0.32	8.28 ± 1.1	8.73 ±0.34	10.26 ± 0.32	9.26 ±0.16	-	9.63 ±0.34[a]
Albumin(g/dL)	3.54 ± 0.28	3.54 ±0.28	3.04 ± 0.15	3.94 ±0.09	2.72 ± 0.24	3.75 ±0.32	2.89 ± 0.39	3.67 ±0.05	-	3.84 ±0.16
Globulin(g/dL)	4.65 ± 0.41	4.65 ±0.41	3.77 ± 0.09	4.32 ±0.41	5.56 ± 1.03	4.97 ±0.05	7.37 ± 0.68[a]	5.59 ±0.11	-	5.79 ±0.18
A/G Ratio	0.78 ± 0.13	0.78 ±0.13	0.80 ± 0.03	0.93 ±0.10	0.52 ± 0.12	0.75 ±0.06	0.41 ± 0.09	0.66 ±0.00	-	0.66 ±0.01
Creatinine (mg/dL)	3.67 ± 0.24	3.67 ±0.24	5.13 ± 0.29	8.27 ±0.18[b]	7.67 ± 0.29[c]	9.40 ±0.42[c]	10.33 ± 0.41[c]	10.00 ±0.12[c]	-	12.00 ±0.44[c]
Cholesterol (mg/dL)	82.82 ± 1.36	82.82±1.36	71.80 ±1.28	68.46 ±1.89	65.38 ± 6.45	102.7 ±14.76	123.08±27.65	375.41±34.88[c]	-	549.31 ± 11.68[c]
ALT (IU/L)	27.33 ± 3.71	27.33±3.71	29 ± 3.06	31 ±0.58	31 ± 3.06	35 ±2.89	35±1.53	52.33±3.48[c]	-	63.33 ±3.18[c]
AST (IU/L)	33.67 ± 2.96	33.67±2.96	39.33 ±3.71	26 ±3.00	41.33 ± 3.21[a]	31.5 ±1.61	57±2.08[b]	33.33 ±1.86	-	37.83 ±2.68
Antioxidents										
LPO RBC	7.38 ± 1.10	7.38 ±1.10	5.65 ± 0.48	6.15 ±0.63	4.64 ± 0.48[a]	4.08 ±0.04[b]	4.03 ± 0.12[b]	1.56 ± 0.28[c]	-	1.56 ±0.30[c]
(nM.MDA/ml) Liver	12.65± 1.72	12.65 ±0.72	21.15± 2.40	18.04 ± 2.50	24.57 ± 1.42[a]	24.58 ±2.68[a]	27.08 ± 2.22[b]	20.66 ±1.88	-	21.29 ±2.77
Kidney	20.16± 1.14	20.16 ±1.14	16.69± 1.22	18.35 ±5.91	22.31 ± 2.12	12.85 ±2.91	28.65 ± 1.40	32.08 ±1.98[a]	-	32.25 ±2.97[a]
Brain	15.61± 0.69	15.61 ±0.69	15.58± 1.94	21.54 ±4.27	14.96 ± 1.80	20.15 ±2.91	14.65 ± 2.32	17.43 ±2.73	-	17.66 ±2.17
GSH RBC	0.34 ± 0.02	0.34 ±0.02	0.42 ± 0.04	0.47 ± 0.02	0.50 ± 0.01[b]	0.73± 0.02[b]	0.60 ± 0.01[c]	0.92 ± 0.02[c]	-	0.93 ±0.02[c]
(mM/ml) Liver	0.54 ± 0.11	0.54 ±0.11	0.35 ± 0.01[b]	0.54 ±0.07	0.40 ± 0.02	0.54 ±0.07	0.50 ± 0.07	0.39 ±0.05	-	0.26 ±0.02[b]
Kidney	0.83 ± 0.04	0.83 ±0.04	0.88 ± 0.10	0.88 ±0.12	0.93 ± 0.03	0.71 ±0.01	0.92 ± 0.14	1.07 ±0.04	-	0.89 ±0.13
Brain	0.54 ± 0.01	0.54 ±0.01	0.50 ± 0.03	0.54 ±0.04	0.49 ± 0.02	0.55 ±0.04	0.48 ± 0.02	0.59 ±0.06	-	0.75 ±0.15

Values in Table are Mean ± S.E. (n = 3), a Significant (P< 0.05) as compared to Control group within the same row. b Significant (P< 0.01) as compared to Control group within the same row. c Significant (P< 0.001) as compared to Control group within the same row. *All the rats died in this group.

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Research Article

COMPARATIVE STUDY AND CLASSIFICATIONS OF ANAEMIA CAUSED BY *TRYPANOSOMA BRUCEI* AND DFMO (DIFLUORO METHYL ORNITHINE) IN RATS

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ABSTRACT

The effect of *Trypanosoma brucei* and difluoromethyl ornithine (DFMO), a trypanocidal, agent on packed cell volume (PCV), haemoglobin (Hb) concentration, red blood cells (RBC) count, body weights and types of anaemia induced during therapy were studied in wistar albino rats of mixed sexes. Rats in group A were used as control, group B was orally administered 2% difluoromethyornithine (DFMO) W/V only, group C inoculated with concentration of 5 X 10^5/µL of *Trypanosoma brucei* intraperitoneally whereas group D was inoculated with 5 X 10^5/µL of *Trypanosoma brucei* and later treated with 2% DFMO W/V at the dosage of 400mg/kg daily for 21 days, respectively. Decrease in PCV, RBC, Hb, progressive weight loss and anaemia were noticed in all the experimental groups. Normocytic normochromic anaemia was observed in DFMO treated rats. Trypanosoma *brucei* infected rats showed macrocytic normocytic anaemia which later changed to normochromic normocytic anaemia while microcytic hypochromic anaemia was observed in rats of group D infected with *T. brucei* and treated with difluoromethyl ornithine (DFMO). Body weights of experimental rats decreased in all the experimental rats from 6 – 21days, though appreciation in body weights of rats treated with DFMO was observed by day 21. Conclusively, treatment of trypanosomosis with difluoromethylornithine DFMO had untoward effect on haematology of the experimental rats due to the effect of this drug on b wt and bone marrow activity.

Keywords: Comparative studies, anaemia, difluoro methyl ornithine, *Trypanosoma brucei*, rats.

INTRODUCTION

Trypanosomosis is a debilitating disease condition affecting man and livestock production in sub-Saharan Africa caused by haemo - flagellates genus of trypanosomes and transmitted principally by tsetse fly species Glossina. In other regions of the world including Asia and South America where trypanosomosis also occurred, it is thought to be maintained in those areas through mechanical transmission by other haematophagous flies such as *Tabanus,* haematopota, liperosia and *Stomoxys* (Leeflang, 1998). The disease is characterized by anaemia, intermittent fever, progressive weight loss, high mortality (Abbott *et al.*, 2006), reduced productivity and economic losses due to poor hide / skin, meat and milk production in livestock sector (Soulsby, 1994).

The treatment, control and eradication of trypanosomosis in domestic animals in tsetse infested zone of Africa still remains a problem due to wide spread vector hosts and central nervous system involvement especially in infection due to *T.brucei*. Once, the parasites cross the blood brain barrier, they are safely shielded away from the effects of trypanocides (Mbaya *et al.*, 2007). The economic losses due to this disease alone throughout Africa were estimated to be 5 Million dollars / year, which amounts to financial losses of about 300 Million naira annually (Nawathe *et al.*, 1988).

DFMO was found to be effective when combined with suramin, berenil, and pentamidine (Anosa, 1983), though the drug is found to have some inhibitory effect on erythropoiesis. It was also reported that human patients treated with oral doses of DFMO, developed anti-proliferative properties on living cells, varying degree of gastro-intestinal disorders such as diarrhoea, vomition, dizziness and convulsions were also reported (Vanneuvenhave *et al.*, 1995). The objective of this research is to ascertain the effects of *Trypanosoma brucei* and DFMO (Difluoro methyl Ornithine) on haematological parameters and types of anaemia caused in wistar strain albino rats.

MATERIALS AND METHODS

Experimental animals

Twenty (20) healthy wistar albino rats of both sexes weighing 149 - 255 grams kept in fly-proof clean cages were used. They were fed growers mash and provided water *ad libitum* and screened using wet mount and giemsa stained blood film for haemoparasites before experiment. The rats were grouped into four (4) experimental groups (A, B, C and D) of 5 rats each. Rats in group A were used as control, those in group B were orally administered 2% difluoromethyornithine (DFMO) W/V only, those group C were inoculated with 5 X 10^5/µL of *Trypanosoma brucei* intraperitoneally, whereas rats in group D inoculated with 5 X 10^5/µL of *Trypanosoma brucei*

and later treated with 2% DFMO W/V at the dosage of 400mg/kg respectively. The concentration of *Trypanosoma brucei* organisms used for inoculation was estimated using the Dark ground / phase contrast buffy coat (DG) method.

Haematological examination

The packed cell volume (PCV, %), red blood cell count ($\times 10^6 mm^3$) and haemoglobin (Hb, %) were determined using the method of Coles (1986).

The haemoglobin concentration was calculated using the formula;

$$Hb\ (g/dL) = \frac{OD\ (test) \times Conc\ of\ STD\ (mg/L) \times 250}{OD\ (standard) \quad 1000}$$

Where, OD = Optical density, STD = Standard.

The mean corpuscular volume (MCV) was calculated by dividing the volume of packed red cell per 1ml of blood by the total red blood cell in millions, per cubic millimetre (MCV = PCV X 10 / RBC X 10^6) and the results were then expressed in fentolitre (fl).

The mean corpuscular haemoglobin was calculated by dividing the haemoglobin present in grams per ml of blood by the total red blood cell count in millions per cubic millimetre (MCH = Hb X 10 / RBC X 10^6).

The mean corpuscular haemoglobin concentration is calculated by dividing the haemoglobin in grams per 1ml of blood by the packed cell volume per 1ml of blood (MCHC = Hb X 100 / RBC). The results were finally expressed in percentage of haemoglobin of total weight of erythrocyte.

The results obtained were presented as means ± standard deviation. Difference between means was assessed using analysis of variance (ANOVA) and post-test using Dunnett comparison test (Mead and Curnow, 1982).

RESULTS

Values of haematological parameters and body weight have been given in Table 1. No clinical changes were observed in the rats in control (group A). The rats in group B exposed to DFMO showed ocular discharge, loss of appetite, weight loss and diarrhoea. Rats in group C exposed to *T. Brucei* alone manifested weakness, facial oedema, paraphimosis and anorexia. Rats in group D infected with T. brucei and treated with DFMO manifested, dullness, lacrimation, facial oedema, anorexia, mental derangement, weakness, diarrhoea and death. The rats in groups B, C, D treated with DMFO, infected with *Trypanosoma brucei* and infected with *T. brucei* & treated with DFMO did not show effect on their PCV between day zero – three, six days into the research, rats in groups B, C and D showed significant ($P < 0.05$) decrease in their PCV when compared to rats in control group A. The rats in groups B, C, D (Table 2, 3 and 4) treated with DMFO, infected with *Trypanosoma brucei* and infected with *T. brucei* & treated with DFMO did not show effect on their RBC, HB and PCV count from day 0 – 3, but showed significant ($P < 0.05$) decrease from day 6 - 21 when compared to the rats in the control group (A). The haematological indices of all the groups of rat involved in this *In vivo* study were computed and presented in Table 2. Progressive loss in the b wt in all the rats in groups B, C and D were observed from day six – twenty one when compared to the rats in the control group (A).

DISCUSSION

This study revealed progressive weight loss, dullness, aggressiveness, facial oedema, emaciation, anorexia, convulsions, anaemia and death in the rats exposed to *Trypanosoma brucei* and Difluoro-methylornithine (DFMO). Microscopically, numerous immature red blood cells initially dominated by macrocytes and later large numbers of microcytes were observed in the blood stained slides of the experimental rats. The presence of numerous immature RBCS observed is suggestive of regenerative nature of anaemia in the experimental animals due to destruction of red blood cells in wistar rats by *Trypanosoma brucei* and difluoro-methylornithine. Microcytes appeared with poorly stained centre which is suggestive of microcytic hypochromic anaemia that could have developed as a result of acute iron and cyanocobalamin deficiencies as a result of the effect of *Trypanosoma brucei* and difluoromethylornithine observed on the stained blood film.

Trypanosoma brucei infected rats presented macrocytic normocytic anaemia in the acute phase which could be as a result of haemolysis of red blood cells due to initial appearance of trypanosomes in the blood leading as a result of production of proteases, neuraminidase, phospholipases, free fatty acids (FFA), pyruvate and aromatic byproducts in the peripheral circulation or due to mechanical injury to the erythrocytes by trypanosomes due to lashing action of powerful locomotory flagella and microtubule reinforced bodies of the millions of the trypanosomes during parasitaemia as reported in earlier studies which demonstrated normocytic normochromic anaemia in the acute phase of the disease (Anosa and Obi, 1980; Mbaya & Ibrahim, 2011).

This variation may be as a result of strain and host difference used in this research. Aggressiveness, diarrhoea, facial oedema, emaciation, anorexia, progressive weight loss and exhaustion were also clinical signs observed in the rats treated with DFMO. The facial oedema encountered in these rats may be due to release of vaso active substances from blood vessels of the experimental rats caused by both Trypanosoma brucei (Clerkson *et al.*, 1984). Diarrhoea, weight loss, dullness, lacrimation and normocytic normochromic anaemia was observed in DFMO treated rats (Mbaya *et al.*, 2010).

The diarrhoea and weight loss may be due to

Table 1.
Effects of DFMO, *T. brucei* and *T. brucei* plus DMFO treatment on haematological parameters and mean body weight in rats.

Groups	Treatment days							
	0	3	6	9	12	15	18	21
Packed Cell Volume (%)								
A	42.0 ± 1.41	42.0 ± 1.41	42.4 ± 1.52	42.8 ± 1.48	43.4 ± 1.67	43.0 ± 0.17	43.6 ± 1.52	44.0 ± 1.23
B	44.6 ± 1.52	44.6 ± 1.52	40.8 ± 1.52*	38.0 ± 1.87*	35.2 ± 3.11*	35.4 ± 3.21*	40.2 ± 0.45*	47.6 ± 1.14
C	44.2 ± 1.30	44.2 ± 1.30	33.0 ± 2.25*	32.0 ± 0.83*	30.8 ± 5.26*	25.8 ± 0.84*	22.8 ± 2.28*	20.0 ± 3.67*
D	43.2 ± 0.84	43.2 ± 0.84	30.2 ± 1.09*	30.0 ± 2.44*	25.2 ± 2.78*	22.6 ± 1.34*	20.0 ± 1.23*	18.8 ± 1.30*
Red Blood Cell Count ($x10^6 mm^3$)								
A	5.4 ± 0.71	5.4 ± 0.71	5.3 ± 0.71	5.3 ± 0.64	5.3 ± 0.64	5.2 ± 0.46	5.4 ± 0.64	5.4 ± 0.61
B	6.0 ± 0.48	6.0 ± 0.48	4.5 ± 0.72*	4.6 ± 0.41*	4.8 ± 0.36*	4.7 ± 0.31*	4.8 ± 0.24*	7.9 ± 0.27
C	5.1 ± 0.56	5.1 ± 0.56	4.4 ± 0.20*	4.5 ± 0.31*	4.3 ± 0.19*	3.9 ± 0.27*	3.7 ± 0.13*	3.6 ± 0.34*
D	5.3 ± 0.27	5.3 ± 0.27	4.2 ± 0.57*	3.3 ± 0.43*	2.8 ± 0.08*	2.8 ± 0.08*	2.8 ± 0.08*	2.8 ± 0.08*
Haemoglobin (%)								
A	14.3 ± 0.42	14.3 ± 0.42	14.3 ± 0.43	14.6 ± 0.46	14.7 ± 029	14.8 ± 0.24	14.8 ± 0.24	14.8 ± 0.24
B	14.1 ± 0.13	14.1 ± 0.13	10.8 ± 0.45*	10.9 ± 1.67*	10.7 ± 0.52*	10.3 ± 2.45*	9.90 ± 2.77*	15.40 ± 3.02
C	14.0 ± 0.71	14.0 ± 0.71	13.2 ± 0.47*	11.4 ± 0.59*	10.5 ± 0.87*	10.4 ± 0.89*	10.2 ± 0.45*	10.2 ± 0.45*
D	14.6 ± 0.49	14.6 ± 0.49	12.7 ± 0.42*	9.50 ± 0.80*	9.00 ± 0.71*	8.8 ± 0.46*	8.80 ± 0.46*	8.80 ± 0.46*
Mean body weight (g)								
A	115.00±13.64	114.74±13.16	114.09±11.00	113.56±18.00	114.33±13.36	114.08±10.08	114.33±13.36	115.08±10.08
B	111.37±10.11	100.80±11.11	101.91±18.00*	98.00 ±14.01*	87.93±12.12*	85.32±11.04*	81.93±12.11*	120.32±11.00
C	110.54±11.04	109.86±16.21	109.91±19.00*	100.39±11.00*	90.91±19.05*	90.39±11.01*	89.91±19.05*	79.39±11.01*
D	103.30±14.52	101.19±18.00	100.30±15.11*	100.62±17.03*	90.30±15.02*	86.62±17.33*	82.30±15.36*	80.62±17.39*

KEY: X = Mean ± SD, N = 5, A = Control Group, B = Rats treated with DMFO, C = Rats infected with *Trypanosoma brucei*, D = Rats infected with *T. brucei* & treated with DFMO, * = significant (P < 0.05) decrease as compared to control

Table 2.
Comparision of haematological indices in rats treated with *T. brucei* and *T. brucei* plus DMFO in rats.

Groups	Haematological indices of wistar albino rats over 24 days of In vivo studies		
	MCH	MCV	MCHC
A	27.20 ± 0.29	71..60 ± 0.92	33.90 ± 0.24
B	26.40 ± 0.90	80.30 ± 6.84	33.60 ± 2.41
C	26.20 ± 0.78	84.10 ± 14.02	31.60 ± 5.37
D	31.90 ± 2.03	93.10 ± 11.65	33.60 ± 2.41

KEY: X = Mean ± SD, N = 5, A = Control Group, B = Rats treated with DMFO, C = Rats infected with *Trypanosoma brucei*, D = Rats infected with *T. brucei* & treated with DFMO

irritation or increase in the influx of fluids into the gastro-intestinal tract (GIT) induced by the drug orally administered and the weight loss due to inappetance. The dullness was suggestive of anaemia and nervous system disorder due to depressive effect of the drug on the central nervous system in the rats. The anaemia in these animals may be attributed to the inhibition of enzyme, ornithine decarboxylase (ODC) that is an enzyme that catalyses the bio-synthesis of poly-amine from putrescine, spermine or spermidine of the host vascular system thereby interfering with the precursors of RBC and biosynthesis of haemoglobin (Mbaya *et al.*, 2011).

The anaemia caused by DFMO was found to be temporal as the rats improved soon after the withdrawal of the drug as was observed in rats treated with DFMO. Microcytic hypochromic anaemia is observed in rats in group D infected with *T. brucei* & treated with DFMO. Diarrhoea, dullness, lacrimation, facial oedema acute anaemia, loss of weight, and lethargy were also observed in rats in this group (D). The microcytic hypochromic anaemia observed may be due to excess haemolysis of RBC, erythrophagocytosis, and bone marrow hypofunction due to combined effect of *T. brucei* and DFMO. The hypochromic anaemia observed may be suggestive of iron and vitamin B_{12} deficiency caused by *T. brucei* and DFMO.

In this research, progressive decrease in PCV, RBC count, Hb concentration and various forms of anaemia were observed. These effects were more observed in rats in group D (infected with *T. brucei* & treated with DFMO), due to the combined effect of trypanosomes and DFMO on haematological parameters, when compared to the rats in groups B (treated with DMFO alone) and C (infected with *Trypanosoma brucei* alone). This finding therefore discourages the therapeutic application of DFMO in the treatment of animals with clinical cases of trypanosomosis.

Conclusively, this study revealed the untoward effect of DFMO and pathological effect of *Trypanosoma brucei* on haematological parameters in rats.

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Research Article

EFFECT OF *HEDYCHIUM SPICATUM* ON RHYTHMICITY, GLUCOSE UPTAKE AND ACHE ACTIVITY OF *FASCIOLA GIGANTICA*

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ABSTRACT

Hedychium spicatum (kapur kachari) is an herb found in subtropical Himalayas, growing abundantly in Punjab and Nepal. The effect of *H. spicatum* alcoholic extract (HSAE) was investigated *in vitro* on the spontaneous muscular activity, glucose uptake, glycogen content, AChE activity and lactic acid production activity of *Fasciola gigantica*. The HSAE at 100 and 300µg/ml concentration increased both frequency and amplitude of contractions and at 1000 µg/ml cumulative concentration it produced complete irreversible paralysis of *F. gigantica*. HSAE at 30 and 100 µg/ml significantly ($p<0.01$) inhibited glucose uptake. At 100 µg/ml HSAE produced significant depletion of glycogen reserve in *F. gigantica* and inhibited AChE activity of the liver fluke significantly($p<0.01$). HSAE did not alter lactic acid production significantly. Thus, HSAE produced paralytic effect on *F. gigantica* probably by inhibition AChE activity and also its effect on glucose uptake dependent mechanism.

Keywords: *Hedychium spicatum*; *Fasciola gigantica*; rhythmicity; glucose uptake, AChE.

INTRODUCTION

Fascioliasis in animals is a chronic disease known as distomatosis or liver rot and causes anaemia, lethargy, weight loss and lower fertility (Chiezey *et al.*, 2013). Its economic importance is mostly obvious when the disease causes mortality, but even subclinical infections have been shown to cause high losses from reduced feed efficiency, weight gains, milk production, reproductive performance, carcass quality and work output in draught animals, and from condemnation of livers at slaughter (Vassilev and Jooste 1991). Synthetics anthelmintic are the most effective means for the control of fascioliasis, but these anthelmintics have serious disadvantages such as risk of misuse, development of drug resistant, adverse drug reactions, high cost, environmental pollution and residual effect. Plant based anthelmintics offer an alternative that can overcome some of these problems and are sustainable and environmentally acceptable (Singh *et al.,* 2002).

Hedychium spicatum (Hindi-Kapur kachari)Buch.-Ham. belonging to the family Zingiberaceae, commonly known as spiked ginger lily and used traditionally to treat several GIT and respiratory ailments (Sahu, 1979). Rhizomes possess hypoglycaemic, vasodialator, spasmolytic, hypotensive, antioxidant, antimicrobial, anti-inflammatory and anthelmintic properties (Giri *et al.,* 2010; Sravani and Paarakh, 2011). There is no study reported on effect *H. spicatum* rhizomes on liver fluke *F. gigantica*. Therefore, the present study was undertaken to evaluate the effect of *H. spicatum* alcoholic extract on *in vitro* spontaneous muscular activity, glucose uptake, glycogen content and AChE activity of *F. gigantica*.

MATERIALS AND METHODS

Plant material and preparation of extract

H. spicatum rhizomes were procured from the local market and were identified botanically for their authenticity before use. The rhizomes were dried under shade, powdered with pulverizing machine and was extracted with 70% ethanol under reflux. Yield of *H. spicatum* alcoholic extract (HSAE) of rhizome was 6.23%. Stock solutions of the extract having strength of 10 mg/ml in tween-80 and distilled water were prepared.

Collection of parasites

Mature and healthy flukes were collected from the bile ducts of the freshly slaughtered buffaloes from local abattoir in insulated container containing sterile modified and warm Hedon-Fleig (H.F.) solution (NaCL-119.82mM; KCL-4.01mM; $MgSO_4$-0.29mM; $CaCl_2$-0.40mM; $NaHCO_3$-17.8mM; Glucose-22.3mM; Streptomycin sulphate-6900 units @ 10mg/liter and benzyl penicillin-9900 units/liter). Flukes were maintained at 38±1ºC in BOD incubator until use.

Effect of HSAE on rhythmicity of *Fasciola gigantica in vitro*

Rhythmicity or spontaneous muscular activity (SMA) of mature flukes was recorded by mounting worms isometrically in the tissue bath, using force displacement transducer connected to a pen writing recorder (Polyrite, Medicare, India) as per the method of Fairweather *et al.* (1983) for *F. hepatica* and modified by Kumar *et al.* (1995) for *F. gigantica*. Briefly, the flukes were mounted with the help of two fine steel hooks. One hook was inserted to the

posterior end of the fluke and tied to the tip of the aeration tube. The other hook was fixed posterior to the ventral sucker and connected to the force-displacement transducer. The fluke was left for a period of about 15 min in the tissue vessel before passive tension was applied.

The SMA was recorded, 30 min after equilibration under the resting force of 0.5g. After 15 min of equilibration period, the fluke was exposed to different cumulative concentration (100, 300 and 1000µg/ml) of HSAE and responses were recorded. Each concentration was allowed to act for a period of 15 min. Six flukes were mounted isometrically to examine effect of cumulative concentration of HSAE. Isometrically mounted flukes were also exposed to Tween-80 (Final concentration 0.1%) at an interval of 15 min and for a period of 1h to eliminate the possibility of its effect on SMA of the flukes.

Estimation of glucose uptake, glycogen content, AChE and lactic acid production

The *F. gigantica* were incubated in different concentrations (30 and 100µg/ml) of HSAE in H-F solution for 4h. The control group was also taken with only normal H-F solution. Following 4h incubation, flukes were used for estimation of glucose uptake and glycogen content (Hultman, 1959), AChE activity (Fishman and Green, 1961) and lactic acid production (Barker and Summerson, 1941). Minimum six observations were made for each of the four groups.

Statistical analysis

All the values are expressed as mean ± SEM. The statistical analysis of the data was carried out by one way ANOVA with Studentized Range Post-Hoc test. A probability level of $P<0.05$ and $P<0.01$ were considered significant.

RESULTS AND DISCUSSION

Effect on rhythmicity of *F. gigantica*

The alcoholic extract caused statistically significant concentration dependent increase in frequency of SMA at 100 (4.46±0.07 contractions/min) and 300 (5.30±0.14 contractions/min) µg/ml concentrations as compared to the control frequency (4.26±0.19 contractions/min). The amplitude of contractions was increased significantly and in concentration dependent manner (0.52±0.04g at 100µg/ml and 0.53±0.04g at 300µg/ml conc.) as compared to control (0.41±0.02g). At highest cumulative 1000µg/ml concentration, the frequency became nil, and irreversible rapid paralysis of worm was produced (Table 1). At this concentration, flukes did not recover from the paralysis with two successive washes with normal H-F solution at 15 min interval. From the reported literature it appears that acetylcholine (ACh) is a major inhibitory transmitter in fasciola (Probert and Durrani, 1977). In earlier studies ACh has been shown to produce inhibitory effect on the rhythmicity of *F. gigantica* caused flaccid paralysis of many flatworm parasites (Ribeiro *et al.*, 2005). Similarly, in the present study, the extract produced flaccid paralysis of the fluke. In the absence of substantial information on the effect on neurotransmitters on the spontaneous muscular activity of trematode, it can be speculated that the paralytic effect caused by HSAE might be due to its effect on cholinergic transmission mechanism of fluke.

Effect on glucose and glycogen content of *F. gigantica*

HSAE (30 and 100 µg/ml) inhibited glucose uptake significantly ($p<0.01$). HSAE found more effective in inhibiting glucose uptake than the reference drug hexaclorophen. The extract produced depletion of glycogen

Table 1.

Effect of cumulative doses of *H. spicatum* alcoholic extract (HSAE) on Spontaneous muscular activity of *F. gigantica.*

Groups	Dose	Frequency /minute	Amplitude/ tension (g)
Control	Tween-80 (1%)	4.26±0.19	0.41±0.02
HSAE	100 µg/mL	4.46±0.07	0.52±0.04*
	300 µg/mL	5.30±0.14**	0.53±0.04*
	1000 µg/mL	0.00	0.00

n=6; values are expressed as mean±SEM; tension 0.5 g; *= p<0.05; **= p<0.01 as Compared with control

Table 2.

Effect of different doses *H. spicatum* alcoholic extract on glucose uptake, glycogen content, AChE and Lactic acid production activity of *F. gigantica*

Groups	Dose	glucose uptake (mg/100 mg of tissue/h)	Glycogen content (mg/100mg of tissue)	AChE activity (mM of ACh hydrolysed/ g wet weight/h)	Lactic acid production (µg/g of tissue)
Control	Tween-80 (1%)	1.61±0.10	2.06±0.10	71.34±5.51	22.19±4.03
Hexachlorophene	10^{-5} M	1.08±0.07**	2.11±0.09	72.80±5.84	113.07±10.43**
HSAE	30 µg/mL	0.85±0.06**	2.03±0.10	66.52±4.87	37.89±4.03
	100 µg/mL	0.72±0.05**	1.01±0.06**	52.60±5.06*	26.08±3.19

n=6; Values are expressed as Mean±SEM; *= p<0.05; **= p<0.01 as compared with control

content in concentration related manner and caused significant ($p<0.01$) depletion of glycogen content at 100 µg/ml. Glucose and glycogen are the only energy sources utilized by the fasciola (Lloyd and Barrett, 1983). *F. gigantica* contains up to 3.7 to 6.3% glycogen of wet weight fluke (Goil, 1961). In the present study glycogen content was found to be 2.06% of wet weight fluke while glucose uptake was found to be 1.61mg/100mg of tissue/h (Table 2). This suggest that like benzimedazole group anthelmintics, anthelmintic action of HPAE may be due to loss of transport of vesicles of fluke, which leads to decrease in glucose uptake and increased utilization of stored glycogen.

Effect on AChE activity of *F. gigantica*

HSAE showed dose dependent marked inhibitory effect on AChE activity of treated flukes as compared to controls following 4 hour incubation. However, HSAE at 100 µg/ml concentration produced significant ($p<0.01$) inhibition of AChE activity of *F. gigantica*. AChE activity has earlier been reported in *F. hepatica* and *F. gigantica* (Probert and Durrani, 1997) as well and inhibitors of the enzyme have been reported to markedly reduce rhythmicity of flatworm parasites (Ribeiro *et al.,* 2005). Many conventional anthelmintic including those used against *F. hepatica* and against *F. gigantica*. viz., oxyclozanide and rafoxanide act through inhibiting AChE enzyme of the parasite (Probert and Durrani, 1997). As higher concentration of ACh cause flaccid paralysis, inhibition of AChE might also cause increase in concentration of endogenous ACh which might finally cause flaccid paralysis. Clearly, HSAE inhibited the activity of AChE enzyme of the fluke. Thus, it is interpreted that HSAE might possess some AChE inhibitory compounds.

Effect on lactic acid production by *F. gigantica*

The lactic acid production by *F. gigantica* in HSAE treated flukes did not altere significantly compared to control flukes at any of the dose levels (Table 2).

Thus, it is concluded that *H. spicatum* alcoholic extract has paralytic effect on *F. gigantica* which might be due to its interference on glucose uptake mechanism and also inhibition of AChE activity of the fluke resulting into flaccid type of paralysis. The observations of this study could further be exploited to explore the possibility to develop herbal-based anthelmintic of future.

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Research Article

PHARMACOKINETICS OF LEVOFLOXACIN IN CROSSBRED COW CALVES FOLLOWING INTRAVENOUS ADMINISTRATION

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ABSTRACT

Pharmacokinetic study of levofloxacin was carried out in six crossbred cow calves of 9-12 months of age weighing 70-90 kg. Levofloxacin was given intravenously at the dose rate of 4 mg/kg b wt in six crossbred cow calves. Levofloxacin concentration was estimated at different intervals (0.042 to 24 h) by microbiological assay method using *E. coli* (ATCC 25922) as test organism. Kinetic parameters were calculated by using two compartment open models. Attempts were made to calculate the rational dosage regimens of levofloxacin on the basis of kinetic data and maintenance of therapeutic concentrations in plasma. Following intravenous administration of levofloxacin, the extrapolated zero time concentration during distribution phase (65.34 ± 5.56 µg/ml), elimination phase (0.848 ± 0.008 µg/ml) and theoretical zero timed concentration (66.19 ± 5.56 µg/ml) were observed. The area under curve (10.43 ± 0.14 µg/ml/h) and area under first moment curve (58.21 ± 1.77 µg/ml/h^2) were observed. The mean residence time (MRT) was noted to be 5.56 h. For maintaining therapeutic concentration of 0.2 µg/ml, a loading dose (D*) of around 4.42 mg/kg and maintenance dose (D_0) of 3.78 mg/kg may be used at the dosage interval of 16 h for treating systemic infections in crossbred cow calves.

Keywords: Pharmacokinetics, levofloxacin, cow calves

INTRODUCTION

Levofloxacin [(-)-9-Fluoro-3-methyl-10-(4-methyl-1-piprazinyl)-7-oxo-2, 3-dihydro-7H- pyrido [1, 2, 3-de] [1,4]-benzoxazine-6-carboxylic acid] a recently introduced third-generation fluoroquinolone, possesses excellent activity against Gram-positive, Gram-negative and anaerobic bacteria (North *et al.*, 1998). As compared to ofloxacin and ciprofloxacin, it has more pronounced bactericidal activity against organisms like *Pseudomonas*, Entero-bacteriaceae and *Klebsiella* (Klesel *et al.*, 1995). The drug distributes well to target body tissues and fluids in the respiratory tract, skin, urine and prostate and its uptake by cells makes it suitable for use against intracellular pathogens (Langtry and Lamb, 1998). The mechanism of action levofloxacin and other quinolone antibacterials involves inhibition of bacterial topoisomerase II (DNA gyrase) and topoisomerase IV. Topoisomerases are essential in controlling the topological state of DNA replication, transcription, repair and recombination.

The pharmacokinetic studies of the levofloxacin have been conducted in adult cattle and buffaloes (Kumar *et al.*, 2009; Khutale, 2010), however, little literature is available on pharmacokinetics of levofloxacin in young animals, this study has been undertaken for pharmacokinetics of levofloxacin in cow calves.

MATERIALS AND METHODS

The present study was undertaken to determine the pharmacokinetics of levofloxacin administered by intravenous routes in healthy crossbred cow calves.

Experimental animals

The study was conducted on six healthy crossbred cow calves ranging 9-12 months of age and weighing between 70-90 kilograms. The animals were maintained at the Instructional Farm, College of Veterinary Science and Animal Husbandry, Mhow, Madhya Pradesh in separately 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. The experimental protocol for use of animals for conducting the present study was approved by IAEC.

Levofloxacin infusion (500 mg/100 mL; ZILEE®) was administered @ 4 mg/ kg B Wt by intravenous route in each crossbred cow calves. Intravenous injection of the drug was given through a jugular vein. Six healthy crossbred cow calves were employed to investigate the pharmacokinetics of levofloxacin following intravenous administration in crossbred cow calves.

Collection of blood samples

The blood samples (4-5 ml) from cow calves in clean sterilized previously added anticoagulant (10 % EDTA solution) test tube were collected with the help of an intravenous catheter (Teflon, 22 × 0.9 × 25 mm) fixed into the contra lateral jugular vein. Following intravenous administration of levofloxacin, the blood samples were collected at 0 minute (before drug administration) 0.042, 0.083, 0.167, 0.25, 0.333, 0.50, 0.75, 1, 2, 4, 6, 8, 10, 12, 16 and 24 h. Plasma was separated soon after collection by centrifugation at 5000 revolution per min (rpm) for 10 minutes (Eppendorf 5804 R, Germany). Separated plasma samples were transferred to labeled cryovials and stored

in deep freezer at -20°C.

Estimation of levofloxacin

The concentration of levofloxacin in plasma was determined by microbiological assay technique (Arret *et al*, 1971) using *E. coli* (ATCC 25922) as test organism.

Pharmacokinetic analysis

The plasma concentration-time profile of levofloxacin for each animal was used to determine the pharmacokinetics. The data of levofloxacin was subjected to two compartment open model (Gibaldi and Perrier, 1982).

RESULTS AND DISCUSSION

Concentrations of levofloxacin in plasma at various time intervals following its single intravenous (i.v.) injection at the dose rate of 4 mg/kg b wt have been shown in Figure 1. The mean plasma concentration of the drug at 0.042 h was found to be 17.02 ± 0.03 μg/ml and the values ranged from 16.89 to 17.14 μg/ml. The drug was detectable in all the six animals up to 24 h and the mean concentration at 24 h was noted to be 0.06 ± 0.003 g/ml however, the effective therapeutic concentration (≥ 0.1 μg/ml) of levofloxacin maintained with mean value of 0.11 ± 0.006 μg/ml up to 16 h of levofloxacin administration. The drug was not detectable in all the six animals at 30 h. Plasma drug concentration versus time profile has confirmed the two compartment open model for levofloxacin.

Table 1.
Pharmacokinetic parameters of levofloxacin after single intravenous administration (4 mg/kg) in crossbred cow calves.

Parameters (Unit)	Mean ± SE
A (μg/ml)	65.34 ± 5.56
B (μg/ml)	0.848 ±0.008
Cp0 (μg/ml)	66.19 ± 5.56
± (h-1)	13.77 ± 0.386
t1/2± (h)	0.051 ± 0.001
b (h-1)	0.121 ± 0.002
t1/2² (h)	5.72 ± 0.077
AUC (μg/ml.h)	10.43 ± 0.14
AUMC (μg/ml.h2)	58.21 ±1.77
MRT (h)	5.56 ± 0.158
K12 (h-1)	7.98 ± 0.049
K21 (h-1)	0.299 ± 0.006
Kel (h-1)	5.61 ± 0.363
Fc	0.022 ± 0.001
TH"P	45.06 ± 2.590
VdC(L/kg)	0.06 ± 0.005
VdB (L/kg)	4.70 ± 0.043
Vdarea (L/kg)	3.17 ± 0.051
Vdss (L/kg)	2.14 ± 0.062
ClB (L/kg/h)	0.38 ± 0.005
AUC/MIC	104.33 ±1.406

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

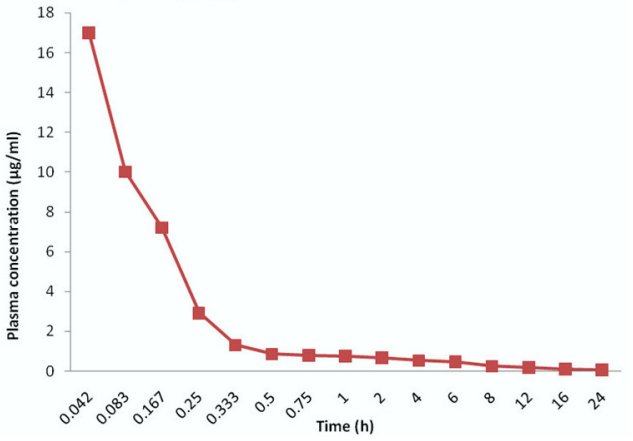

Fig 1.
Shows plasma concentrations (μg/ml) of levofloxacin following intravenous administration (4 mg/kg) in crossbred cow calves.

Table 1 shows the values of different kinetic parameters in healthy crossbred cow calves calculated by the compartment model.

The dosage regimens required to maintain the different levels of therapeutic concentration (C_p° min = 0.1, 0.2 and 0.3 μg/ml) in plasma for i.v. route in healthy crossbred cow calves at different dosage intervals (τ) of 8, 12 and 16 h are presented in Table 2.

In the present study, after single intravenous dose of levofloxacin in crossbred cow calves, mean peak plasma concentration of levofloxacin at 2.5 min was 17.02 ± 0.03 μg/ml and it was detected up to 24 h (0.06 ± 0.003 μg/ml).

The mean therapeutic concentration (≥ 0.1 μg/ml) of levofloxacin was maintained from 2.5 min to 16 h in plasma of calves. In contrast to present findings (16 h), Dumka (2007) observed that therapeutic concentration of levofloxacin in plasma was maintained only up to 10 h after i.v. administration of the drug (5 mg/kg) with paracetamol in crossbred calves. Dumka *et al.* (2008) stated that levofloxacin level above MIC_{90} (≥ 0.1 μg/ml) in plasma which was detected up to 10 h on single intravenous administration of levofloxacin (4 mg / kg) concurrently with meloxicam (0.5 mg / kg) in calves.

Plasma levofloxacin concentrations versus time disposition curves after intravenous administration were best fitted to the two compartment open model reported in calves (Dumka and Srivastava, 2007). In the present study, the value for extrapolated zero time concentration of levofloxacin during distribution phase and theoretical zero time concentration were observed (Table 1). In contrast, the mean value for theoretical zero time concentration (0.848 ± 0.008 μg/ml) during elimination phase was noted.

Table 2.
Dosage regimens of levofloxacin for intravenous route in crossbred cow calves

C_P^{∞} min (µg/ml)	τ (h)	Dose (mg/kg)	Levofloxacin
0.1	8	D*	0.84± 0.011
		D_0	0.52± 0.008
	12	D*	1.35± 0.02
		D_0	1.04± 0.02
	16	D*	2.21± 0.04
		D_0	1.89± 0.04
0.2	8	D*	1.67± 0.02
		D_0	1.04± 0.02
	12	D*	2.72± 0.04
		D_0	2.08± 0.04
	16	D*	4.42± 0.09
		D_0	3.78± 0.09
0.3	8	D*	2.51± 0.03
		D_0	1.56± 0.03
	12	D*	4.08± 0.06
		D_0	3.13± 0.06
	16	D*	6.62± 0.13
		D_0	5.68± 0.13

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

The lower distribution rate constants (α) and higher distribution half life ($t_{1/2}\alpha$) denote that higher rate of distribution of the drug occurred in crossbred cow calves. The present findings are more or less in close agreement with the findings of Dumka (2007) who also reported a very low $t_{1/2}\ \alpha$ of 0.04 ± 0.01 h in levofloxacin with paracetamol and 0.06 h when drug given alone in buffalo calves.

The higher elimination half life value (5.72 ± 0.08 h) was observed for levofloxacin in crossbred cow calves, which is also supported by shorter total body clearance (Cl_B). It indicates that retention time of levofloxacin is more in crossbred cow calves. This is further supported by the lower value of elimination rate constant of drug from central compartment (Kel) for levofloxacin (Table 1).

The value of area under plasma concentration time curve (AUC) and area under first moment of plasma drug concentration time curve (AUMC) of levofloxacin were noted to be 10.43 ± 0.14 µg/ml.h and 58.21 ±1.77 µg/ml.h^2 in crossbred cow calves (Table 1). A high values of AUC of levofloxacin have been reported in crossbred cow calves (23.94 ± 2.61; Goudah and Abo el-sooud, 2008). A low value of AUC of 9.02 ± 0.34 µg/ml.h and MRT (2.58 ± 0.11 h) were recorded by Dumka *et al.* (2008) in crossbred calves after i.v. administration of levofloxacin with meloxicam.

All values of volume of distribution calculated by different methods (Vd_c, Vd_B, Vd_{area} and Vd_{SS}) were noted to be 0.06 ± 0.005, 4.70 ± 0.043, 3.17 ± 0.051 and 2.14 ± 0.062 L/Kg, respectively. Indicating good penetration of levofloxacin into various body fluids and tissues was noted after intravenous administration in crossbred cow calves. A moderately high value of Vd_{area} obtained in the present study may be attributed to wide distribution of levofloxacin in the body because of its amphoteric nature (Vancutsem *et al.*, 1990) and high lipophilicity (Prescott and Baggot, 1994). The statement is further supported by higher tissue to plasma concentration ratio (45.06 ± 2.59) for levofloxacin was observed in the present study.

An average plasma concentration of 0.032 - 0.5 µg/ml has been reported to be the minimum therapeutic concentration (MIC_{90}) of levofloxacin against most gram positive, gram negative and atypical bacteria (Chulavatnatol, *et al.*, 1999). 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 0.1 µg/ml of levofloxacin has been taken into consideration. Levofloxacin possessed excellent antibacterial activity (MIC for 90% of tested strains i.e. $MIC_{90} \leq 0.5$ µg/ml) against most common gram-negative aerobic pathogens, including *E. coli*, *K. pneumoniae*, *Enterobacter sp*, and *H. influenza* (Thornsberry *et al.,* 1999). Watts *et al. (*1997) reported that most of veterinary fluoroquinolones are active at $MIC_{90} \leq 0.17$ µg/ml against sensitive strains isolated from field of veterinary importance. Thus, for maintaining therapeutic concentration of 0.2 µg/ml, a loading dose (D*) of around 4.42 mg/kg and maintenance dose (D_0) of 3.78 mg/kg may be used at the dosage interval of 16 h for treating systemic infections in crossbred cow calves.

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Research Article

EFFECT OF *PANAX GINSENG* ON IMMUNOMODULATORY PARAMETERS FOLLOWING SUBACUTE EXPOSURE TO ACETAMIPRID IN RATS

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ABSTRACT

An immunotoxicity study was conducted to evaluate ameliorating potential of *Panax ginseng* on immunotoxicity following subacute exposure to Acetamiprid in rats. Rats were divided in four groups comprising six animals each. Group I served as control and was administered with groundnut oil (1ml/100gm), group II was administered with Acetamiprid (52.5 mg/kg b wt), group III served as *Panax ginseng* control and group IV comprised of *Panax ginseng* (50 mg/kg b wt) along with Acetamiprid (52.5 mg/kg b wt). TLC, DLC, serum total protein, albumin, globulin, A:G ratio, humoral immune response (HA titer), cell mediated immune (DTH response) response and lymphocyte proliferation assay were estimated. Acetamiprid produced significant ($P<0.05$) reduction in total protein, albumin, globulin, HA titres, foot pad skin thickness and stimulation indices whereas a significant increase in neutrophils and A:G ratio was observed. *Panax ginseng* significantly restored these parameters towards normal. It was concluded that *Panax ginseng* has ameliorating potential on immunotoxicity following subacute exposure to acetamiprid in rats.

Keywords: Pesticide, acetamiprid, haematobiochemical, panax ginseng immunotoxicity, rat model.

INTRODUCTION

Panax ginseng is a traditional Chinese herb used commonly as herbal medicine because of its cardioprotective, antiasthmatic, antidiabetic and potent central nervous system activities including enhancement of memory, concentration, mental alertness and decrease of mental fatigue and other beneficial properties (Mahady *et al.,* 2000). The major effective components of ginseng are ginsenosides. *Panax ginseng* has also established itself as an immunomodulator. Ginsenoside Rg1 is proven to increase humoral and cell-mediated immune responses, antigen-reactive T helper cells, T lymphocytes and natural killer cells (Attele *et al.,* 1999).

Neonicotinoids are a set of nicotine-based insecticides being used tremendously because of their ability to replace most of the organophosphates (Fasnabi *et al.,* 2012). Acetamiprid is a member of neonicotinoid synthetic chlorinated insecticide family and chemically it is N^1-[(6-chloro-3-pyridyl) methyl]-N^2-cyano-N^1-methyl acetamidine. Studies have been conducted regarding subacute toxicity of acetamiprid (20% suspension) in female rats indicated that acetamiprid suppressed both CMI and antibody forming ability of lymphocytes (Mondal *et al.,* 2009).

MATERIALS AND METHODS

The plant was procured from commercial firm (JKH Herbs and Spcies, Navi Mumbai). The dried root of *Panax ginseng* was powdered and soaked in distilled water for 24 hours with continuous stirring at 40ºC. The mixture was filtered through muslin cloth and Whatmann filter paper no. 42 and concentrated in a rotary vacuum evaporator at 40-50ºC. The final extract was produced after drying the filtrate in incubator with fan (40ºC) and lyophilized. The percent yield (w/w) of aqueous extract of *Panax ginseng* was 11.5%.

Wistar rats of 2 to 2.5 month old age, weighing between 150 to 250 gm, were used in this study. The animals were kept in plastic cages and acclimatized for two weeks in the experimental lab of animal shed under standard managemental conditions. Standard rat feed and water was provided *ad libitum* throughout the experimental period. All the experimental animals were kept under constant observation during entire period of study. All studies were performed in accordance with the guidelines on regulation of scientific experiments as approved by the Institutional Animal Ethics Committee.

Technical grade acetamiprid (96.8% pure) was used to prepare the desired concentration of acetamiprid was made in groundnut oil while the extract of *Panax ginseng* was dissolved in water.

Evaluation of haematobiochemical parameteers TLC and DLC were determined by using standard method (Jain, 1986) and biochemical parameters by using diagnositic kits.

Blood was collected in centrifuge tube containing Alsever's solution with the help of syringe and needle from jugular vein of sheep under sterile conditions. Sheep's blood was mixed with Alsever's solution in proportion of 1:1 and washed thrice in Dulbecco's phosphate buffer saline (DPBS) at 1500 rpm/10 min. From final RBC pack,

1:10 and 1:100 dilutions were prepared for the purpose of counting RBCs. Final volume of RBCs was adjusted as $5x10^9$ cells/ml.

Evaluation of humoral immune response

Blood was collected in centrifuge tube containing Alsever's solution with the help of syringe and needle from jugular vein of sheep under sterile conditions. Sheep's blood was mixed with Alsever's solution in proportion of 1:1 and washed thrice in Dulbecco's phosphate buffer saline (DPBS) at 1500 rpm/10 min. From final RBC pack, 1:10 and 1:100 dilutions were prepared for the purpose of counting RBCs. Final volume of RBCs was adjusted as $5x10^9$ cells/ml.

For evaluation of HA titre, rats from various group were immunized by i.p. injection of SRBCs (0.5×10^9 cells/ rat, 100µl/rat) in saline seven days before completion of the treatment period as per the method given by Shukla *et al.* (2009).

The microtitre HA technique as described by Puri *et al.,* (1994) was employed to determine the serum antibody titer.

On day 18 of the exposure period, rats were sensitized by subcutaneous injection of 50 µl SRBCs suspended in Freund's complete adjuvant (FCA) in the ratio of 1:1 on back region. After 10 days (i.e. on day 28), sensitized rats were challenged by injecting (1.5×10^9 cells/ml) 100 µl of SRBC in right hind foot. Before injecting SRBCs in foot pad, rats were lightly anaesthetized and skin thickness was recorded. Swelling in the right hind foot pad was measured by pressure sensitive micrometer screw gauge, 24 and 48 hours after challenge. Histopathology of footpad was also performed to study cellular changes.

Lymphocyte proliferation test using MTT (3-(4,5-dimethyl thiazol-2-yl)2,5- diphenyl tetrazolium bromide) was performed at the end of experiment (on 29^{th} day of experiment) as per the procedure described by Bounous *et al.,*(1992) with some modifications.

Single cell suspensions of splenocytes without red blood cells were prepared as per the procedure described by Bounous *et al.,*(1992) with some modifications. Cell viability was determined by 0.5% trypan blue dye exclusion test. Finally, the viable cells number was adjusted to $2x10^6$ cells/ml with the help of complete medium. The test was performed in flat bottom 96 wells tissue culture plates (Grenier Bio-one, Germany).

To calculate the number of cells per ml volume, following formula was applied:

No. of cells/ml = Average number of cells x 10^4 x dilution factor

The blastogenic response of splenocytes was assessed by MTT colorimetric method as described by Mosmann (1983). The intensity of colour development was measured by taking optical density (OD) at 540 by Multiskan Ex Microplate reader.

Stimulation index (SI) was calculated using the following formula:

$$\text{Stimulation index (SI)} = \frac{\text{Mean OD of stimulated cell culture}}{\text{Mean OD of unstimulated cell culture}}$$

RESULTS AND DISCUSSION

TLC and DLC values in rats following 28 days exposure to Acetamiprid (ACE), *Panax ginseng* and their combination are shown in Table-1. In acetamiprid treated group, significantly ($P<0.05$) lower TLC and lymphocyte percentage, comparable monocytes and basophils and significantly ($P<0.05$) higher percentage of neutrophils as compared to control group was observed. In acetamiprid and Panax combination group, the values ranged in between control and acetamiprid treated group except for basophils for which values were comparable among the treatment groups. The observed values for control and Panax treated groups were comparable to each other for all TLC and DLC values.

In acetamiprid treated group total leukocyte count (TLC) and lymphocyte (differential) count were significantly lower than control group which reflects its immunosuppressant effect in the present study. A similar decrease in counts because of acetamiprid treatment has been reported by Mondal *et al.,* (2009). The immunomodulatory effect of Panax co-treatment significantly improved the TLC and lymphocytes counts in comparison to Acetamiprid alone group and was almost similar to control. Increase in total leukocytes and lymphocytes counts by intake of *Panax ginseng* in rats has been also cited by Simsek *et al.,* (2007).

The effect on total protein, albumin, globulin and A:G ratio in rats following 28 days exposure to acetamiprid (ACE), *Panax ginseng* and their combination is shown in Table-2. There was significant ($P<0.05$) decrease in level of total protein, albumin and globulin in acetamiprid treated groups and A:G ratio was significantly ($P<0.05$) higher. Panax co-treatment with acetamiprid had significant ($P<0.05$) ameliorating effect by preventing the decrease in level of total protein, albumin and globulin. All parameters in control, Panax and acetamiprid-Panax combination group were comparable among themselves except globulin, which was significantly ($P<0.05$) higher in acetamiprid and Panax combination group than acetamiprid group but still significantly ($P<0.05$) lower than control and Panax group. In our finding, exposure of rats to acetamiprid significantly decreased the total serum protein, albumin and globulin indicative of its toxic effect on various organs particularly liver and kidney. A significant decrease in serum protein, albumin and globulin has been reported in subacute toxicity of Acetaprimid in rats by Doltade *et. al.* (2012). In the present study, in acetamiprid plus Panax group, total

Table 1.
Effect of *Panax ginseng* on TLC and DLC values in rats following 28 days exposure to Acetamiprid (Mean±SE).

Group	TLC (×10⁹/L)	Lymphocyte(%)	Neutrophil(%)	Monocyte(%)	Basophils(%)
Control(Groundnut Oil)	9.72±0.043^{c}	74.50±0.22^{b}	21.83±0.17^{a}	3.17±0.17^{ab}	0.50±0.22
ACE (52.5mg/kg)	8.06±0.044^{a}	72.67±0.42^{a}	23.17±0.17^{b}	3.67±0.21^{b}	0.50±0.22
ACE + *Panax ginseng*	9.25±0.063^{b}	74.17±0.31^{b}	22.17±0.17^{a}	3.33±0.21^{b}	0.33±0.21
Panax ginseng	9.67±0.040^{c}	75.00±0.036^{b}	21.83±0.17^{a}	2.67±0.21^{a}	0.50±0.22

Values (Mean+ SEM, n=6, bearing different superscripts in the same rows differ significantly (P<0.05) in Duncun Multiple comparison post hock test)

Table 2.
Effect of *Panax ginseng* on Total protein, albumin, globulin and A:G ratio in rats following 28 days exposure to Acetamiprid (Mean±SE).

Group	Total protein(gm/dl)	Albumin(gm/dl)	Globulin(gm/dl)	A:G ratio
Control(Groundnut Oil)	6.86±0.032^{b}	3.86±0.017^{b}	2.99±0.014^{c}	1.29±0.004^{a}
ACE (52.5mg/kg)	5.47±0.036^{a}	3.27±0.019^{a}	2.19±0.016^{a}	1.48±0.002^{b}
ACE + *Panax ginseng*	6.79±0.083^{b}	3.83±0.052^{b}	2.89±0.055^{b}	1.32±0.029^{a}
Panax ginseng	6.89±0.029^{b}	3.88±0.023^{b}	2.99±0.017^{c}	1.29±0.009^{a}

Values (Mean+ SEM, n=6, bearing different superscripts in the same rows differ significantly (P<0.05) in Duncun Multiple comparison post hock test)

serum protein, albumin and globulin showed improved levels indicative of prophylactic action of *Panax ginseng*. Fattah *et al.,* (2011) reported that administration of combination of wheat germ oil and *Panax ginseng* to irradiated rats improved serum levels of total protein, albumin and globulin.

Haemagglutination titre against sheep RBCs in terms of log (2)/0.05ml are shown in Fig 1. HA titres were significantly (P<0.05) decreased in acetamiprid alone treatment group as compared to control. Panax plus acetamiprid treatment caused increase in HA titre towards normalization and was comparable to all acetamiprid, control and Panax group. Present study also showed significantly lower HA titers as compared to control in acetamiprid treated group which are in accordance with the findings of Mondal *et al.,* (2009) and Doltade *et al.,* (2012). A significant decrease in the antibody titers in the hemagglutination test against SRBCs is indicative of reduction of immunoglobulin levels in the serum of rats of this group because of immunosuppressive effect of acetamiprid. Multiple reports have cited reasons for decline in HA titre to SRBC (Ghosh and Chauhan, 1991). Combination of Acetamiprid and Panax in the present study exhibited almost comparable HA titre as that of control group which establishes the immunopotentiation by Panax. Immunomodulatory activity of *Panax ginseng* has been reported by Kumar *et al.,* (2012). Zhang *et al.,* (2010) explained the immunopotentiation on murine spleen lymphocytes induced by polysaccharide fraction of *Panax ginseng* via upregulating calcineurin activity (Ca^{2+}–CN–NFAT–IL-2 signaling pathway).

Cell mediated immune response was evaluated employing delayed type hypersensitivity reaction to sheep RBCs. The thickness of skin of foot pad was measured at 24, 48 and 72 hr after the challenge with sheep RBCs in different groups (Figure 2). Acetamiprid with Panax resulted in significant increase in foot pad skin thickness as compared to Acetamiprid alone treatment group in the present study reflecting the immune restoration because of Panax ginseng. Ginseng saponins can ameliorate delayed type hypersensitivity reactions by inhibiting nitric oxide and prostaglandin-E2 expression in macrophages. These compounds also enhance the phagocytic activity of macrophages. Ginseng saponins enhance the activation of T and B lymphocytes, and Natural killer cells. In addition, other animal models have demonstrated that these saponins can also promote interferon induction. Suppression of both CMI and antibody forming ability of lymphocytes due to Acetamiprid has also been cited by Mondal *et al.,* (2009). The reduction in cell mediated immunity thus confirms the toxic potential of Acetamiprid. This may be attributed either to inhibition of cell proliferation (Marshall *et al.,* 2011), inhibition of expansion of primal and activated antigen specific T cell (Dhasarathan *et al.,* 2011), T cell cytotoxicity (Liu *et al.,* 2009) or induction of apoptosis in activated as well as in resting T cell population, which had reduced T cell count (Strauss *et al.,* 2002).

The stimulation indices, following con-A induced stimulation of lymphocytes collected from rats treated with acetamiprid alone and in combination with Panax for 28 days are presented in Figure 3. The stimulation indices were found to be significantly reduced (P<0.05) in acetamiprid treated group in comparison to control. Panax co-treatment significantly(P<0.05) increased stimulation index as compared to acetamiprid treated group. The stimulation index values in combination acetamiprid and Panax group was comparable to both control and Panax alone groups. The lymphocyte stimulation index in

acetamiprid treated group in the present study was significantly ($p<0.05$) lower than control which could be attributed to the depletion of lymphocytes in the spleen and Peyer's patches and lymphocytopenia. The decrease in stimulation index because of imidacloprid, a neonicotinoid insecticide has been cited by Badgujar *et al.,* (2013) and according to them it had cytotoxic effects against T cells (particularly TH cells). A decrease in proliferation index due to acetamiprid in human peripheral blood lymphocytes has been reported (Kocaman *et al.,* 2007). Co treatment with *Panax ginseng* significantly improved the stimulation index to almost same as that of control. Differential regulation of lymphocyte proliferation by ginsenosides from *Panax ginseng* has also been reported by Cho *et al.* (2002). Soowon and Hyeyoung (2012) have reported increased T and B lymphocyte proliferation in response to concanavalin A by *Panax ginseng*.

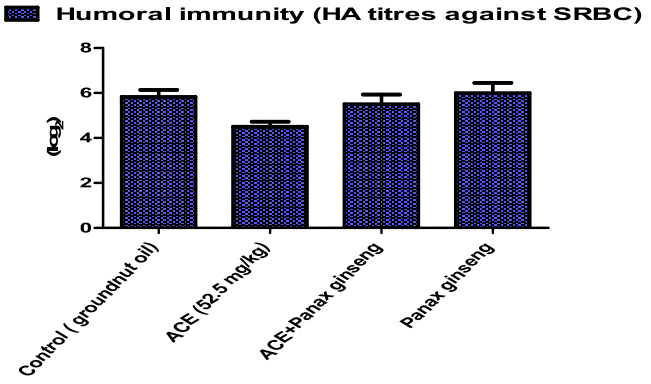

Fig-1
Effect of *Panax ginseng* on humoral immunity (HA titres against SRBC) in rats following 28 days exposure to Acetamiprid (ACE)

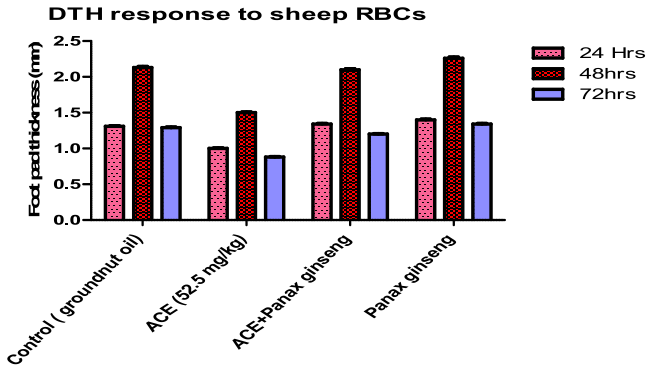

Fig-2
Effect of *Panax ginseng* on DTH response to sheep RBCs in rats following 28 days exposure to Acetamiprid (ACE)

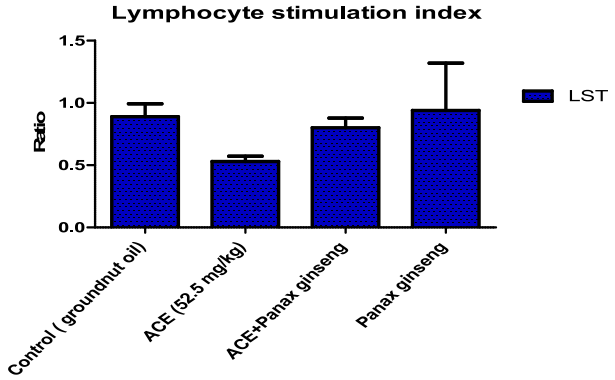

Fig-3
Effect of *Panax ginseng* on Lymphocyte stimulation index in rats following 28 days exposure to Acetamiprid (ACE)

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Research Article

PHARMACOKINETICS OF LEVOFLOXACIN ON CONCOMITANT ADMINISTRATION OF KETOPROFEN IN SHEEP

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ABSTRACT

The pharmacokinetics of levofloxacin was evaluated in female sheep following its single intravenous (i.v) and subcutaneous (s.c) administration (3 mg kg^{-1}) on concomitant intramuscular (i.m.) administration of ketoprofen (3 mg kg^{-1}). Levofloxacin concentration was determined using the High Performance Liquid Chromatography. Following i.v. administration, distribution of the drug was rapid ($t_{1/2(\alpha)}$:0.21 ± 0.02 h) and wide as reflected by the high value of volume of distribution. The drug was eliminated relatively faster with a total body clearance (Cl_B) of 0.56 ± 0.02 $Lh^{-1}kg^{-1}$. The elimination half-life ($t_{1/2\beta}$) was 2.39 ± 0.21 h. Following s.c. administration, the drug was absorbed faster and C_{max} of 2.32 ± 0.07 μgmL^{-1} was achieved at 1 h post administration. The values of volume of distribution (Vd_{area}), total body clearance (Cl_B) and elimination half-life ($t_{1/2\beta}$) were 1.42 ± 0.05 L kg^{-1}, 0.55 ± 0.03 L $h^{-1}kg^{-1}$ and 1.70 ± 0.03 h, respectively. Nearly complete absorption of drug from the site of s.c administration resulted high bioavailability of 91.34 ± 3.66 %. Based on pharmacokinetic-pharmacodynamic indices calculated in the present study,levofloxacin may be a potentially useful drug at prescribed dose along with ketoprofento treat bacterial diseases in sheep.

Keywords: Pharmacokinetics, levofloxacin, ketoprofen, intravenous, subcutaneous, sheep.

INTRODUCTION

Levofloxacin, a chiral fluorinated carboxyquinolone, is newer generation of fluoroquinolone. The drug is active against Gram-positive aerobic organisms such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, Gram-negative bacteria such as *Haemophilus influenzae, Klebsiella pneumonia,Escherichia coli*, *Moraxella catharralis,* and *Pseudomonas aeruginosa*, responsible for pneumonia, including *Mycoplasma* spp., *Legionella* spp*., Chlamydia* spp. and *Mycobacterium*spp., as well as active against anaerobes (Martin *et al.*1998). The drug is also effective against intracellular pathogens as it can readily distribute in body fluids and tissues of various body systems (Langtry and Lamb, 1998). The potential value of levofloxacin was described by previous studies on its pharmacokinetic profiles in domestic animals(Arvind Kumar *et al.*, 2012; Patel *et al.,* 2012; Patel *et al.,* 2013).

Concomitant use of NSAIDs may invariably affect disposition of the quinolones with enhancement of the convulsant activity of quinolones. The alteration in disposition of levofloxacin on concomitant administration of paracetamol and meloxicam in calves have been evaluated previously (Dumka, 2007). Ketoprofen (KTP), an aryl propionic acid derivative, non-selective COX inhibitor NSAID which is useful as anti-inflammatory, analgesic and antipyretic drug for the treatment of inflammatory conditions including rheumatoid arthritis and osteoarthritis in animals (Green, 2001). Pharmacokinetics of ketoprofen have been evaluated in many species of animals.

Sheep, a minor species of animals is reared for wool and mutton production. Pneumonia and other bacterial infections in sheep are conditions which require antimicrobial drugs likely to be used alone or in combination with NSAID like ketoprofen. Information on the disposition of levofloxacin on concurrent administration with ketoprofen in sheep is not available. Therefore, the present study was undertaken to determine the pharmacokinetics of levofloxacin following its single dose intravenous (i.v) and subcutaneous (s.c) injection on concomitant intramuscular (i.m.) administration of ketoprofenin sheep.

MATERIALS AND METHODS

Experimental animals

The study was conducted on six healthy female Patanwadi non-lactating sheep of 2-3 years old age ranging in b wt from 23.5 to 30.0 kg obtained from and maintained at the Instructional Farm, College of Veterinary Science and Animal Husbandry, AAU, Anand, India. The animals were then housed in separate pen and were provided standard ration with water as provided *ad libitum.*Constant observation for two weeks prior to commencement of the experiment was followed with clinical examination in order to exclude the possibility of any disease.The experimental

protocol of the present study was approved by the IAEC.

Drug administration and sampling

Levofloxacin (Tavanic®, 100 mL vial of solution of levoûoxacin hemihydrate equivalent to 500 mg levofloxacin, Aventis Pharmaceutical Ltd, Bangalore) was randomly injectedby the i.v.or s.c. routein all animals according to a crossover design along with i.m. injection of ketoprofen (Neoprofen®, Vetnex Ranbaxy Fine Chemicals Limited, New Delhi, India) at dose rate of 3 mg kg^{-1} for both drugs. The levofloxacin was administered throughi.v.ands.c routes via the left jugular vein and at neck region, respectively. Intramuscular injection of ketoprofenwas given in deep gluteal muscle of thigh region. The washout period of 15 days was observed between two treatments of levofloxacin to rule out possibility of drug residue. Blood samples (3 mL) were collected from i.v. catheter (Venflon, 22 × 0.9 × 25 mm) fixed into the right jugular vein into heparinized centrifuge tube. Following i.v. administration of levofloxacin along with i.m injection of ketoprofen, blood samples were collected at 0 (prior to treatment), 0.033, 0.083, 0.166, 0.33, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 18, 24 and 36 h post-treatment. Whereas following s.c administration of levofloxacin along with i.m. injection of ketoprofen, blood samples were collected at 0 (prior to treatment), 0.083, 0.166, 0.33, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 18, 24, 36 and 48 h post-treatment. Plasma was separated by centrifugation at 3000 g for 15 min and taken into labeled cryovialsfor storage at -35 °C until assayed for levofloxacin concentration using high performance liquid chromatography (HPLC) procedure which was usually done with in 24 to 36 h.

Levofloxacin assay and pharmacokinetic analysis

Levofloxacin concentrations in the plasma samples were determined by HPLC with UV detection according to the method described previously (Patel *et al.* 2012[a]). The plasma concentrations vs. time curves obtained after treatment in each individual animal were semi-logarithmically fitted with PK Solutions software program (Version 2.0, Summit research services, USA). The peak concentration (C_{max}) and time to peak concentration (T_{max}) were taken directly from the curve. A non-compartmental model was used to determine the area under the concentration-time curve (AUC) and the area under the first moment curve (AUMC), using the linear trapezoidal rule with extrapolation of infinity. The mean residence time (MRT) was calculated as AUMC/AUC (Gibaldi and Perrier, 1982). The distribution and elimination half-lives were calculated as ln 2 divided by the distribution and elimination rate constants, respectively. The estimated plasma concentration of the drug at zero time (Cp^0) after its i.v. administration was the sum of the extrapolated zero-time concentrations of the coefficient A and B. Total body clearance (Cl_B), apparent volume of distribution (Vd_{area}) and volume of distribution at steady state (Vd_{ss}) were calculated using following formulas: Cl_B = Dose*F/AUC; Vd_{area} = Dose*F/(AUC)() where for i.v., 100% bioavailability (F=1) was considered and Vd_{ss}= Dose*AUMC/$(AUC)^2$. The absolute bioavailability (F) following s.c. administration of the drug was calculated as ($AUC_{s.c.}/AUC_{i.v.}$) X 100.Student's t-test was used to test the pharmacokinetic parameters for significant difference between pharmacokinetic parameters in ketoprofen treated and normal sheep according to Snedecor and Cochran (1980).

PK/PD integration

Efficacy predictors like C_{max}/MIC_{90} and $AUC_{(0-\infty)}/MIC_{90}$ for concentration dependent antibiotic levofloxacin were calculated using the values of peak plasma drug concentration (C_{max}) and area under the curve ($AUC_{(0-\infty)}$)after s.c. administration. MIC_{90} data of levofloxacin against ovine bacterial isolates have not been reported earlier. Thus, to cover most of the susceptible organisms the MIC_{90} of 0.12 µg mL^{-1} of levofloxacin has been taken into consideration as described by Goudah and Hasabelnaby (2010).

RESULTS AND DISCUSSION

No local or systemic adverse reactions were observed after intereaction of test drugs in clinical examination of all animals under study. Pharmacokinetic parameters (Mean ± S.E.) estimated after each route of drug administrations are depicted in Table 1. The mean plasma concentration-time profile of levofloxacin following single i.v. and s.c. administration on concomitant administration of ketoprofenat 3 mg kg^{-1}bwtis presented graphically in Figure 1.

Following i.v. administration of levofloxacin in the present study, plasma concentration-time profile showed a rapid initial distributive phase, followed by relatively slower elimination phase with average elimination half-life which was proximate to half-lives of the drug reported in normal sheep. The drug was distributed faster andextensive in body organs as reflected by low value of distribution half-life and higher volume of distribution in ketoprofen treated sheep. The higher volume of distribution with levofloxacin was also observed in normal sheep (Patel *et al.,* 2012).

The Vd_{ss} is not clearance-dependent and is used to calculate the drug amount in the body under equilibrium conditions and is high with lipid soluble drugs like fluoroquinolones (Brown, 1996). The Vd_{ss} for levofloxacin in sheep was also relatively high in ketoprofen treated sheep which is proximate to the Vd_{ss}observed in normal sheep (Patel *et al.,* 2012[a]). Due to its lipid solubility and low plasma protein binding, the drug has tendency to penetrate into various body fluids and tissues.Plasma protein binding of 23.74% has been found with levofloxacin in sheep (Goudah and Hasabelnaby, 2010). Rapid transfer of levofloxacin from central to peripheral compartment in sheep treated with ketoprofen was evident from the high values of distribution rate constants and ratio of K_{12}/K_{21}

(2.09 ± 0.11). It indicates that distribution of levofloxacin remains unaltered on simultaneous administration of ketoprofeneven though having high plasma protein binding.

The elimination half-life of the levofloxacinin ketoprofen treated sheep is proximate to those observed in normal sheep. The total body clearance has been found similar to that observed in our previous study in normal sheep (Patel *et al.,* 2012).

Following s.c. administration oflevofoxacin along with ketoprofen injection, the drug was absorbed rapidly as indicated by short absorption half-life in sheep. The maximum drug concentration (C_{max})achieved at 1 h is comparable to the C_{max} observed following extravascular administration of the drug in normal sheep (Patel *et al.* 2012). The value of C_{max} in ketoprofen treated sheep compared to normal sheep (Patel *et al.,* 2012) has been found significantly ($P < 0.01$) higher. The high volume of distribution in ketoprofen treated sheep in the present study is found similar to that observed in normal sheep and goat (Patel *et al.* 2012 and 2013). The elimination half-life and total body clearance of levofloxacin with the presence of ketoprofen in body of sheep have not been altered when compared to the values previously observed in sheep treated alone with levofloxacin (Patel *et al.,* 2012).

The significant differences in most of all pharmacokinetic parameters following i.v. administration have not been observed when compared the values obtained after s.c administration of the drug along with ketoprofen injection in sheep.The nearly complete absorption with high systemic bioavailability has been observed following s.c. administration of the drug in ketoprofen treated sheep which is similar to that observed in normal sheep. Nearly complete absorption has also been documented for levofloxacin in ewes and goats after extravascular injection (Patel *et al.,* 2012 and 2013).

Success of concentration dependant antibacterial drug like levofloxacin is usually predicted from C_{max}/MIC_{90} and AUC/MIC_{90}. High ratio of the C_{max}/MIC_{90} has also been associated with a lower incidence of the development of resistance (Dudley, 1991).The values of C_{max}/MIC_{90} and AUC/MIC_{90} in the present study following s.c administration of the drug were calculated to be 19.33 and 39.58,

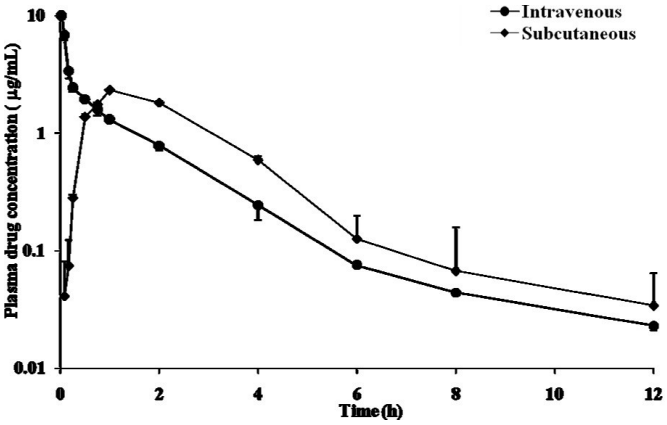

Fig. 1
Semilogarithmic plot of plasma concentration-time profile of levofloxacin following its single dose (3 mg kg^{-1}) intravenous and subcutaneous administration along with single intramuscular injection of ketoprofen (3 mg kg^{-1}) in sheep. Values are presented as mean ± S.E. of 6 animals.

Table 1.
Pharmacokinetic parameters (Mean ± S.E.)of levofloxacin in sheep following single intravenous and subcutaneous administration at a dose of 3 mg kg^{-1} b wt subsequently after single intramuscular injection of ketoprofen (3 mg kg^{-1}).

Parameters	Unit	Intravenous	Subcutaneous
Cp^0	µg mL^{-1}	7.63 ± 0.54	—
$t_{½\alpha}$	h	0.21 ± 0.02	—
$t_{½\beta}$	H	2.39 ± 0.21	1.70 ± 0.03
$t_{½Ka}$	H	—	0.29 ± 0.02
$AUC_{(0-\infty)}$	µg h mL^{-1}	5.40 ± 0.21	4.75 ± 0.11
AUMC	µg h^2 mL^{-1}	8.95 ± 0.49	13.75 ± 0.89
Vd_{area}	L kg^{-1}	1.94 ± 0.21	1.42 ± 0.05
Vd_{ss}	L kg^{-1}	0.93 ± 0.08	—
C_{max}	µg mL^{-1}	-	2.32 ± 0.07
T_{max}	H	-	1.00 ± 0.00
K_{12} / K_{21}	Ratio	2.09 ± 0.11	—
$Cl_{(B)}$	L h^{-1} kg^{-1}	0.56 ± 0.02	0.55 ± 0.03
MRT	H	1.66 ± 0.10	2.71 ± 0.01
F	%	—	91.34 ± 3.66

Cp^0: concentration at time 0; $t_{1/2\alpha}$: half-life of distribution phases; $t_{1/2\beta}$: elimination half-life; $t_{1/2ka}$: absorption half-life; $AUC_{(0-\infty)}$: area under the curve from zero to infinity; AUMC: area under first of moment curve; Vd_{area}: apparent volume of distribution;Vd_{ss}:volume of distribution at steady state; C_{max}: maximum drug concentration; T_{max}: time to time to peak plasma drug concentration; K_{12} and K_{21}: First order rate constants for drug distribution from central and peripheral compartments, respectively; $Cl_{B,}$: total body clearance; MRT: mean residence time; F: bioavailability.

respectively. The value of most important surrogate marker (C_{max}/MIC_{90}) exceeds the recommended ratio.In conclusion, levofloxacin can be administered with ketoprofen to treat bacterial diseases which are caused by susceptible bacteria in sheep.

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Research Article

SINGLE DOSE INTRAVENOUS AND INTRAMUSCULAR PHARMACOKINETICS OF ORBIFLOXACIN IN BROILER CHICKENS

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ABSTRACT

The present study was planned to investigate pharmacokinetics of orbifloxacin in broiler (n = 6) at the dose rate of 2.5 mg kg^{-1} b wt following single dose intravenous and intramuscular administration, using HPLC with fluorescence detector. Initial plasma concentration of orbifloxacin after single dose IV administration (2.5 mg kg^{-1}) showed the level of 5.717 ± 0.053 μg ml^{-1} achieved at 0.0833 h (5 min), whereas, after IM dosing, the mean peak plasma concentration of orbifloxacin (1.533 ± 0.050 μg ml^{-1}) was achieved at 1 h, which rapidly declined to 0.521 ± 0.023 μg ml^{-1} at 6 h.

The mean ± SE values of elimination rate constant (β), elimination half-life ($t_{1/2}$), area under curve (AUC), area under the first moment of the plasma drug concentration (AUMC), mean residence time (MRT), apparent volume of distribution (Vd_{area}), volume of distribution at steady state (Vd_{ss}) and total body clearance (Cl_B), following IV and IM administration, were 0.361 ± 0.035 and 0.264 ± 0.018 h^{-1}, 2.034 ± 0.231 and 2.698 ± 0.211 h, 6.905 ± 0.202 and 7.583 ± 0.340 μg h ml^{-1}, 20.045 ± 2.730 and 29.500 ± 2.064 μg h^2 ml^{-1}, 2.908 ± 0.383 and 3.883 ± 0.166 h, 0.498 ± 0.070 and 0.974 ± 0.167 L kg^{-1}, 0.519 ± 0.076 and 1.286 ± 0.060 L kg^{-1} and 0.364 ± 0.011 and 0.333 ± 0.014 L h^{-1} kg^{-1}, respectively. The calculated intramuscular bioavailability of orbifloxacin in broilers was 109.81 %, and plasma orbifloxacin concentrations were maintained above its therapeutic concentration up to 10 h after IM administration.

Key Words: Pharmacokinetics, Orbifloxacin, Broiler chickens, Intramuscular bioavailability

INTRODUCTION

Orbifloxacin, an antimicrobial drug, is a third generation synthetic fluoroquinolone, developed for the exclusive use in veterinary medicine and it exhibits high bactericidal activity against Gram-positive, Gram-negative and *Mycoplasma Spp.* (Papich and Riviere, 2001). Orbifloxacin is indicated for the treatment of skin, soft tissue, and urinary tract infections in dogs, and skin and soft tissue infections in cats, and in some countries, orbifloxacin has been used for the treatment of gastrointestinal and respiratory infections in cattle, swine and other animals (Cazedey and Salgado, 2013). It also has potential use in poultry species and its pharmacokinetic studies have been investigated in Hubbard chickens and ducks (Tohamy, 2011[a&b]), goat (Ghanshyam *et al.*, 2015), sheep (Dudhatra *et al.*, 2013), buffalo calves (Tohamy, 2011c), cattle (Elias *et al.,* 2009), horses (Davis *et al.,* 2006) and dogs (Gebru *et al.*, 2009). However, in chickens, only oral pharmacokinetics report is available for orbifloxacin (Tohamy, 2011a), therefore the present study was planned to investigate the intravenous and intramuscular pharmacokinetics of orbifloxacin following its single dose administration in broiler chickens.

MATERIALS AND METHODS

Experimental animals

Six healthy Caribro broiler chickens aged between 06-10 weeks and having b wt between 1.5 - 2.5 kg were employed for the present study. Birds were procured from IPDP, Ahmedabad, Government of Gujarat, and experiment was conducted at Department of Pharmacology and Toxicology, College of Veterinary Science and A.H., S.D.A.U., Sardarkrushinagar, after getting approval from Institutional Animal Ethics Committee (IAEC). Birds were housed in individual cages and provided antibiotic free standard grower and finisher broiler feeds and *ad. lib.* fresh drinking water.

Drug and reagents

Orbifloxacin pure powder and injectable solution (50 mg ml^{-1}) was obtained from Intas pharmaceuticals Ltd., Ashram road, Ahmedabad. Drugs, reagents and chemicals, solvents and water used were of HPLC/ analytical grade and were purchased from S. D. Fine Chem. Ltd, Mumbai.

Experimental design and sample collection

Pharmacokinetics of orbifloxacin were studied following two different routes IV and IM at the similar dose rate of 2.5 mg kg^{-1} and between two phases wash-out period of 15 days was kept. Orbifloxacin (5 %) solution was administered single at a dose rate of 2.5 mg kg^{-1} b wt, intravenously in wing vein and intramuscularly in breast muscle in broiler chickens.

Sample preparation and HPLC assay

About 2-3 ml of blood samples were collected from wing vein with help of intravenous catheter in heparinized test tubes at 0 min (pre-administration), 5, 10, 15 and 30 min, and 1, 2, 4, 6, 8, 10, 12 and 24 h post

administration of drug. The plasma were separated by centrifugation and stored in cryo-vials at –20°C until assayed using HPLC system (Knauer, Germany) equipped with isocratic solvent delivery pump (Model K 501) and fluorescence detector (Model RF 10 AXL). Chromatographic separation was performed by using reverse phase Thermo C_{18} column at room temperature.

Samples for HPLC assay were prepared by protein precipitation of plasma samples (1 ml) with acetonitrile (1 ml), then vortexed for 30 seconds and centrifuged at 4°C for 10 minute at 3000 rpm. Finally, 150 µl of supernatant was transferred to HPLC vial and 850 µl of diluent (0.067-M-disodium hydrogen phosphate buffer; pH 7.5) was added to it.

Mobile phase was prepared by mixing buffer and methanol in the ratio of 43:57. Buffer was prepared by dissolving 6.8 gm of potassium dihydrogen orthophosphate in 1000 ml water and by adding 3 ml of triethylamine, then pH was adjusted to 2.5 with orthophosphoric acid. Flow rate was 1.5 ml min^{-1} at ambient temperature. Detection was performed at an excitation wavelength of 290 nm and an emission wavelength of 450 nm. The sensitivity of assay method for orbifloxacin was 0.01 mg ml^{-1}. The assay was responsive, reproducible and linearity was observed from 0.01 to 6.0 µg ml^{-1}. The mean correlation coefficient (R^2) was 0.9990.

Pharmacokinetic analysis

The plasma concentration–time curves of individual birds were analyzed for pharmacokinetic parameters with the PK Solution 2.0 software (Summit Research Services, USA) based on non-compartmental method.

RESULTS AND DISCUSSION

The semi logarithmic plot of plasma orbifloxacin concentrations versus time after single dose intravenous and intramuscular administrations at the dose rate of 2.5 mg kg^{-1} b wt in broiler chickens is presented as Figure 1. Post IV administration of orbifloxacin in broilers, the initial plasma orbifloxacin concentration was 5.717 ± 0.053 µg ml^{-1} at 5 min and 5.190 ± 0.130 µg ml^{-1} at 10 min. Following IM dosing, the mean peak plasma concentration of orbifloxacin (1.533 ± 0.050 µg ml^{-1}) was achieved at 1 h, which declined to 1.208 ± 0.060 µg ml^{-1} at 2 h. Plasma levels of orbifloxacin further diminished and was detected (0.062 ± 0.012 µg ml^{-1}) up to 12 h after drug administration. Orbifloxacin was not detectable in sample collected at 24 hours after both IV and IM administration. Mean values of important pharmacokinetic parameters following intravenous and intramuscular administration of orbifloxacin in broiler chickens have been given in Table 1.

In present study, following single dose IV administration of orbifloxacin (2.5 mg kg^{-1} b. wt.) in Caribro broiler chickens., the mean initial plasma level was found to be 5.717 µg ml^{-1} at 5 min., whereas, following single dose IM administration, the mean peak plasma drug concentration (C_{max}) of 1.533 µg ml^{-1} was observed at 1 h. Similar value of C_{max} following oral administration of orbfloxacin at same dose rate was reported as 1.63 µg ml^{-1} in chickens (Tohamy, 2011a).

The mean value of elimination half-life ($t_{1/2\beta}$) after IV administration of orbifloxacin was 2.03 h which was lower than the values reported as in domestic species (Dudhatra *et al.*, 2013) and 8.63 h in goat (Ghanshyam *et al.*, 2015).

Following single dose IM administration of orbifloxacin, mean $t_{1/2\beta}$ was calculated to be 2.698 h in broiler chickens. This value was lower than that reported in chickens following oral administration as 4.92 h (Tohamy, 2011a). A higher $t_{1/2\beta}$ value was also reported in healthy ducks as 4.18 h following single dose IM administration of

Table 1

Pharmacokinetic parameters (mean ± S.E.) of orbifloxacin after single dose intravenous and intramuscular administrations in broiler chickens (n=6).

Pharmaco-kineticparameters	Unit	Intravenous	Intramuscular
Cp^o	µg ml^{-1}	5.717 ± 0.053	——-
β	h^{-1}	0.361 ± 0.035	0.264 ± 0.018
C_{max}	µg ml^{-1}	——-	1.533 ± 0.050
T_{max}	h	——-	1.000 ± 0.000
$t_{½\alpha}$	h	0.976 ± 0.076	——-
$t_{½\beta}$	h	2.034 ± 0.231	2.698 ± 0.211
AUC	g h ml^{-1}	6.905 ± 0.202	7.583 ± 0.340
AUMC	g h^2 ml^{-1}	20.045 ± 2.730	29.500 ± 2.064
Vd_{area}	L kg^{-1}	0.498 ±0.070	0.974 ± 0.167
Vd_{ss}	L kg^{-1}	0.519 ±0.076	1.286 ± 0.060
Cl_B	ml h^{-1} kg^{-1}	0.364 ± 0.011	0.333 ± 0.014
MRT	h	2.908 ± 0.383	3.883 ± 0.166
F	%	——-	109.81 ± 3.66

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

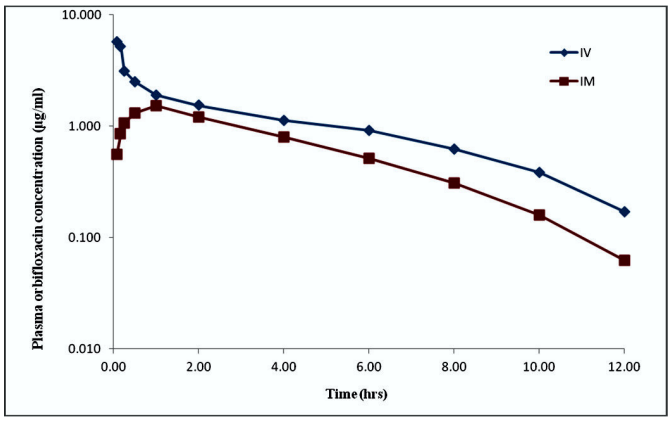

Fig. 1

The Semi-logarithmic plot of mean plasma concentration of orbifloxacin against time following IV and IM administrations in broiler chickens (n=6).

orbifloxacin but at higher dose rate of 5.0 mg kg^{-1} (Tohmay, 2011). The result indicates moderate distribution of the drug into various body fluids and tissues of broiler chickens following IM administration.

Mean total body clearance (Cl_B) derived as 0.36 L h^{-1} kg^{-1} after IV administration of the drug in the present study, was lower than its corresponding value reported as 0.60 L h^{-1} kg^{-1} in Japanese Quail (Tohamy, 2011b). Following single dose IM administration Cl_B value of the drug was found to be 0.33 L h^{-1} kg^{-1} which was higher to those reported as 0.12 h in both sheep and goat (Dudhatra *et al.*, 2013; Ghanshyam *et al.*, 2015).

In present study, IM bioavailability in chicken was calculated as 109.81 ± 3.66 %,. The similar bioavailability of orbifloxacin was also reported in Japanese quail as 102 and 117 % following its oral administration at dose of 15 and 20 mg kg^{-1}, respectively.

On the basis of therapeutically effective concentration (0.12 µg ml^{-1}; Haines *et al.,* 2001), it was concluded that the clinically effective concentration of orbifloxacin would be maintained up to 12 h after IV (0.171 mg Kg^{-1}) and 10 h after IM (0.159 mg Kg^{-1}) administration in broiler chickens given at dose rate of 2.5 mg Kg^{-1} .

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Research Article

COMPARATIVE STUDY BETWEEN NEEM (*AZADIRACHTA INDICA*) LEAF AQUOUS EXTRACT AND AMPROLIUM HYDROCHLORIDE FOR TREATMENT OF COCCIDIOSIS IN FOWL (*GALLUS GALLUS*)

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ABSTRACT

The present work was carried out for comparative study between Neem (Azadirachta indica) leaf aquous extract and amprolium for treatment of coccidiosis in fowl. Forty (40) broiler chickens aged 4 weeks were divided equally randomly in five groups. Group I was taken as control, 2nd group was infected but without treatment, 3rd and 4th groups were treated with neem leaf extract @ 500 mg/ kg b wt and 800 mg/ kg b wt whereas, the 5th group was treated with standard drug Amprolium hydrochloride @ 1.2g/lt. The haemoglobin, PCV, neutrophils (%), total serum protein (g/dL), SGPT (I .U/dL), SGOT (I.U/dL) values significantly (P<0.01) decreased and the value of TLC (%), lymphocytes (%), eosinophils (%) and the serum glucose (mg/dl) significantly increased in infected untreated group 2 birds from 5th day of infection. All the values returned toward the normal range in birds of treatment groups. 100% anticoccidial efficacy was found in birds treated with neem leaf extract 800 mg / kg b wt at the end of experiment, whereas, amprolium was 100 % effective on 15th days of treatment against *Eimeria spp*. High growth rate was observed in healthy birds of control group followed by amprolium and neem treated group. Heavy lesion score (++++) was recorded in infected and untreated group. The clinical signs and symptoms such as loss of appetite, unthriftiness, reddish diarrhea, comb and wattles pale and anaemic from 7th day were recorded in infected and untreated group. The aqueous neem leaf extract and amprolium hydrochloride for five consecutive day treatment was found most effective against *Eimeria spp*.

Key words: Coccidiosis, SGPT, Fowl, neem, Amprolium, Boby weight, lesion score.

INTRODUCTION

Coccidiosis is a common infectious disease in poultry, causing major economic losses. The protozoan parasite of the genus *Eimeria* multiplies in the intestinal tract of poultry and produces tissue damage, resulting in reduced growth and increased susceptibility to pathogens (McDougald, 2003). Neem (*Azadirachta indica*) is perhaps the most useful traditional medicinal plant in India. Each part of the neem tree has some medicinal property and is thus commercially exploitable. *A. indica* contains at least 35 biologically active principals of which Nimbin and azadirachtin are the most active insecticidal ingradients and are present predominantly in the seeds, leaves and other parts of the neem tree (Mulla *et al.,* 1999). Comparative study between was conducted using neem leaf water extract and amprolium hydrochloride for the treatment of *Eimeria* spp. in poultry.

MATERIALS AND METHODS

A total of 40 broiler chickens aged 4 weeks were randomly divided into five equal groups. Feed and water were allowed *ad- libitum* to all birds throughout the period of experiment except that they were withdrawn 6 hours prior of experimentally infection the birds group 2, 3 ,4 and 5 with an oral dose of 1ml of coccidian suspension (containing 5000 oocysts). Birds of group 3 and 4 were treated with aqueous neem leaf extract @ 500 mg/ kg b.wt. and 800 mg/ kg b.wt. orally, respectively, whereas, the birds of 5th group were treated with amprolium hydrochloride @ 1.2g/lt. in water orally. Birds of all the three treatment groups were treated for five consecutive days. The research work was approved by the IAEC.

Haematological observations like Hb, PCV, TLC and DLC were done as per the method described by Schalm *et al.* (1975). The biochemical parameter like serum glucose, total serum protein, SGOT and SGPT were estimated through Auto analyzer (15 Biosystem) using standard kit. The OPG was estimated as per method Stoll (1930). All the haemato-biochemical and OPG were estimated at five days interval and b wt along with clinical sign taken at weekly interval for 20 days of experiment. Lesion scoring was done by sacrifing one bird of each group on 10th day of the experiment as per technique suggested by Johnson and Reid (1970). The data was statistical analyzed as per Snedecor and Cochran (2004).

RESULTS AND DISCUSSION

Effects of Hemato-biochemical profile, OPG, B wt and lesion score are given in the Table 1. The results revealed significantly (P<0.01) decreased hemoglobin and

PCV levels in group 2, while in group 3, 4 and 5 showed the positive increase in the Hb and PCV level 5th days onward and the Hb and PCV levels returned towards the normal value at the end of experiment. Similar findings were reported by Patra *et al.* (2010). They also reported haemoglobinaemia and reduced packed cell volume during coccidia infection in poultry, where the values returned toward the normal range treated with mushroom or amprolium. The significant increase in TLC % was recorded in group 2, whereas, fowl of group 3 and 4 showed significant decrease. While in group 5 as the treatment days progressed TLC level returned towards the normal value at end of experiment. This is in agreement with the findings of Hirani *et al.* (2007) where they reported the high value of total leucocytes count during coccidial infection in poultry bird. Significantly increased lymphocytes and slightly increased eosinophils and significantly decreased neutrophills or heterophils and monocytes were recorded in fowls of group 2, whereas fowls of group 3, 4 and 5 showed the value at about normal range in the end of experiment.

There was significant (P<0.01) increase in serum glucose, SGPT and SGOT in group 2. The values of TSP slightly reduced from 5th day to the end of experiment. Whereas, in treated groups the birds had significantly decreased serum glucose, SGPT and SGOT and significantly increased the value of TSP. It came in the range of the healthy birds of control group at the end of experiment. Such finding is in agreement with the work of Biu *et al.* (2006). Biochemical serum analysis of coccidial infected chickens showed a significant increase (P<0.05) in SGOT. Mondal *et al.* (2011) also reported significantly increased serum glucose, AST, ALT and reduced TSP values. Patra *et al.* (2010) reported an increase in the values of ALT and AST in *Eimeria spp*. infection in boiler birds. The biochemical profile alteration might be due to dysfunction of liver caused by the presence of parasite in organ (*Eimeria* spp.) during infection and subsequent recovery of the organ after treatment indicated an immunogenic effect of the parasite which was observed in *Eimeria* spp. of infection. Though the enzymes SGPT and SGOT are primarily cellular enzyme of hepatocytes and

Table 1.
Effects of Hemato-biochemical profile, oocysts per gram (OPG), B wt and leaves on lesion score of *Eimeria spp*. infection and treated by aqueous neem (*Azadirachta indica*) leaf extract and amprolium hydrochloride in fowl.

Parameters	Days of observation	Groups				
		1	2	3	4	5
Hb(gm %)	0	10.86±0.07	$10.91±0.08^{e}$	$10.96±0.08^{d}$	$10.93±0.07^{d}$	$10.83±0.07^{bc}$
	5	$^{D}10.88±0.07$	$^{A}6.30±0.10^{d}$	$^{B}7.06±0.07^{a}$	$^{B}7.25±0.11^{a}$	$^{C}8.39±0.10^{a}$
	10	$^{C}10.95±0.08$	$^{A}5.11±0.11^{c}$	$^{B}8.60±0.13^{b}$	$^{C}8.98±0.11^{b}$	$^{D}10.65±0.12^{b}$
	15	$^{C}10.99±0.06$	$^{A}4.21±0.12^{b}$	$^{B}10.25±0.10^{c}$	$^{B}10.30±0.15^{c}$	$^{C}10.83±0.08^{bc}$
	20	$^{C}11.08±0.07$	$^{A}3.61±0.11^{a}$	$^{B}10.80±0.07^{d}$	$^{B}10.86±0.10^{d}$	$^{BC}11.04±0.09^{c}$
PCV (%)	0	33.54±0.12	$33.56±0.12^{e}$	$33.78±0.17^{d}$	$33.76±0.16^{c}$	$33.54±0.12^{b}$
	5	$^{E}33.65±0.17$	$^{A}20.10±0.48^{d}$	$^{B}24.85±0.15^{a}$	$^{C}26.20±0.34^{a}$	$^{D}27.80±0.18^{a}$
	10	$^{C}33.75±0.17$	$^{A}17.20±0.34^{c}$	$^{B}29.81±0.44^{b}$	$^{B}30.30±0.43^{b}$	$^{C}33.09±0.39^{b}$
	15	$^{B}33.76±0.16$	$^{A}14.50±0.38^{b}$	$^{B}32.85±0.34^{c}$	$^{B}33.11±0.23^{c}$	$^{B}33.55±0.37^{b}$
	20	$^{B}33.80±0.17$	$^{A}11.70±0.34^{a}$	$^{B}33.15±0.37^{cd}$	$^{B}33.48±0.30^{c}$	$^{B}33.61±0.38^{b}$
TLC(%)	0	$10.96±0.08^{a}$	$10.95±0.10^{a}$	$11.03±0.08^{a}$	$10.98±0.09^{a}$	$10.95±0.14^{a}$
	5	$^{A}10.98±0.09$	$^{C}12.60±0.10^{b}$	$^{B}11.90±0.11^{c}$	$^{B}11.80±0.10^{b}$	$^{B}11.68±0.15^{b}$
	10	$^{A}10.96±0.13$	$^{C}14.60±0.15^{c}$	$^{B}11.41±0.12^{b}$	$^{A}11.00±0.09^{a}$	$^{A}10.99±0.09^{a}$
	15	$^{A}10.99±0.12$	$^{B}16.78±0.34^{d}$	$^{A}11.00±0.15^{a}$	$^{A}10.99±0.08^{a}$	$^{A}10.98±0.15^{a}$
	20	$^{A}10.99±0.13$	$^{B}17.76±0.39^{e}$	$^{A}10.96±0.14^{a}$	$^{A}10.90±0.12^{a}$	$^{A}10.95±0.10^{a}$
Lymphocytes (%)	0	56.25±0.67	$56.25±0.49^{a}$	$56.25±0.56^{a}$	$56.50±0.53^{a}$	$55.88±0.52^{a}$
	5	$^{A}56.38±0.42$	$^{B}64.63±2.03^{b}$	$^{B}63.50±0.42^{c}$	$^{B}63.13±0.67^{c}$	$^{B}62.25±0.53^{d}$
	10	$^{B}56.75±0.59$	$^{C}72.25±0.45^{c}$	$^{B}59.75±0.45^{b}$	$^{B}59.25±0.25^{b}$	$^{B}58.50±0.42^{c}$
	15	$^{A}56.50±0.78$	$^{B}76.88±0.58^{d}$	$^{A}57.63±0.50^{a}$	$^{A}57.38±0.38^{a}$	$^{A}57.38±0.38^{bc}$
	20	$^{A}56.63±0.50$	$^{B}79.50±0.63^{e}$	$^{A}57.25±0.53^{a}$	$^{A}57.13±0.44^{a}$	$^{A}56.50±0.50^{ab}$
Monocytes (%)	0	9.50±0.19	$9.50±0.19^{b}$	9.88±0.23	9.75±0.25	9.75±0.25
	5	9.63±0.18	$9.63±0.18^{b}$	9.50±0.19	9.50±0.19	9.88±0.13
	10	$^{B}9.50±0.27$	$^{A}8.50±0.19^{a}$	$^{B}9.63±0.18$	$^{B}9.63±0.18$	$^{B}9.63±0.18$
	15	$^{B}9.63±0.26$	$^{A}8.38±0.18^{a}$	$^{B}9.63±0.18$	$^{B}9.63±0.18$	$^{B}9.63±0.18$
	20	$^{B}9.75±0.25$	$^{A}8.38±0.18^{a}$	$^{B}9.75±0.16$	$^{B}9.50±0.19$	$^{B}9.50±0.19$
Eosinophils (%)	0	1.75±0.16	$1.88±0.13^{a}$	$1.88±0.23^{ab}$	$1.50±0.19^{a}$	$1.88±0.13^{bc}$
	5	$^{A}1.63±0.18$	$^{AB}2.00±0.27^{a}$	$^{B}2.38±0.18^{b}$	$^{B}2.25±0.16^{b}$	$^{AB}2.13±0.13^{c}$

Means bearing different small and capital superscripts differ significantly (P < 0.05) within groups and between group respectively.

important marker of liver damage. It is also present in other tissue including intestine, hence increase in SGPT is rendered to hepatic disturbance mediated due to coccidial enteritis. Moreover, it can also be due to degenerative changes in intestinal mucosa.

The body weights were found to have significantly increased in all groups but a high growth rate was observed in healthy group 1 followed by 5, 4 and 3. Whereas, in group 2 the lowest bofy weight was recorded. The present finding indicated that growth of treated group was satisfactory. Durrani *et al.* (2008) reported neem leaf treated birds had better b wt gain as compared to control. The body weight alteration might have been due to loss of blood through bloody diarrhoea, decrease feed conversion ratio and weakness in infected group.

The OPG count of infected group significantly increased from 5th days and at the end of experiment. In amprolium treated group 100% efficacy was observed on 15th day, whereas, in neem leaf treated group 100% efficacy observed in 20th day of observation. Biu *et al.* (2006) showed that anticoccidial efficacy of the aqueous neem leaf extract dose of 800mg/kg in comparison to amprolium compared favourably with 10mg/litre of amprolium in treating the disease, both showed 100% survival rates for infected and treated chickens with zero oocyst per gram at day 4 post treatment.

The lesion score value of group 2 showed heavy lesion score (++++) and in group 3 and 4 showed moderate lesion score (+), whereas, group 1 and 5 (0) showed no lesion. Owai and Gloria (2010) reported that increase in the dose rate of neem leaves extract decreased the level of infection.

In group 2 the clinical signs and symptoms were recorded as loss of appetite, unthriftiness, reddish diarrhea, comb and wattles pale and anaemic from 7th day. Whereas, in group 1, 3, 4 and 5 no clinical sign and symptom were observed. No mortality was observed in all groups, but morbidity was observed in group 2 birds. These findings are in accordance with Soomro *et al.* (2001) where they observed the symptoms of loss of appetite, unthriftiness, greenish or reddish diarrhoea. Affected birds were mostly found showing their comb and wattles pale and anaemic.

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Research Article

PHARMACOKINETICS OF CEFTIZOXIME FOLLOWING SINGLE DOSE INTRAVENOUS ADMINISTRATION IN SHEEP

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ABSTRACT

Ceftizoxime is a third generation cephalosporin drug, used in veterinary medicine. The present study was planned to investigate pharmacokinetics of ceftizoxime in sheep (n = 6) at the dose rate of 10 mg kg^{-1} b wt following single intravenous dose.The ceftizoxime plasma concentrations as versus time data after intravenous administration was described by non-compartment model of pharmacokinetics with the use of "PK Solver Software". The LOQ of ceftizoxime was found to be 0.1 µg ml^{-1} in plasma. Following single dose intravenous administration of ceftizoxime (10 mg kg^{-1} b. wt.), therapeutically effective concentration of ceftizoxime as1.56 ± 0.27 µg ml^{-1} persisted in plasma up to 24 h. The average mean values of elimination rate constant, half-life, area under curve, mean residence time and total body clearance were found as 0.08 h^{-1}, 7.31 h ,126.29 µg h ml^{-1},11.42 h and 0.09 L h^{-1} kg^{-1}, respectively.

Key Words: Pharmacokinetics, ceftizoxime, intravenous, sheep

INTRODUCTION

Ceftizoxime is a third generation cephalosporin having high bactericidal activity against a wide range of Gram-positive and Gram-negative microorganisms including *Streptococci, Staphylococci, Proteus, Bacillus, Klebsiella, Clostridium, Salmonella* and *Shigella spp*. (Mandell, 1979). It is commonly used for the treatment of the infections of respiratory tract, urogenital tract, skin, soft tissues, bones and joints. It has better activity against anaerobes, broader spectrum of activity against Gram negative bacteria, penetrates the cerebrospinal fluid in sufficient concentration due to greater lipid solubility, and is resistant to hydrolysis by β-lactamase (Mandell and Sande, 1991). Ceftizoxime is not metabolized in the body and is excreted unchanged predominantly by glomerular filtration (Facca *et al.*, 1998). Previously, pharmacokinetic studies of ceftizoxime were carried out in sheep (Rule *et al.*, 2000), goats (Karmakar *et al.*, 2011) and, cross breed calves (Singh *et al.*, 2008). Due to paucity in such research work in Indian breed of sheep, the present study was designed to determine the pharmacokinetic data of ceftizoxime in Patanwadi sheep (*Ovis aries*) after its single dose intravenous administration, under tropical environment.

MATERIALS AND METHODS

Animals

Six female healthy sheep of Patanwadi breed having b wt between 25-35 kg and age of 2-4 years were selected for the study. The sheep were housed, in clean experimental pens, two weeks prior to experiment for acclimatization. All essential and standard managemental practices were followed to keep the sheep free from any stress. This study was undertaken after approval of IAEC, College of Veterinary Science and Animal Husbandry, SDAU, Sardarkrushinagar.

Experimental design and sample collection

Injectable ceftizoxime powder formulation was obtained from Intas Pharmaceuticals Ltd., Ashram road, Ahmedabad. Drug was administered intravenously at the dose rate of 10 mg kg^{-1} to each of six sheep through left jugular vein. Blood samples (3-4 ml) were collected into heparinized tubes from IV catheter (Venflon, 22 X 0.9 X 25 mm) fixed into the right jugular vein at 0 (pre-dosing), 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 36 h, 48 h and 96h, post intravenous administration of drug. Plasma was separated after centrifugation (10 min, 5000 RPM, 4^0C) of blood samples and stored at – 40^0C until analyzed.

Sample preparation for ceftizoxime assay

Ceftizoxime was extracted from plasma using ice-cold acetonitrile. Exactly, 300 µl of plasma sample was mixed with 300 µl ice-cold acetonitrile. After thorough mixing with vortex mixture (1 minute), samples were centrifuged (10 min) at 10,000 RPM at 4^0C using refrigerated centrifuge machine. Supernatant thus obtained was collected and 20 µl was injected into UHPLC machine.

Chromatographic conditions and validation

UHPLC apparatus (Thermo Fisher, Germany) consisting of UV detector (Dionex ultimate 3000), Gradient Solvent Delivery Pump (Dionex ultimate 3000) and manual injector was used for this study. Chromatographic separation was performed by using reverse phase C_{18} column (ODS; 25 cm x 4.6 mm ID, 4.5 µ) at room temperature. The data integration was performed by "Chromeleon" software version 6.8. The mobile phase was

Table 1:
Pharmacokinetic parameters of ceftizoxime in plasma after single dose IV administration (10 mg.kg^{-1} b. wt.) in female Patanwadi sheep (n=6).

Pharmacokinetic parameters	Unit	Values (IV route)
β	h^{-1}	0.08 ± 0.01
C_{max}	μg ml^{-1}	48.95 ± 2.31
$t_{½β}$	h	7.31 ± 1.05
AUC	μg h ml^{-1}	126.29 ± 16.02
AUMC	μg h^2 ml^{-1}	1459.49 ± 231.06
Vd_{area}	L kg^{-1}	1.10 ± 0.22
Vd_{ss}	L kg^{-1}	0.97 ± 0.17
Cl_B	ml h^{-1} kg^{-1}	0.09 ± 0.01
MRT	h	11.42 ± 1.30

β, Elimination rate constant; C_{max}, maximum drug concentration; $t_{½β}$, elimination half life; AUC, area under plasma drug concentration-time curve; AUMC, area under first moment of curve; Vd_{area}, apparent volume of distribution; Vd_{ss}, volume of distribution at steady state; Cl_B, total body clearance; MRT, mean residence time.

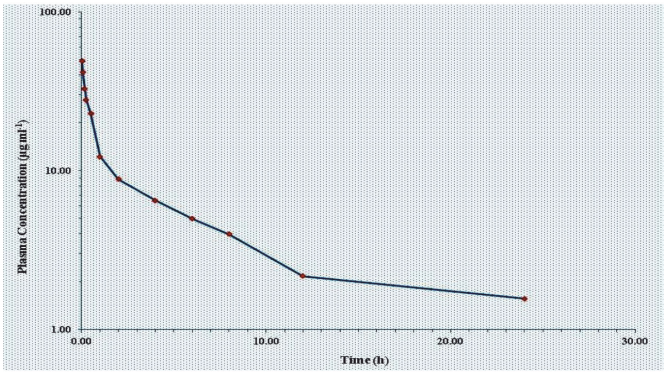

Fig. 1:
Semi logarithmic plot of mean ceftizoxime concentrations in plasma versus time following single dose IV administration (10 mg.kg^{-1} b. wt.) in Patanwadi sheep (n=6).

a mixture of 286 µl glacial acetic acid in 250 ml HPLC water and acetonitrile (60:40). Mobile phase was filtered through 0.45 µm filter paper by using vacuum pump and degassed using sonicator and pumped into column at a flow rate of 0.8 ml min^{-1} at ambient temperature. The effluent was monitored at 260 nm wavelength. The retention time of ceftizoxime in plsama samples was 3.2 min.

Calibration curve was prepared by adding known amount of ceftizoxime to blank unfortified (drug-free) pooled sheep plasma in the concentration range from 0.1 to 80 μg ml^{-1}, which were processed exactly as done for samples, described above. The assay was responsive, reproducible and linearity was observed from 0.1 to 80 μg ml^{-1} with mean correlation coefficient (R^2) of 0.9976. The mean recovery percentage of ceftizoxime in plasma was 98.41 % at 80 μg ml^{-1}. Intraday and interday precision and accuracy were calculated and at all concentrations, the value of the RSD was less than 10 %.

Pharmacokinetic analysis

The plasma concentration - time curves of individual sheep were analyzed for obtaining pharmacokinetic parameters with the software 'PK Solver', a freely available menu-driven add-in program for Microsoft Excel written in Visual Basic for Application (VBA) in solving basic problems in pharmacokinetic (Zhang *et al.*, 2010).

RESULTS AND DISCUSSION

The graphical representation of mean plasma concentration of ceftizoxime against time, after its single dose IV administration (10 mg kg^{-1} b. wt.), is presented in Figure-1 as a semi-logarithmic plot. Following IV administration, the initial plasma ceftizoxime concentration was found as 48.95 ± 2.31 μg ml^{-1} at 0.033 h (2 min), which nearly halved to 22.83 ± 1.96 μg ml^{-1} at 0.50 h (30 min). Thereafter, plasma level of ceftizoxime diminished gradually (1.56 ± 0.27 μg ml^{-1} at 24 h) and was not detectable post 24 h of drug administration.

Various pharmacokinetic parameters calculated from plasma concentrations of ceftizoxime after its single dose IV administration (10 mg.kg^{-1} b. wt.) in female Patanwadi sheep are shown in Table-1.

In present study, the plasma levels of ceftizoxime were estimated by UHPLC system. Following single dose IV administration of ceftizoxime at the rate of 10 mg kg^{-1} b. wt., the mean peak plasma level found at 0.033 h (2 min) was 48.95 µg ml^{-1}. Comparative higher peak plasma level (88.78 µg ml^{-1}) of ceftizoxime was reported in goat at same dose rate following IV administration (Karmakar *et al.*, 2011), whereas similar peak level (44.40 µg ml^{-1}) was observed in goats in another study at same dose rate (Bhatiya, 2015). Rule *et al.* (2000) also observed higher peak levels as 116.4 and 78.85 µg ml^{-1} in sheep and goats, respectively, which may be attributable to higher dose rate (20 mg kg^{-1} b.wt.) used in both the studies. For a successful therapeutic effect, the plasma concentrations of an antimicrobial agent should not fall below MIC during the course of treatment. In present study, the plasma ceftizoxime level at 24 h was detected as 1.56 µg ml^{-1} which was well above MIC range of 0.2 – 1.0 µg.ml^{-1}.

In present study, elimination rate constant (β) observed was 0.08 ± 0.01 h^{-1} with corresponding elimination half life ($t_{1/2β}$) of 7.31 ± 1.05 h, which demonstrate long elimination phase of ceftizoxime kinetics in sheep. However, higher mean $t_{1/2β}$ value of 8.92 h was reported by Bhatiya (2015) in goats following ceftizoxime IV administration. In other species like goat (Karmakar *et al.*, 2011), lower values of ceftizoxime $t_{1/2β}$ were reported as 6.24 and 5.86 h, respectively. In present study, the mean apparent volume of distribution calculated by area method (Vd_{area}) and volume of distribution at steady state (Vd_{ss}) were 1.10 and 0.97 L kg^{-1}, respectively, which

indicate wider distribution of the ceftizoxime in sheep body. The mean values of Vd_{area} were reported lower in goats as 0.45 (Karmakar *et al.*, 2011) and 0.329 L kg^{-1} (Bhatiya, 2015), whereas, similar value (1.10 L kg^{-1}) was reported in buffaloes (Kumar *et al.*, 2016). In present study, mean values of AUC was found as 126.29 µg h ml^{-1}. In sheep (Rule *et al.,* 2000), dog and monkey (Murakawa *et al.*, 1986), lower values of drug AUC were noticed as 100.00, 73.30, and 56.2 µg h ml^{-1}, respectively, However, Karmakar *et al.* (2011) found higher drug AUC as 185.22 µg h ml^{-1} in goats. Longer value of mean resident time (MRT) for ceftizoxime observed as 11.42 h, in present study shows long persistence of this drug in sheep body. Comparatively, similar values of MRT were reported in goats as 11.44 h (Bhatiya, 2015) and 7.58 h (Karmakar *et al.,* 2011), however, Prashant *et al.* (2014) found lower value of MRT as 3.63 h in goats after IV administration of ceftizoxime (20 mg kg^{-1} b. wt.).

Total body clearance (Cl_B) was found as 0.09 L h^{-1} kg^{-1} after IV administration of ceftizoxime (10 mg kg^{-1} b. wt.) in present study, whereas, higher values of Cl_B as 0.24 L h^{-1} kg^{-1} in sheep (Rule *et al.*, 2000) and lower values of Cl_B as 0.05 L h^{-1} kg^{-1} in goats (Karmakar *et al.*, 2011) were observed after IV administration of ceftizoxime. Murakawa *et al.* (1986) found higher values of Cl_B in dog, mouse, rat and monkey as 0.196, 3.67, 1.16 and 3.57 L h^{-1} kg^{-1}, respectively.

Present study of ceftizoxime pharmacokinetics in Patanwadi sheep reveals the favorable pharmacokinetic outcomes, like $t_{1/2\beta}$, Vd_{area}, AUC and Cl_B, suggesting the rational usefulness of this drug in sheep reared in tropical environment to combat infections disorders.

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Research Article

AMELIORATING POTENTIAL OF BACOPA MONNIERI IN ACETAMINOPHEN AND ALCOHOL INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

The objective of the study was to evaluate the hepatoprotective efficacy of 50% hydroethanolic extract of *Bacopa monnieri* (HEBM) against alcohol and acetaminophen induced toxicity in rats. The rats were divided into seven groups (n=5). During 28 days study, alcohol @ 1ml of 50% v/v solution, acetaminophen @ 1000 mg/kg p.o., silymarin @ 100 mg/kg b wt and hydroethanolic extract of *Bacopa monnieri* (HEBM) in 2 dose rates (100 & 200 mg/kg b wt) were given as per the experimental design. $HEBM_{200}$ significantly ($p<0.05$) restored reduced liver weights and hematological parameters towards normal. HEBM at both dose levels improved the levels of ALT, AST, GGT and ALP significantly ($p<0.05$) as compared to negative control. The changes observed in levels of total protein, albumin, globulin, serum cholesterol, triglyceride, total and direct bilirubin, urea, creatinine, uric acid and calcium were restored significantly ($p<0.05$) by $HEBM_{200}$. It can be concluded that hydroethanolic extract of *Bacopa monnieri* @ 100 and 200 mg/kg po possessed protective efficacy activity against ethanol and acetaminophen induced hepatotoxicity in rats.

Key words: Antioxidant activity, Hepatoprotective activity, *Bacopa monnieri,* rats.

INTRODUCTION

Liver is the major organ for metabolism and disposition of endogenous substances and plays an important role in the maintenance and regulation of homeostasis of the body. Being the chief site for metabolism, it is highly exposed to xenobiotics and is prone to hepatotoxicity. Among NSAIDs, acetaminophen is one of the commonly prescribed over the counter drug which is well reported for its hepatotoxic action after chronic use or overdosing. acetaminophen toxicity is aggravated in alcoholics as ethanol is a potent inducer of CYP2E1 and increases the metabolism of acetaminophen, leading to increased production of its intermediate metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) causing depletion of glutathione reserves along with increased NAPQI formation which ultimately interferes with protein and nucleic acid functioning(Mitchell *et al.*,1973).

Bacopa monnieri, commonly known as Brahmi (Family S*crophulariaceae*) is a profusely branched herb growing in marshy areas, with succulent stem and leaves and small-white flowers. The plant has indistinct odour and a slight bitter taste (Aiyer and Kolammal, 1964; Chopra *et al.*, 1956). *Bacopa monnieri* is reported to be rich in many phytocompounds including alkaloids like brahmine and herpestine, saponins like d-mannitol, hersaponin, acid A and monnierin, flavonoids (luteolin and apigenin) and sterols responsible for its potent antioxidant activity (Hossain *et al.,* 2012). Various reports reveal its neuroprotective, sedative, tranquillising, anticancer, immunomodulatory, anti-inflammatory and antimicrobial activity. Since, sufficient literature is not available on its putative hepatoprotective activity, the aim of the study was to evaluate the hepatoprotective potential of the plant against acetaminophen and alcohol induced toxicity in rats. The objective of the study was to evaluate the hepatoprotective potential of the plant when challenged against severe damage produced by administering ethanol and acetaminophen.

MATERIALS AND METHODS

Plant material was identified, collected and authenticated from Medicinal Plant Research and Developmental Centre (MRDC), GBPUAT, Pantnagar, Uttarakhand. Acetaminophen (Paracip, Cipla) and silymarin (Silybon-140, Microlabs) were purchased from a medical store at Pantnagar.

Hydroethanolic extract of *Bacopa monnieri* was prepared using 50% ethanol. The cold extracts were prepared by the method described by Singh (2008) with slight modifications. According to this method, powdered material was soaked in 50% hydroethanolic solution (1gm/ 10ml of 50% hydroethanolic solution) for 24 hours with continuous stirring at 37 °C. The mixture was filtered through several layered muslin cloth and centrifuged to separate the supernatant. The final extract was produced after drying the filtrate in fan incubator (JSGW, India) at 35°C. The percentage yield of extract was calculated. The dried extract was scrapped and kept in air tight bottles in refrigerator at 4°C.

Qualitative chemical analysis of various extracts of *Bacopa monnieri* was done to detect major phytochemical groups viz., alkaloids, anthraquinones,

flavonoids, saponins, tannins, sterols, reducing sugars, glycosides, resins, triterpenes and proteins by standard methods(Harborne, 1973; Sofawara, 1982)

Thirty five albino Wistar rats weighing between 150-200 gm (1-1.5 months) were procured from Experimental Animal House, Pantnagar for the study. The animals were kept in plastic cages under standard managemental conditions in experimental house of the department. They were acclimatized to animal house conditions for two weeks before the commencement of experiment. The animals were maintained on the standard rat feed and water *ad libitum*. All the experimental animals were kept under constant observation during entire period of study. All studies were performed in accordance with the guidelines on regulation of scientific experiments as approved by the Institutional Animal Ethics Committee (registration no.IAEC/VPT/CVASc/133)

Experimental design

Thirty five rats were divided randomly and equally into seven groups and were treated as given below during 28 days study:

Group I : Vehicle control (1ml distilled water daily po).

Group II : Acetaminophen was given @ 1000 mg/kg, p.o., bid, daily for 3 days from 16th to 18th day.

Group III : 50% ethanol @ 1ml, p.o., daily for first 15 days.

Group IV: 50% ethanol @ 1ml p.o. for 15 days followed by acetaminophen for 3 days @ 1000mg/kg b wt po.

Groups V and VI : 50% ethanol @ 1ml p.o. for 15 days followed by acetaminophen at 1000 mg/kg b wt p.o. for 3 days and treatment was given with plant extract @ 100 and 200 mg/kg ($HEBM_{100}$ & $HEBM_{200}$) respectively.

Group VII : 50% ethanol @ 1ml p.o. for 15 days followed by acetaminophen for 3 days at 1000 mg/kg b wt p.o. and silymarin @100mg/kg b wt po.

During 28 day study period, the rats were observed for any toxic signs and symptoms. At the end of study, all the rats were sacrificed humanely and blood and tissue samples were collected to evaluate hematological and biochemical parameters. The biochemical parameters were estimated by using standard kits (Erba Diagnostics) and changes in hematology (Benzamin, 2007and Jain, 1986), were studied using standard methods.

Calculation of percent protection

The percent protection provided by HEBM and silymarin against acetaminophen toxicity in alcoholic rats was calculated by the following formula,

$$\text{Percent protection} = \frac{(\text{Negative control} - \text{Sample})}{(\text{Negative control} - \text{Vehicle control})} \times 100$$

where, Negative control= group IV, Sample = groups V, VI and VII, Vehicle control = group I

Statistical analysis of data was done by using one-way ANOVA technique using Duncun's method in SPSS 16 statistical software (Snedecor and Cochran, 1967).

RESULTS

The recovery of the extract was found to be 22.33%. The phytochemical analysis revealed the presence of alkaloids, flavonoids, glycosides, proteins, resins, sterols, saponins, tannins and terpenes. Anthraquinones and reducing sugars were not found.

Drowsiness and dullness was observed in rats of groups III, IV, V, VI and VII after ethanol administration and dull hair coat in groups II, IV, V, VI and VII after acetaminophen administration. Administrations of ethanol lead to moderate increase in b wt during first 15 days. There was a rapid decline in b wt of rats after acetaminophen administration but rats showed progressive increase in b wt after treatment with extract and silymarin in their respective groups.

A significant reduction in liver weight was seen in groups given acetaminophen alone and ethanol-acetaminophen. There was an increase in liver weight in ethanol treated rats in the present study (Table 1). There was a significant reduction in liver weight in acetaminophen and ethanol-acetaminophen receiving groups. The loss in liver weight was significantly improved in HEBM treated rats providing 74.24%, 108%, and 78.66% protection by $HEBM_{100}$, $HEBM_{200}$ and silymarin, respectively.

As depicted in table 2 groups II, III and IV showed a significant ($p<0.05$) decrease in Hb, TLC, lymphocytes and eosinophils and a significant ($p<0.05$) increase in PCV, TEC, neutrophils and monocytes in comparison to rats of control group. $HEBM_{100}$ caused non-significant changes in TEC, monocytes and eosinophils count but $HEBM_{200}$ significantly ($p<0.05$) restored the value of above mentioned parameters towards control group in comparison to groups II, III and IV and the results were equivalent to that of silymarin. The percent protection given by HEBM at lower and higher doses was 91.07% and 96.42%, respectively.

Ethanol and acetaminophen administration increased the activity of hepatic enzymes like AST, ALT, ALP and GGT in rat serum indicating hepatic damage. Hepatoprotective potential of *Bacopa monnieri* plant extract was substantiated by the significant decrease in the elevated levels of AST, ALT, ALP and GGT in groups V and VI. HEBM at both dose levels decreased these values significantly ($p<0.05$) as compared to the values in groups II, III and IV; however, $HEBM_{200}$ showed more decline in enzyme activities, approaching towards normal as compared to $HEBM_{100}$. $HEBM_{200}$ provided 96.84% protection for increased ALT levels, 89.21% for AST, 92.53% for GGT and 89.39% for ALP as shown in table 3.

Ethanol and acetaminophen administration

Table 1:
Effect of HEBM on body and liver weight measured weekly in rats administered ethanol for 15 days followed by acetaminophen for 3 days (Mean±S.E., n=5)

Groups	B wt (gms)**				Wet liver weight (gm/100gm body wt.)
	I WEEK	II WEEK	III WEEK	IV WEEK	
I.CONTROL	160.00±4.47	162.00±4.63	164.00±4.58	167.00±4.63	6.23±0.18
II.APAP *ONLY	152.00±10.67	153.00±10.44	136.00±7.96	138.00±7.84	4.79±0.29[a]
III.ETHANOL ONLY	168.00±5.83	168.00±5.83	188.00±5.83	204.00±4.00	7.25±0.04[a,b]
IV. E*+ APAP	146.00±5.09	150.00±5.24	135.00±4.74	136.00±5.53	4.32±0.05[a,c]
V.E+APAP+ $HEBM_{100}$	134.00±5.09	128.00±6.44	117.00±8.88	141.00±5.78	5.74±0.30 [b,c,d](74.24%)
VI.E+APAP+ $HEBM_{200}$	126.00±7.48	132.00±8.74	119.00±8.57	131.00±6.96	6.38±0.37[b,c,d](108.00%)
VII.E+APAP+S^*_{100}	123.00±10.19	145.00±7.41	139.00±6.40	158.00±5.61	5.82±0.17 [b,c,d](78.66%)

Note: Percent values inside parentheses in bold letters below the means of parameters of group V, VI, VII depict percentage protection given by treatments. *(APAP=Acetaminophen; E=Ethanol and S =Silymarin)
Mean value bearing superscript a,b,c,d differ significantly (p<0.05) when compared within group in a column.
a= Significantly (p<0.05) different when compared with group I., b= Significantly (p<0.05) different when compared to group II.
c= Significantly (p<0.05) different when compared to group III., d=Significantly (p<0.05) different when compared to group IV

Table 2.
Effect of HEBM on haematological profile in rats administered ethanol for 15 days followed by acetaminophen for 3 days (Mean±S.E., n=5)

Groups	Parameters							
	Hb(gm%)	PCV (%)	TEC(10^6/µl)	TLC(10^3/µl)	Lymphocytes	Neutrophils	Eosinophils	Monocytes
I. CONTROL	14.2±0.56	35.90±1.38	2.93±0.35	11.22±1.09	52.4±4.31	12.00±3.34	35.40±2.99	3.20±0.58
II. APAP *ONLY	10.72±1.09[a]	44.30±1.28[a]	3.26±0.18	4.2±0.16[a]	37.6±4.44[a]	30.60±2.22[a]	27.20±0.73	4.60±0.40
III. ETHANOL ONLY	11.94±0.27[a]	41.76±0.56 [a]	2.99±0.35	3.86±0.11 [a]	33.20±0.37 [a]	30.60±2.60 [a]	30.20±3.81	2.80±0.20
IV. E*+ APAP	9.72±0.57[a,c]	44.86±0.50 [a]	4.28±0.26 [a,b,c]	2.91±0.22 [a]	14.4±0.87 [a,b,c]	68.80±3.76 [a,b,c]	11.00±2.30[a,b,c]	5.20±0.58[a,b,c]
V. E+APAP+ $HEBM_{100}$	13.8±1.03[b,d] (91.07%)	40.20±1.85[d] (52.00%)	4.13±0.11 [a,b,c] (11.39%)	9.18±1.93[b,c,d] (75.45%)	43.00±3.03[c,d] (75.26%)	22.00±1.54[b,c,d] (82.39%)	19.80±3.00[a] (36.06%)	3.80±0.80 (70.00%)
VI. E+APAP+$HEBM_{200}$	14.04±0.56[b,d] (96.42%)	38.80±2.48[b,d] (67.63%)	3.17±0.23[d] (81.95%)	9.59±1.42 [b,c,d] (80.38%)	48.20±6.00 [c,d] (88.94%)	19.00±5.49 [b,c,d] (87.67%)	36.20±6.62[d] (103.27%)	3.20±0.48[d] (100%)
VII. E+APAP+S^*_{100}	13.04±0.17[b,d] (74.10%)	36.70±2.00[b,c,d] (91.07%)	2.41±0.07[b,d] (138.75%)	9.42±0.26 [b,c,d] (78.33%)	46.00±4.25 [c,d] (83.15%)	18.4±0.74 [b,c,d] (88.73%)	34.80±1.28 [d] (97.54%)	3.20±0.20 [d] (100%)

Note: Percent values inside parentheses in bold letters below the means of parameters of group V, VI, VII depict percentage protection given by treatments. * (APAP=Acetaminophen; E=Ethanol and S=Silymarin)
Mean value bearing superscript a,b,c,d differ significantly (p<0.05) when compared within group in a column.
a= Significantly (p<0.05) different when compared with group I. b= Significantly (p<0.05) different when compared to group II.
c= Significantly (p<0.05) different when compared to group III. d= Significantly (p<0.05) different when compared to group IV.

Table 3.
Effect of HEBM on serum enzyme profile in rats administered ethanol for 15 days followed by acetaminophen for 3 days (Mean±S.E., n=5)

Groups	Parameters			
	ALT (IU/L)	AST (IU/L)	GGT (IU/L)	ALP (IU/L)
I.CONTROL	31.11±3.28	35.00±1.03	13.15±1.82	61.85±1.58
II.APAP *ONLY	57.99±2.92[a]	70.72±2.01 [a]	29.26±3.38[a]	88.44±1.62[a]
III.ETHANOL ONLY	51.27±1.76 [a,b]	68.24±1.63 [a]	27.99±2.95 [a]	87.35±4.73 [a]
IV. E*+ APAP	64.70±1.82 [a,b,c]	71.07±1.96 [a]	41.57±2.07 [a,b,c]	97.66±3.09 [a,b,c]
V. E+APAP+ $HEBM_{100}$	36.42±2.27[b,c,d](84.21%)	48.08±1.88 [a,b,c,d](63.72%)	19.51±1.03[b,c,d](77.61%)	69.45±3.04 [b,c,d](78.78%)
VI. E+APAP+ $HEBM_{200}$	32.17±1.41 [b,c,d](96.84%)	38.89±1.11[b,c,d](89.21%)	15.27±1.94 [b,c,d](92.53%)	65.65±0.54 [b,c,d](89.39%)
VII. E+APAP+S^*_{100}	33.94±2.04 [b,c,d](91.57%)	38.18±1.06 [b,c,d](91.17%)	13.99±2.73 [b,c,d](97.01%)	65.65±3.14 [b,c,d](89.39%)

Note: Percent values inside parentheses in bold letters below the means of parameters of group V, VI, VII depict percentage protection given by treatments. *(APAP=Acetaminophen; E=Ethanol and S=Silymarin)
Mean value bearing superscript a,b,c,d differ significantly (p<0.05) when compared within group in a column.
a= Significantly (p<0.05) different when compared with group I. b= Significantly (p<0.05) different when compared to group II.
c= Significantly (p<0.05) different when compared to group III. d= Significantly (p<0.05) different when compared to group IV.

caused a significant (p<0.05) decrease in total protein, albumin and globulin level but significantly (p<0.05) increased cholesterol, triglycerides, total and direct bilirubin, urea, creatinine, uric acid and calcium levels. $HEBM_{200}$ restored all biochemical parameters, significantly (p<0.05) as compared to ethanol and acetaminophen (Table 4).

DISCUSSION

Drowsiness and dullness observed in rats of groups III, IV, V, VI and VII after ethanol administration might be due to alcohol intake. Dullness of hair coat after

Table 4:
Effect of HEBM on serum biochemical profile in rats administered ethanol for 15 days followed by acetaminophen for 3 days (Mean±S.E., n=5)

Group	Parameters											
	Total prot. (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G	Total cholesterol (mg/dl)	TGs (mg/dl)	Total bilirubin (mg/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Uric acid (mg/dl)	Glucose (mg/dl)	Calcium (mg/dl)
I.	8.82±0.28	3.74±0.04	5.03±0.25	0.76±0.04	25.66±1.56	54.45±1.29	0.58±0.05	0.04±0.01	14.16±3.91	2.11±0.26	70.64±5.71	7.37±2.30
II.	6.78±0.65^{a}	2.97±0.14^{a}	3.81±0.54^{a}	0.96±0.23	55.96±0.91^{a}	200.37±3.02^{a}	1.35±0.11^{a}	0.19±0.05^{a}	33.05±1.48^{a,c}	7.51±0.85^{a,c}	115.09±5.98^{a}	11.19±1.38
III.	6.54±0.25^{a}	3.03±0.21^{a}	3.51±0.41^{a}	0.94±0.03	64.29±2.63^{a}	200.17±0.47^{a}	0.99±0.36	0.11±0.04	17.87±4.03	4.05±0.23	108.39±0.91^{a}	11.41±1.95
IV.	5.63±0.32^{a}	2.88±0.11^{a}	2.14±0.21^{a,b,c}	1.67±0.32^{a}	65.49±1.61^{a}	206.08±1.54^{a}	1.38±0.08^{a}	0.25±0.01^{a,c}	43.10±1.21^{a,c}	8.91±2.50^{a,c}	169.33±1.75^{a,b,c}	14.86±1.91^{a}
V.	6.80±0.16^{a} (36.78%)	3.19±0.19^{a,d} (33.67%)	3.61±0.10^{a,d} (50.99%)	0.88±0.07^{d}	26.61±6.37^{b,c,d} (97.60%)	76.93±3.76^{a,b,c,d} (85.17%)	0.71±0.06^{b,d} (84.30%)	0.14±0.02^{d} (52.20%)	22.27±6.84^{d} (71.96%)	5.47±0.94^{d} (50.52%)	68.48±3.86^{b,c,d} (102.19%)	10.17±1.17 (62.65%)
VI.	7.95±0.51^{d} (72.71%)	3.31±0.06^{a,d} (47.68%)	4.63±0.46^{c,d} (86.31%)	0.73±0.05^{d}	24.98±6.58^{b,c,d} (101.69%)	58.38±4.97^{b,c,d} (97.40%)	0.68±0.03^{b,d} (88.37%)	0.07±0.005^{b,d} (85.94%)	18.43±6.93^{b,d} (85.26%)	4.16±0.61^{b,d} (69.85%)	48.16±7.13^{a,b,c,d} (112.78%)	9.51±0.56^{d} (71.47%)
VII.	7.81±0.45^{c,d} (68.11%)	3.40±0.03^{a,b,d} (57.28%)	3.63±0.11^{a,d} (51.59%)	0.82±0.08^{d}	29.08±1.76^{b,c,d} (91.41%)	57.21±3.12^{b,c,d} (98.17%)	0.74±0.14^{b,d} (80.81%)	0.09±0.004^{b,d} (74.28%)	16.31±5.24^{b,d} (92.57%)	3.01±0.24^{b,d} (86.71%)	60.85±7.66^{b,c,d} (109.92%)	9.68±1.46 (69.19%)

Note: Percent values inside parentheses in bold letters below the means of parameters of group V, VI, VII depict percentage protection given by treatments. * (APAP=Acetaminophen; E=Ethanol and S=Silymarin). Mean value bearing superscript a,b,c,d differ significantly ($p<0.05$) when compared within group in a column.
a= Significantly ($p<0.05$) different when compared with group I. b= Significantly ($p<0.05$) different when compared to group II.
c= Significantly ($p<0.05$) different when compared to group III. d= Significantly ($p<0.05$) different when compared to group IV.

acetaminophen administration in groups II, IV, V, VI and VII might be attributed to its toxicity.

The appetite stimulant action of ethanol may be considered as the reason behind moderate increase in b wt during first 15 days, which kindled the rats to consume more food. There was a rapid decline in b wt of rats after acetaminophen administration which might be due to hepatic damage which has caused off feeding in them. Rats showed progressive increase in b wt after treatment with HEBM. The weight gain in HEBM treated rats might be due its hepatoprotective action leading to restoration of normal feeding pattern in rats.

The restoration of hematological parameters by HEBM could be attributed to the phytoconstituents, saponins and flavonoids which combated hypoxia and reverted acetaminophen induced damage (Rohini and Devi, 2008; Anila and Vijayalakshmi, 2002).

The rise in serum levels of hepatic marker enzymes due to induced toxicity has been attributed to necrosis and loss of structural integrity of hepatocytes, resulting in release of these cytoplasmic enzymes into circulation after cellular damage. The improvement in serum enzymatic profile after HEBM administration could be due to free radical-scavenging properties of saponins present in high concentration of the extract (Ghosh, 2007).

Hypoalbuminemia produced by ethanol and acetaminophen is indicative of severe hepatocellular liver disease because of defective albumin synthesis. The improvement in protein levels by $HEBM_{200}$ might be attributed to the presence of proteins in the extract which have also been confirmed through phytochemical screening. Globulins are intermediate proteins which are involved in antibody formation. The higher level of globulins in group fed with *Bacopa monnieri* may be correlated with the immunomodulatory property of the herb (Imsungnoen, 2009).

The increased cholesterol level observed in ethanol-acetaminophen intoxicated rats might have been be due to increased HMG-CoA reductase activity which is the rate-limiting step in cholesterol biosynthesis(Ashakumary and Vijayammal, 1993) and could also be due to deficient lipid metabolism in the liver(Gauda, 1985).The flavonoids present in *Bacopa monnieri* may be responsible in reducing the level of serum cholesterol (Anila and Vijayalakshmi, 2002). The increase in levels of serum bilirubin (total and direct) is associated with functional state of the liver reflecting the level of jaundice. The significant inhibition in bilirubin levels by HEBM indicated early improvement in secretory mechanism of hepatic cells. *Bacopa monnieri* is rich in saponins which are known to possess antidiabetic activity (Yoshikawa et. al., 2001) and lowered glucose levels might be due to the saponins present in the extract.

Ethanol and acetaminophen affected renal function characterized by increased accumulation of the serum urea and creatinine since the rate of production exceeded the rate of clearance due to the defect in renal function[14]. The nephroprotective effect by HEBM could be attributed to presence of phytocompounds mainly flavonoids, alkaloids, sterols and proteins in the extract. The presence of saponins, flavonoids, alkaloids, sterols and proteins has also been confirmed by phytochemical study.

Thus, based on the findings, it can be concluded that *Bacopa monnieri* possesses potential hepatoprotective activity

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Research Article

AMELIORATIVE POTENTIAL OF *ECLIPTA ALBA* IN ARSENIC EXPOSED COCKERELS

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ABSTRACT

The present study was designed to assess the protective effects of *Eclipta alba* in arsenic intoxicated cockrels by determining haematological, biochemical and serum enzymatic parameters. Thirty five white leghorn (WLH) cockerels of 4 to 6 weeks old age, were randomly divided into five groups of seven birds each. After two weeks of adaptation period, the II group was offered feed mixed with arsenic@100ppm, III group with arsenic@ 100ppm + silymarin and IV and V groups with arsenic@100ppm+ *Eclipta alba*@1000ppm and arsenic@100ppm +*Eclipta alba*@ 2000ppm respectively for 90 days. Group I was kept as control and was given normal feed. Arsenic exposure led to significant depletion (P<0.05) of Hb, PCV, TEC and TLC in cockerels fed on 100ppm arsenic. There was a significant increase (P<0.05) in AST, ALT and ALP levels along with increased blood glucose, cholesterol, triglycerides, blood urea nitrogen, creatinine, total bilirubin, indirect bilirubin and uric acid levels. Significant reduction P<0.05) was observed in total protein, albumin and globulin levels indicative of hepatic and renal damage. Coadministration of *Eclipta alba* was quite effective in reducing arsenic induced haematological, biochemical and serum enzymatic alterations like silymarin.

Key words: Arsenic, *Eclipta alba,* haemato-biochemical, serum enzymatic,cockerels, toxicity

INTRODUCTION

Arsenic is a common environmental contaminant distributed around the world. This metalloid has not only been used in medicine but also has various applications in agriculture, livestock and industrial sector. Water and soil contamination by arsenic is a serious threat to mankind all over the world water (Singh *et al.*2011). Arsenic causes several toxic effects on hepatic, cardio-pulmonary, renal, immunological and reproductive systems. In addition to embryotoxicity and teratogenecity, tumors of skin, lung, liver, bladder and oxidative stress have also been reported following prolonged exposure of arsenic in man and animals including poultry. *Eclipta alba*, has been reported to have various pharmacological effects (Prabhu *et al.* 2011). Despite wide therapeutic potential of *Eclipta alba,* little information is available on ameliorative effect against arsenic induced toxicity in avian species, the present endeavour was thus undertaken to investigate the phytotherapeutic role of *Eclipta alba* in arsenic intoxicated poultry birds.

MATERIALS AND METHODS

Preparation of the dried powder of *Eclipta alba* (DPEA)

The plant procured from MRDC Pantnagar was chopped and shade dried followed by drying in incubator at 35-40°C for 3-4 days to remove excess moisture. The dried plant was then grinded in mixer to obtain a fine homogeneous powder. The powder of *Eclipta alba* plant was light green in colour and was stored in sealed plastic container in a dry place at room temperature till further use.

Experimental design

Thirty five WLH cockerels of 4 to 6 weeks old age, were procured from Instructional Poultry Farm Nagla, Pantnagar and maintained in the experimental poultry shed under standard managemental conditions. All the birds were fed with starter ration for initial 2 weeks followed by grower ration till the end of the study.

Arsenic (Loba Chemie) and silymarin (Silybon-140, Microlabs) were used in this study.

After two weeks of adaptation period, the II group was fed on arsenic@100ppm, III group with arsenic@ 100ppm + silymarin, IV and V groups with arsenic 100ppm + DPEA @1000ppm and arsenic @ 100ppm +DPEA @ 2000ppm respectively for 90 days. Group I was kept as control and was given normal feed. After every 30 days blood was taken from each bird for study of different parameters upto 90 days trial.

Haematobiochemical assay

The blood samples were transferred to heparin coated tubes for haematological examination, and in plain tubes for serum separation. 1.0 ml of blood was collected from each bird in clean heparin coated tube and haematological parameters such as TEC, TLC, PCV and haemoglobin were estimated.

Biochemical parameters viz. total proteins, albumin, globulin, total cholesterol, bilirubin, glucose, triglycerides, creatinine and uric acid using ERBA diagnostic kits. Serum enzymes viz. aspartate aminotransferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) were estimated using ERBA diagnostic kits.

Statistical analysis

Statistical analysis of the data was done by using ANOVA technique for significant difference in the values of different groups as 5% level of significance (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

There was a significant (P < 0.05) reduction in haemoglobin concentration of cockerels in all groups as compared to control at 60 and 90 days interval Hb concentration declined in all treatment groups in duration dependent manner.At 60 and 90 days, group II, IV and V showed significant (P<.05) decline in PCV concentrations as compared to control (Table 1). Comparison at different time intervals showed that group II, III, IV and V revealed a significant deduction (P<0.05) in TEC between the groups as compared to control. The highest decline was observed in cockerels of group II whereas high DPEA group V revealed restoration in TEC like silymarin group at all intervals as.compared to others. The highest decline in TLC was observed in group II as compared to other groups whereas group III, and high DPEA group V revealed almost parallel TLC values. DPEA treatment groups showed restoration in values of haematological biomarkers.

Ramsajan (2009) reported significant alterations in haematological parameters in cockerels The reduction in haematological profile might have occurred due to action of arsenic as a capillary poison which increases the fragility of red blood cells. Mandal and Suzuki *(*2002) also reported anaemia and leucopenia to be the common effects of arsenic poisoning which can be due to the direct haemolytic or cytotoxic effects on the blood cells and suppression of erythropresis. Findings of the study indicated that DPEA2000ppm ameliorated the haematological parameters like silymarin. Protective effect of *Eclipta alba* on haematological parameters was also been reported by Pandey *et al. (*2012).

The total protein concentration in serum was significantly (P < 0.05) reduced in all groups as compared to control (4.47±0.09) at 90 day feeding interval. The significant (P < 0.05) reduction in concentration of serum protein was observed in group was less than group II, III, IV and V at 60 and 90 days of feeding trial (Table 2).

The decrease in protein profile indicates hepatic dysfunction which could be attributed to the reduced capacity of liver to synthesize them. This could also be due to the peroxidative damage of liver which is the exclusive site of protein synthesis. The liver is the exclusive site of albumin synthesis, so decline in the total protein content and albumin is considered as a useful index of severity of cellular dysfunction in chronic liver disease (Kaneko, 2008). The altered protein profile might have been due to the hepatic damage caused by arsenic induced oxidative stress, which induces apoptosis in the hepatocytes by activating various pathways (Jain *et al.*2011)**.** Globulins are intermediate protein involved in antibody formation. The significantly (P < 0.05) higher levels of globulin in the DPEA treated group V as compared to group II indicated the immunomodulatory property of the herb *Eclipta alba* (Banji *et al.*2007). A significant (P < 0.05) increase in serum glucose level of all groups was observed at 60 and 90 days of feeding trial as compared to control. Treatment group III, IV and V showed significant (P < 0.05) reduction in serum glucose levels as compared to arsenic treated group II at 60 and 90 days interval.

A significant (P < 0.05) increase was observed in serum cholesterol level in group II and IV at 30 days feeding interval and II, III, IV and V group at 60 and 90 days feeding interval as compared to control group. Group III and V showed a significant (P < 0.05) decline in cholesterol values when compared with group II and IV throughout the trial. A significant (P < 0.05) increase was observed in triglyceride concentration in group II, III, IV and V at 60 and 90 days feeding interval as compared to control.

Hyperglycemia might have resulted due to increased glycogenesis by the activation of sympathetic component and release of adrenaline from adrenal medulla and secretion of glucocorticoids from adrenal cortex under stress conditions (Kaneko, 2008), produced as a toxic effect of arsenic. Arsenic induced hyperglycemia has also been reported by Rama Sajan (2009) in cockerels. Arsenite has high affinity for SH groups and hence forms covalent bonds with the disulfide group of insulin, insulin receptors, glucose transporters and enzymes involved in glucose metabolism (Singh *et al.*, 2011).

Administration of DPEA caused lowering of glucose levels in cockerels. Thus, lowered glucose levels might be due to saponins present in the herb. Liver is the major site of cholesterol, bile acid and phospholipid synthesis and metabolism. There was a significant (P < 0.05) increase in the level of total cholesterol and serum triglycerides. The elevation in cholesterol might be due to altered metabolism of fat in the liver (Hochgraf *et al.* 2000). Damage to liver might have lead to disturbances in total cholesterol and serum triglycerides level. The increased cholesterol level in arsenic intoxicated cockerels could be due to deficient lipid metabolism in the liver (Gauda *et al.* 1985). Our findings are in corroboration with the findings of Ram Sajan (2009) reported similar findings in arsenic intoxicated cockerels.

There was a significant (P < 0.05) increase in the serum creatinine levels in all the groups at 60 and 90 days feeding interval. Group II recorded the highest level at 90 days as compared to control. The serum creatinine levels were significantly (P < 0.05) low in group III and V as compared to group II and IV at 60 and 90 days feeding intervals. A significant (P < 0.05) increase in the serum uric acid and total bilirubin level was observed in all the

Table 1 :
Effect on haematological parameters following oral administration of different dietary levels of arsenic and DPEA in feed for 90 days in cockerels (n=7)

Parameters	Control			Arsenic (100 ppm)			Arsenic (100 ppm) + Silymarin			Arsenic (100 ppm) + DPEA (1000 ppm)			Arsenic (100 ppm) + DPEA (2000 ppm)		
	Interval (days)														
	30	60	90	30	60	90	30	60	90	30	60	90	30	60	90
Haemoglobin (g/dl)	11.46 ±0.27	11.5 ±0.24	11.54 ±0.36	11.32 ±0.41^{Ax}	8.89 ±0.35^{y}	7.89 ±0.25^{z}	11.46 ±0.46	10.64 ±0.28	10.32 ±0.18^{By}	11.61 ±0.30^{Ax}	9.39 ±0.48^{By}	8.11 ±0.27^{Cz}	11.57 ±0.38^{Ax}	10.21 ±0.37^{y}	10.07 ±0.26^{z}
PCV (%)	32.57 ±0.71	32 ±0.87	32.43 ±0.75	32.71 ±0.60^{Ax}	27.29 ±0.52^{By}	23.14 ±0.80^{Cz}	32.43 ±0.75^{x}	30.86 ±0.70	30 ±1.07^{y}	32.43 ±0.61^{Ax}	29.3 ±0.57^{By}	25.14 ±0.96^{Cz}	32.86 ±0.73^{x}	30.57 ±0.61	28.86 ±0.73^{Bz}
TEC (10^6/µl)	3.57 ±0.12^{x}	3.59 ±0.09	3.53 ±0.13	3.51 ±0.09^{Bx}	2.39 ±0.10^{y}	2.13 ±0.07^{z}	3.52 ±0.09x	3.24 ±0.11^{y}	3.19 ±0.09^{z}	3.5 ±0.13^{Bx}	3.03 ±0.12^{Cy}	2.73 ±0.13^{Dz}	3.57 ±0.09^{Ax}	2.92 ±0.10^{Dz}	3.02 ±0.17^{Cy}
TLC (10^3/µl)	32.07 ±0.30	32.26 ±0.55	32.49 ±0.61	32.56 ±0.42^{Ax}	27.66 ±0.66^{Dy}	23.377 ±0.90^{z}	32.04 ±0.36	31.03 ±0.11	30.48 ±0.26^{Bx}	32.5 ±0.63^{Ax}	29.43 ±0.33^{y}	27.72 ±0.45^{z}	32.07 ±0.34^{Ax}	30.82 ±0.31^{x}	30.44 ±0.40^{y}

Values bearing A,B,C,D superscripts in a row differ significantly (P < 0.05) among groups and x,y,z (P < 0.05) superscripts in a row differ significantly among time intervals

Table 2 :
Effect on biochemical and serum enzymatic parameters following oral administration of different dietary levels of arsenic and DPEA in feed for 90 days in cockerels (n=7)

Parameters	Control			Arsenic (100 ppm)			Arsenic (100 ppm) + Silymarin			Arsenic (100 ppm) + DPEA (1000 ppm)			Arsenic (100 ppm) + DPEA (2000 ppm)		
	Interval (days)														
30	60	90	30	60	90	30	60	90	30	60	90	30	60	90	
Total protein (g/dl)	4.25 ±0.08^{By}	4.28 ±0.05^{A}	4.47 ±0.09^{x}	4.46 ±0.08^{Ax}	3.62 ±0.06^{Cy}	2.58 ±0.05^{Ez}	4.34 ±0.03^{A}	4.28 ±0.03^{x}	4.07 ±0.05^{By}	4.46 ±0.09^{Ax}	3.68 ±0.06^{Cy}	2.76 ±0.07^{Dz}	4.27 ±0.04^{Bx}	4.17 ±0.02	3.86 ±0.05^{Cy}
Albumin (g/dl)	1.51 ±0.03^{y}	1.53 ±0.03^{x}	1.62 ±0.065	1.54 ±0.04^{Ax}	1.26 ±0.03^{Cy}	1.11 ±0.03^{Dz}	1.53 ±0.04^{A}	1.59 ±0.03	1.48 ±0.02^{B}	1.53 ±0.03^{Ax}	1.35 ±0.02^{By}	1.22 ±0.03^{Cz}	1.54 ±0.05^{Ax}	1.39 ±0.03^{By}	1.48 ±0.02^{Bx}
Globulin (g/dl)	2.74 ±0.07^{C}	2.74 ±0.08^{B}	2.85 ±0.11^{A}	2.92 ±0.10^{Ax}	2.36 ±0.09^{Dy}	1.47 ±0.07^{Ez}	2.81 ±0.06^{Bx}	2.69 ±0.05^{C}	2.59 ±0.05^{By}	2.93 ±0.10^{Ax}	2.32 ±0.08^{Ey}	1.54 ±0.07^{Dz}	2.73 ±0.05^{Cx}	2.78 ±0.04^{A}	2.39 ±0.05^{y}
Glucose (mg/dl)	291.16 ±12.15^{Ax}	296.06 ±8.14^{Bc}	289.67 ±12.66^{x}	271.32 ±10.85^{z}	334.9 ±11.78^{y}	380.53 ±11.10^{x}	283.73 ±16.35^{Ax}	263.93 ±9.50^{C}	238.35 ±7.83^{y}	285.41 ±11.18^{Ax}	259.43 ±13.85^{Cy}	196.77 ±11.63^{Dz}	285.16 ±6.91^{Ax}	253.55 ±8.20^{Cy}	227.87 ±7.18^{z}
Cholesterol (mg/dl)	150.2 ±1.74^{B}	152.32 ±1.69^{D}	156.33 ±1.28^{E}	176.49 ±1.63^{z}	208.02 ±2.52^{y}	227 ±7.56^{x}	150.94 ±0.90^{B}	156.55 ±0.78^{C}	171 ±1.34^{Dx}	173.55 ±3.14^{Az}	190.1 ±2.48^{By}	204.02 ±2.47^{x}	151.41 ±1.24^{Bz}	162.84 ±2.62^{Cy}	188.12 ±1.75^{x}
Triglycerides (mg/dl)	85.22 ±2.58^{B}	85.86 ±1.62^{C}	83.63 ±3.08^{D}	91.91 ±1.79^{z}	120.8 ±1.20^{y}	143.1 ±2.98^{x}	85.37 ±1.13^{Ay}	86.38 ±1.47^{Cx}	92.08 ±2.08	87.26 ±1.14^{Az}	97.41 ±2.78^{By}	108.07 ±4.04^{x}	88.33 ±2.07^{A}	92.14 ±1.67^{B}	93.36 ±3.43^{C}
Creatinine (mg/dl)	0.38 ±0.012^{A}	0.38 ±0.013^{B}	0.38 ±0.016^{D}	0.4 ±0.019^{z}	0.71 ±0.062^{y}	1.57 ±0.094^{x}	0.36 ±0.017^{Ay}	0.44 ±0.060^{Bx}	0.53 ±0.062^{Cx}	0.36 ±0.013^{Az}	0.79 ±0.094^{y}	1.15 ±0.07^{Bx}	0.36 ±0.031^{Ay}	0.46 ±0.064^{Bx}	0.61 ±0.083^{C}
Uric acid (mg/dl)	6.97 ±0.06^{D}	7.04 ±0.04	7.27 ±0.08^{E}	16.74 ±0.11^{Ax}	24.39 ±0.19^{y}	35.2 ±0.19	7.8 ±0.08^{Cz}	11.81 ±0.12^{y}	13.39 ±0.18^{Dx}	8.51 ±0.08^{z}	13.93 ±0.14^{y}	23.52 ±0.72^{x}	8.84 ±0.10^{Bx}	12.2 ±0.09^{y}	17.63 ±0.08
Total bilirubin (mg/dl)	1.18 ±0.03^{B}	1.22 ±0.07^{D}	1.26 ±0.03	1.32 ±0.03^{Az}	1.75 ±0.04^{By}	2.12 ±0.09^{x}	1.45 ±0.03^{z}	2.03 ±0.06^{y}	2.19 ±0.05^{x}	1.19 ±0.04^{Bz}	1.36 ±0.04^{Cy}	1.57 ±0.08^{x}	1.23 ±0.02^{By}	1.38 ±0.05^{C}	1.92 ±0.03^{x}
Indirect (mg/dl)	0.94 ±0.03^{B}	0.99 ±0.07^{D}	0.99 ±0.02	1.09 ±0.03^{Az}	1.5 ±0.05^{By}	1.88 ±0.09^{x}	1.21 ±0.03^{z}	1.76 ±0.06^{By}	1.93 ±0.04^{x}	0.95 ±0.04^{Bz}	1.11 ±0.03^{Cy}	1.34 ±0.08^{x}	1.01 ±0.02^{By}	1.15 ±0.05^{C}	1.67 ±0.03^{x}
AST (IU/L)	116.63 ±1.10^{Cx}	116.05 ±1.13	115.91 ±1.28	145.73 ±1.11^{Az}	155.59 ±0.95	167.12 ±0.51	111.82 ±0.43^{Cx}	113.68 ±1.01	127.52 ±1.18	123.1 ±0.45^{Cx}	130.13 ±1.91	152.14 ±0.77	115.83 ±1.31^{Cx}	121.63 ±0.91	134.92 ±0.87
ALT (IU/L)	25.6 ±0.34^{C}	25.11 ±0.67^{D}	25.3 ±0.31^{E}	34.24 ±0.60^{Z}	40.48 ±0.57^{y}	50.04 ±0.75^{x}	23.33 ±0.65^{z}	25.33 ±0.66^{y}	33.33 ±0.46^{x}	27 ±0.50^{z}	32.09 ±0.45^{y}	39.34 ±0.72^{x}	26.53 ±0.73^{z}	27.96 ±0.42^{y}	36.27 ±0.86
ALP (IU/L)	187.78 ±1.70^{Cx}	187.24 ±1.31^{D}	189.25 ±1.23^{E}	216.1 ±2.26^{Az}	245.86 ±1.37^{Ay}	256.88 ±1.89A^{x}	186.38 ±1.12^{C}	188.61 ±1.02^{D}	211.84 ±3.20^{x}	209.69 ±1.52^{z}	230.65 ±1.48^{y}	241.11 ±1.85^{x}	184.1 ±1.96^{z}	201.33 ±2.23^{y}	237.45 ±2.08^{x}

Values bearing A,B,C,D superscripts in a row differ significantly (P ? 0.05) among groups and x,y,z (P ? 0.05) superscripts in a row differ significantly among time intervals

groups at 30, 60 and 90 days feeding interval as compared to control.

Blood urea nitrogen, serum creatinine and uric acid levels. These are the indicators of kidney damage (Benzamin, 2010).Due to anomalies in the renal function there is marked accumulation of serum urea and creatinine, as rate of production increases the rate of clearance (Kaneko, 2008). The present study revealed significant ($P < 0.05$) increase in the serum creatinine and uric acid levels in all the groups. The results of this study are in agreement with the findings of toxic effect of arsenic in cockerels**.** The nephron protective effect of DPEA could be attributed due to the presence of phytocompounds as flavonoids and alkaloids.

The increase in the level of serum bilirubin is associated with the functional state of liver indicating the level of jaundice. The elevated levels of indirect bilirubin are usually caused by liver cell dysfunction. The results of the study are in agreement with the findings of other studies on arsenic toxicity in animals (Singh *et al.* 2011). The results in our study demonstrated that DPEA and silymarin caused significant decrease in bilirubin levels indicating early improvement in secretory mechanism of hepatic cells. The reduction in the bilirubin levels could be due to the presence of saponins in the extract.

Serum AST activity was significantly ($P < 0.05$) higher in all the groups as compared to control at 90 days feeding intervals. Increase in AST activity was maximum at 90 days feeding interval. Silymarin treated group III and high DPEA dose group V showed significant ($P < 005$) decline in AST values at all intervals. Serum ALT and ALP levels were significantly ($P < 0.05$) higher in all the groups in comparison to control values at 30, 60 and 90 days feeding intervals in comparison to control values (Table 2).

Elevated enzyme activity of ALT, AST and ALP indicates damage to liver, myocardium and muscles (Benzamin, 2010). Increase in serum activity of ALT, enzyme gives an indication of the extent of hepatocellular necrosis or increased cell permeability. Liver ALT represents 90% of the total enzymes in the body (Kaneko, 2008). AST is useful in assessing the hepatocellular damage.

A rise in the concentration of serum AST and ALT enzymes has been attributed to the catenation in structural and cellular integrity of the hepatocytes. Continued arsenic feeding resulted in fatty liver with increased serum AST and ALT levels and hepatic fibrosis. Thus, it appears from the enzymic profile in the study that arsenic produced hepatotoxic effect and high dose of DPEA revealed ameliorative effect in cockerels.

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Research Article

TOXICITY STUDY OF EXTRACTS OF *MELIA AZEDARACH* AND *CUMINUM CYMINUM* IN WISTAR RATS

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ABSTRACT

M. azedarach and *Cuminum cyminum* are medicinal plants with antioxidant, anti-inflammatory, antiproliferative, anticancer and immunomodulatory properties. In order to assess the toxicity of hydroethanolic extract of *M. azedarach* and *C. cyminum,* these extracts were fed orally to rats @ 800 mg/kg and 1600mg/kg for 14 days. The clinical observation, haematological and biochemical analysis showed signs of toxicity in animals fed with Melia extract at the dose 1600mg/kg. No sign of toxicity was found in the animals fed with Cuminum extract

Keywords:

INTRODUCTION

Melia azedarach and *Cuminum cyminum* are two medicinal plants, proved to have promising antioxidant, anti-inflammatory, antiproliferative, anti cancer and immunomodulatory properties (Takeya, 1996*)*. Traditionally, extracts of melia were used for curing burns, pyrexia and gingivitis. *C. cyminum* belonging to the family Apaiaceae considered as a stimulant, stomachic, astringent, carminative and used in diarrhea, dyspepsia, and gonorrhea. There are no reports suggesting the toxicity of Cumin in animals however consumption of large quantity Melia, fruits or berries have proved to be toxic to humans, animals and birds due to the presence of tetranortriterpenoids which are chemically related to azadirachtin (Azam *et al.,* 2013). Therefore, present study was done in order to investigate the toxicity of hydroethanolic extract of *M. azedarach* leaves (MHE) and *C. cyminum* (CHE) seeds in wistar rats.

MATERIALS AND METHODS

Experimental design

Healthy adult male Wistar rats (120-140g) were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (U.P.), India. The animals, were housed in polypropylene cages on standard ration with access to fresh water. The experimental protocol was approved by the IAEC. All the animals were acclimatized for a period of 7 days prior to the experiment.

The acute toxicity of MHE and CHE was studied at by giving two doses, a lower dose of 800mg/kg body wt and a higher dose of 1600mg/kg body wt orally once daily for 14 days. The animals were divided into four groups of 6 animals each and the extracts were fed orally to each animal for 14 days. The animals were observed throughout the study for signs of toxicity

Body weights and organ weights

The body weights were recorded at weekly interval. The vital organs liver, kidneys, brain and spleen were removed at the end of the treatment period blotted with tissue paper and their weights were recorded. Later on relative organ weights (gm per 100 gm b wt) were calculated as mentioned below.

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight}}{\text{Total b wt}} \times 100$$

Haematobiochemical examination

The blood samples from individual rats were collected from heart at the time of sacrifice. About 2 ml of blood was collected into dry sterilized vials containing anticoagulant, sodium-EDTA. The haematological estimations, such as hemoglobin concentration (Hb), packed cellvolume (PCV), total erythrocytes count (TEC), total leucocytes count (TLC) and differential leucocyte counts (DLC) were carried out. Serum was separated from the blood and stored at -20ºC for estimations of the activity of ALT, AST, LDH and creatinine kinase by using commercially available diagnostic kits (Span Diagnostics Ltd; India).

Statistical analysis

Results are expressed as Mean ± SEM. with n equal to number of animals. Data were analyzed applying one way anova using the GraphPad Prism v4.03 software program (San Diego, CA, USA), and the differences were considered statistically significant at * $P<0.05$ or lower (Snedecor and Cochran, 1989).

RESULTS

Signs of toxicity were observed in the group in which *Melia azedarach* extract was fed orally at a dose of 1600 mg/kg (MHE1600) (Table 1). Two out of six animals died in this group and the remaining animals were weak with reduced food and water intake. Administration of *Cuminum cyminum* extract orally did not produce any mortality in Wistar rats. Animals in this group exhibited normal activities and behavior during the study period.

Autopsy finding did not show any particular drastic change in the body except few hemorrhagic spots on heart, liver and stomach.

As shown in Table 2, the final body weight of all the groups except MHE1600 were found to significantly ($P<0.05$) increased as compared to their initial b wt at the start of the treatment .The final b wt of MHE1600 treated rats was found to increase, however the increase was not statistically significant. Control group showed the highest increase in final b wt followed by CHE 1600. The order of increase in initial and final b wt were control> CHE1600>CHE 800> MHE800>MHE1600. The absolute and relative organ weights of MHE 1600 treated animals were found to be significantly ($P<0.05$) higher as compared to control group. In case of CHE treated animals the significant increase in weight was found only in the spleen of CHE 1600 ($P<0.05$) treated animals. Liver , kidney , brain, heart and spleen of MHE 800 and CHE 800 treated animals were found to be of almost similar weight as control (Table 2).

The effect on hematological parameters in rats following 14 days oral administration of hydroethanolic extracts of *M. azedarach* and *C. Cyminum* for toxicity study are presented in Table 3. Decrease in the values of TEC, TLC , Hb, PCV and DLC were observed in MHE1600 treated animals as compared to control, however, the values were not significantly different.

Table 1:
Experimental protocol for toxicity study of *M.azedarach* and *C. cyminum* hydroethanolic extracts.

Group	Dosage of extracts (mg/kg)	No. of rats	Duration of treatment (days)	No of animals died
CHE extract orally once daily	800	6	14	0
CHE extract orally once daily	1600	6	14	0
MHE extract orally once daily	800	6	14	2
MHE extract orally once daily	1600	6	14	0

Table 2
Effect of hydroethanolic extracts of *M. azedarach* (MHE) and *C. cyminum* (CHE) on b wt (g), absolute organ weights (g) and relative organ weight of wistar rats following oral administration for 14 days

Group	Initial body weight	Final Liver weight	Organ weights									
			body		Kidney		Brain		Heart		Spleen	
			absolute	relative	absolute	relative	absolute	relative	absolute	relative	absolute	relative
control	230.53 ±2.15[a]	269.5 ±1.23	12.28 ±0.026	5.03 ±0.096	1.15 ±0.010	0.49 ±0.32	1.05 ±0.053	0.703 ±1.01	1.12 ±0.008	0.423 ±2.67	0.65 ±0.032	0.256 ±0.26
MHE 800	232.67 ±2.51	249.5 ±3.43 [a]	12.76 ±1.05	5.11 ±0.013	1.25 ±0.56	0.501 ±0.004	1.19 ±0.21	0.712 ±0.023	1.16 ±0.21	0.419 ±0.007	0.67 ±0.03	0.269 ±0.023
MHE 1600	241.23 ±3.23	252.5 ±2.54	13.25 ±2.56[a]	5.22 ±0.011[a]	1.57 ±0.23 [a]	0.531 ±0.023[a]	1.32 ±0.42 [a]	0.743 ±0.05[a]	1.42 ±0.09 [a]	0.421 ±0.006	0.89 ±0.15 [a]	0.352 ±0.006[a]
CHE 800	243.57 ±1.34	278.3 ±0.024	11.78 ±0.11	4.77 ±0.003	1.4 ±0.19	0.515 ±0.008	1.07 ±0.17	0.708 ±0.012	1.08 ±0.11	0.403 ±0.012	0.76 ±1.78	0.273 ±1.71 [a]
CHE 1600	262.83 ±1.67	291.45 ±0.009	12.67 ±0.25	4.33 ±0.008	1.21 ±0.17	0.523 0.034	1.1 ±0.14	0.701± ±0.007	1.23 ±0.27 [a]	0.417 ±0.007	0.79 ±1.89	0.251 ±3.25 [a]

a= Significant ($P<0.05$) as compared to control

Table 3:
Effect of hydroethanolic extracts of *Melia azedarach* (MHE) *and Cuminum cyminum* (CHE) on haematological profile in wistar rats.

Groups	TEC (10^6/µl)	TLC (10^3/µl)	Hb (g/dl)	Pcv (%)	Lymphocyte (%)	neutrophil (%)	Monocyte (%)	Eosinophil (%)
Control	8.27 ±0.324	9.98 ±0.65	13.34 ±0.34	39.56 ±0.31	54.84 ±0.36	44.56 ±0.63	1.47 ±0.25	0.13 ±0.49
MHE 800	7.85 ±0.65	8.77 ±0.152	12.43± 0.209	38.56 ±0.451	54.22 ±1.76	44.73 ±1.59	0.601 ±0.013	0.03 ±0.035
MHE 1600	7.03 ±0.57	8.09 ±0.537	11.07 ±0.22	37.27 ±0.61	44.85 ±0.81	53.12 ±0.90	1.22 ±0.195	0.8 ±0.053
CHE 800	8.18 ±0.906	8.96 ±0.302	13.77 ±0.223	39.13 ±0.271	55.42 ±0.98	43.08 ±0.77	1.48 ±0.25	0.08 ±0.43
CHE 1600	8.32 ±0.530	8.43 ±0.512	13.22 ±0.302	39.33 ±0.413	54.32 ±0.209	44.18 ±0.274	1.37 ±0.055	0.18 ±0.041

a= Significant ($P<0.05$) as compared to control

Table 4:
Effect of hydroethanolic extracts of *Melia azedarach* (MHE) *and Cuminum cyminum* (CHE) on serum biochemical profile in wistar rats

Group	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	AST (U/L)	ALT (U/L)	Creatinine (mg/dl)	LDH (U/L)
cancer control	7.38 ±0.103	4.12 ±0.21	3.26 ±0.110	65.66 ±2.31	30.42 ±3.45	0.714 ±0.034	237.13 ±1.34
MHE800	6.45[a] ±0.321	3.23 [a] ±0.78	3.22 ±0.302	71.34[a] ±4.76	34.78 ±2.91	1.02[a] ±0.067	241.32[a] ±1.98
MHE1600	5.23[a] ±0.87	2.81 [a] ±0.078	2.43 [a] ±0.048	79.78[a] ±3.45	38.12[a] ±3.41	1.14[a] ±0.045	256.34[a] ±1.68
CHE800	6.96 ±0.078	3.67 ±0.34	3.26 ±0.098	68.12 ±2.1	32.67 ±2.56	0.72 ±0.03	236.67 ±1.45
CHE 1600	7.12 ±0.17	3.79 ±0.089	3.39 ±0.081	70.18 ±2.67	32.31 ±2.07	0.79[a] ±0.033	238.55 ±2.78

a= Significant (P<0.05) as compared to contro

The levels of total protein and albumin were found to be significantly decreased while significant increase in serum level of all ALT, AST, LDH and creatinine kinase activities were obseved in MHE800 and MHE1600 treated animals as compared to control. The serum biochemical profile of CHE treated animals (CHE 800 and CHE 1600) were found to be similar to that of control (Table 4).

DISCUSSION

Mortality, reduction in b wt, changes in haematoligical and biochemical parameters were observed in MHE1600 group, indicating that the normal body functions of these animals were compromised due to *Melia* extract feeding. Also the levels of total protein and albumin were found to be significantly decreased while significant increase in serum level of ALT, AST, LDH and creatinine kinase were obseved in Melia extract treated animals as compared to control. The significant variation in these serum biochemical parameters can be associated to toxicity since the activities of these enzymes like AST and ALT plays a major role in disease investigation and liver toxicity (Martins, 2006) . AST and ALT are cytoplasmic and mitochondrial enzymes predominantly found in liver, and increase in the levels of these enzymes is an indication of injury to liver. Also hypoproteinaemia is another common finding for liver damage (Kaneko, 1989). It has been reported that alcoholic and aqueous extracts of *M. azedarach* flowers and berries through oral and intravenous route were toxic in rats and mice and the LD50 of alcoholic extract of berries was found to be 925mg/kg in rats (Rahman *et al.,* 1991). There were also reports of toxicity of berries of this plant in higher animals like cattle and pigs in very high doses.(Oelrichs *et al.,* 1983; Hothi *et al.,* 1976). So based on these data it can be concluded that high doses of Melia extracts are toxic in rats. The group in which Cumin extracts were fed showed no signs of toxicity and all the animals were healthy and active which gave the assumption that Cumin extracts are not toxic in rats at the doses used in this study.

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Research Article

IN-VITRO ANTHELMINTIC EFFICACY OF AQUEOUS AND ETHANOLIC EXTRACTS OF *ARTEMISIA ABSINTHIUM* LINN. ON *HAEMONCHUS CONTORTUS* OF SMALL RUMINANTS

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ABSTRACT

Present study was aimed to investigate the approximate composition and *in-vitro* anthelmintic efficacy of aqueous and ethanolic areal extracts of *Artemisia absinthium* Linn. against *Haemonchus contortus* of small ruminants. Proximate analysis of areal powder of plant showed high concentration of crude protein, calcium, phosphorus, copper and zinc. *In-vitro* anthelmintic trials were conducted to determine the efficacy of aqueous and ethanolic extracts of *A. absinthium* Linn. against *H. contortus*. *In-vitro* trials revealed that aqueous extract exposure in concentration of 50 mg/mL caused little movement at 2 h, very sluggish parasite at 4 h and complete mortality at 6 h. The LC_{50} at 4 and 6 h were 44.96 mg/mL and 11.35 mg/mL, respectively. The ethanolic extract exposure showed very sluggish movement at 30 min post exposure and extremely sluggish with negligible movement at 1 hr post exposure of 100 mg/mL concentration. Observations of the study revealed plant powder contains high concentration of crude proteins, trace elements and minerals. Further, aqueous extract of plant has high *in-vitro* anthelmintic efficacy than the ethanolic extract of *A. absinthium* against *H. contortus* of small ruminants.

Keywords: Anthelmintic; *Artemisia absinthium*; *Haemonchus contortus*; Levamisole.

INTRODUCTION

Gastrointestinal helminths pose serious veterinary and public health problems as they infect animals as well as human beings. *Haemonchus contortus* infection in tropical countries is the major health hazard to the small ruminants especially sheep and goat. The *H. contortus* infection causes damage to the gastrointestinal wall and suckling of blood and nutrients (Githigia *et al.* 2001). High prevalence, ubiquitous distribution and pathological consequences of infection make the parasite as one of the top ten constraints to the profitable sheep and goat rearing throughout the world (Perry *et al.* 2002).

Artemisia absinthium Linn., family *Asteraceae,* is distributed throughout northern half of the world. Grows in Kashmir valley and is locally known as Tethwen. Traditionally, fresh and dried extracts of top foliage and shoots are given to children against infestation of tapeworms and roundworms. Therefore, the present study was aimed to investigate *in-vitro* anthelmintic efficacy of the aqueous and ethanolic extracts of *A. absinthium* Linn. against *H. contortus* of sheep and goats origin.The comparative assessment was based on the motility and mortality of *H. contortus* of sheep and goats.

MATERIALS AND METHODS

Collection of plant materials

The aerial parts of *Artemisia absinthium* Linn. was purchased from Agro Food Processing Emporium, Peerbagh, Srinagar, India. The aerial parts were cleaned of adulterants, shed dried and milled to a fine powder using an electric mixer grinder. The powdered plant material was stored in an airtight container at 4^0C until extraction.

Determination of crude protein and minerals

The powdered plant sample was analyzed for the crude protein, ether extract and minerals viz. calcium, phosphorus as per the standard protocol described in AOAC (1995). The trace minerals like zinc and copperwere analyzed in 1.0 gm plant sample in Polarized Zeeman Atomic Absorption Spectrophotometer (Z-2300, HITACHI) as per method described (Kolmer *et al.* 1951).

Preparation of plant extracts

The aqueous and ethanol extracts of powdered material of *Artemisia absinthium* were prepared as per standard method (Singh *et al.* 2012). In brief, the 100 g of plant powder was placed in conical glass percolators to which 500 ml of distilled water and ethanol solvents were added. Plant material was allowed to macerate for 72 h at room temperature and the percolates were collected by filtering through filter paper (0.45mm). The solvents were removed in a rotary vacuum evaporator under reduced temperature below 60^0C at a rotation speed of 20 rpm yielded aqueous and ethanolic extracts. The extracts were scrapped off, transferred to air tight containers and stored in a deep freezer at -20^0C till subsequent uses.

In-vitro anthelmintic test

Mature live *H. contortus* from sheep and goats were used to determine the effect of aqueous and ethanol extracts of *A. absinthium* Linn. as per method described (Tariq *et al.* 2009). Briefly, the mature worms were collected from the abomasa of freshly slaughtered sheep and goats. The worms were collected, washed and finally suspended in Hank's Balanced Salt Solution (HBSS). The 20 to 25

adult actively moving *H. contortus* worms constituting approximately 100 mg of weight were exposed in triplicate in each petri dish containing 10, 25, 50 and 100 mg/mL concentrations of the aqueous and ethanol extracts prepared in 5 ml of HBSS and HBSS alone as negative control group. The levamisole @ 0.5 mg/mL was used as reference drug of positive control. The petri dishes were kept in an incubator at 37°C. The inhibition of motility, activeness and mortality of the worms were observed at an interval of 30 min, 1, 2, 4, 6 and 8 h after exposure. The number of motile (alive) and non motile (dead) worms were counted and recorded for each concentration. Death of worms was ascertained by absence of motility for an observation period of 30 seconds in the lukewarm fresh HBSS.

Statistical analysis

Statistical analyse was done by one-way ANOVA followed by Dunnet's test at 5% level of significance. LC_{50} were calculated by the method of Probits using the programme SPSS 16 for windows at different hour intervals of each extract.

RESULTS

Table 1 depicted the concentrations of crude protein, minerals (calcium, phosphorus) and trace elements (zinc and copper) in the powder of whole plant of *A. absinthium.* The per cent mortality along with the observations on activeness and motility in each concentration of aqueous extract was recorded at the intervals of 30 min, 1, 2, 4, 6 and 8 h, respectively, and are presented in Table 2. The results revealed that parasites were very sluggish and movement was very little at 1 h post exposure of 100 mg/mL concentration. This

Table 1:

Crude proteins, minerals (calcium, phosphorus) and trace elements (zinc and copper) concentration in powder of areal part of *Artemisia absinthium* Linn.

Parameters	Concentrations
Crude protein (CP)	20.01 ± 0.23
Ether extract (EE)	4.89 ± 0.15
Calcium (Ca)	0.65 ± 0.02
Phosphorus (P)	0.24 ± 0.04
Zinc (Zn)	35.30 ± 3.37
Copper (Cu)	15.52 ± 1.52

Values are expressed as mean ± SE of three replicates.Crude protein, calcium, phosphorus are expressed on dry matter basis.Values of copper and zinc are expressed as ppm

Table 2:

Mean per cent of dead parasites ± S.D. and LC_{50} of different concentrations of aqueous extract of *A. absinthium* in *in-vitro* trial on *Haemonchus contortus* mortality at different time intervals

Time/Conc.(mg/mL)	30 min(s).	1hr	2hrs	4hrs	6hrs	8hrs
LC_{50}	-	-	-	44.96 mg/mL	11.35 mg/mL	-
100	00.00 ±0.00	52.00 ±4.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00
50	00.00 ±0.00	00.00 ±0.00	08.00 ±4.00	60.00 ±8.00	100.00 ±0.00	100.00 ±0.00
25	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	04.00 ±4.44	64.00 ±8.00	100.00 ±0.00
10	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	52.00 ±4.00	96.00 ±4.00
HBSS -ve	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00
Levamisole(0.5 mg/mL)	00.00 ±0.00	20.00 ±4.00	50.00 ±4.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00

Observations: 1. Parasites were very sluggish and movement was very little at 1hr post exposure and all dead at 2 hr post exposure of 100 mg/mL concentration, 2. Movement was little at 2 hr post exposure, very sluggish at 4hrs post exposure at concentration of 50 mg/mL.

Table 3:

Mean per cent of dead parasites ± S.D. and LC_{50} of different concentrations of ethanolic extract of *A. absinthium* in *in-vitro* trial on *Haemonchus contortus* mortality at different time intervals.

TimeConc.(mg/mL)	30 min(s).	1hr	2hrs	4hrs	6hrs	8hrs
LC_{50}	-	-	-	36.52 mg/mL	-	-
100	00.00 ±0.00	60.00 ±4.00	95.00 ±0.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00
50	00.00 ±0.00	00.00 ±0.00	20.00 ±4.00	90.00 ±8.00	100.00 ±0.00	100.00 ±0.00
25	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	05.00 ±4.00	20.00 ±8.00	40.00 ±4.00
10	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00
HBSS -ve	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00	00.00 ±0.00
Levamisole(0.5 mg/mL)	00.00 ±0.00	20.00 ±4.00	50.00 ±4.00	100.00 ±0.00	100.00 ±0.00	100.00 ±0.00

*Observations: 1. Parasites were very sluggish and movement was very little at 30 min post exposure and extremely sluggish with negligible movement at 1 hr post exposure of 100mg/ml concentration. 2. Parasites were sluggish at 1 hr post exposure and very sluggish at 2 hrs post exposure at concentration of 50mg/ml. 3. Movement was sluggish at 2 hr post exposure at concentration of 25mg/*mL *but mortality started at 4 hrs post exposure.*

concentration caused complete mortality of the adult *H. contortus* worms at the time exposure of 2 h. The exposure in concentration of 50 mg/mL caused little movement at 2 h, very sluggish parasite at 4 h and complete mortality at 6 h. At 25 mg/mL concentration mortality of 64.00 ±8.00 percent was recorded at post exposure period of 6 h and complete mortality at 8 h. The LC_{50} at 4 and 6 h were 44.96 mg/mL and 11.35, respectively. Levamisole at concentration of 0.5 mg/mL caused 50 per cent mortality at 2 h post exposure and full mortality at 4 h post exposure. The results confirm the cidal effect of aqueous extract of *A. absinthium* on adult *H. contortus* worms.

As shown in Table 3, the ethanolic extract of *A. absinthium* showed complete mortality of the adult *H. contortus* worms at the concentrations of 100 mg/mL at the time exposure of 4 h and with the concentration of 50 mg/mL parasites were sluggish at 1 h post exposure and very sluggish at 2 h post exposure with complete mortality at the post exposure of 6 h. The movement was sluggish at 2 h post exposure at concentration of 25 mg/mL but mortality started at 4 h post exposure and reached upto 40.00 ± 4.00 at the end of trial.

DISCUSSION

The plant powder contains high crude proteins, minerals and trace elements playing important role in restoring normal physiology and boosting defense mechanism thus minimizing economic losses due to parasitism. The recent studies have shown that oral administration of copper in sheep and goats has an anthelmintic effect against *H. contortus* with extended protection upto 8 weeks (Burke *et al.* 2005).

The efficacy of the aqueous extract of *A. absinthium* in dose dependent concentrations revealed that the aqueous extract has good efficacy against the *H. contortus* of sheep and goat origin in the *in vitro* trials. Though the efficacy was not comparable with the levamisole, the reference drug used for positive anthelmintic control for the control of the haemonchosis of sheep and goat in field conditions but research out is of considerable importance. The present finding is in agreement with the previous published research work (Tariq *et al.* 2009).

The extract of *A. absinthium* have significantly high concentration of total phenolic, flavonoids and tannin contents (Singh *et al.* 2012) and high concentration of crude proteins, minerals and trace elements in the whole plant suggesting it as a good candidate for anthelmintic activity. The combination of *A. absinthium* powder along with other animal feed ingredients, need to be addressed for their wide applications in present time when the use of most of antimicrobial growth promoters in animal feed have been prohibited due to residual effect (Ko *et al.* 2006).

Observations of the study revealed plant powder contains high concentration of crude proteins, trace elements and minerals. Further, aqueous extract of plant has high *in-vitro* anthelmintic efficacy than the ethanolic extract of *A. absinthium* against *H. contortus* of small ruminants.

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Research Article

CLERODENDRUM INFORTUNATUM LINN ACCELERATES WOUND HEALING IN CUTANEOUS EXICISIONAL WOUND MODEL IN RATS

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ABSTRACT

The present study was undertaken to evaluate the wound healing activity of *Clerodendrum infortunatum* LInn in cutaneous wound model in rats. Thirty six healthy male Wistar rats were procured from LARS, IVRI, Izatnagar. Two groups namely control and treated consisting of 18 rats each. Open excision wounds of 2×2 cm^2 were created on the back of the rats to evaluate the time dependent wound healing effects (days 3, 7, and 15) of *C. infortunatum* ointment (4%) which was applied twice a day on wound area. The percent wound contraction, oxidative stress related parameters, hydroxyproline content, and histopathology were done to define wound healing. The percent wound contraction on treated group was significantly higher on days 7 and 15 as compared to control. The levels of SOD, Catalase and GSH were significantly higher whereas MDA was reduced in treated group as compared to control. The levels of hydroxyproline were significantly higher on days 3, 7 and 15 in treated group, as compared to control. H&E stained sections showed that, treated wounds had marked proliferation of fibroblasts with collagen deposition. The new and well formed capillaries and granulation tissue covered by newly formed epithelial layer as compared to control. *C. infortunatum* treatment showed faster and organized healing of cutaneous wounds in rats.

Keywords: *C.infortunatum*; Ointment; Oxidative Stress; Wound Healing; Rat

INTRODUTION

Wounds are physical injuries that result in an opening or break of the skin that causes disturbances in the normal skin anatomy and function (Strodtbeck, 2001). Wound healing is an innate mechanism of action that works reliably most of the time. There are two types of therapy, firstly the phytotherapy and secondly the chemotherapy (Davis *et al.*, 1994). Due to unwanted effects of chemotherapy, shifting to phytotherapy is imperative.

The plant, *Clerodendrom infortunatum* belongs to the family *"Verbenaceae"* and commonly known as "Bhant" grows throughout the country and used for medicinal purpose. The leaves are bitter acrid, thermogenic, laxative, cholagogue, antiseptic, demulcent, anti-inflammatory, vermifuge, expectorant, antipyretic and tonic and are useful in vitiated conditions of Kapha, helminthiasis, ascarides, abscesses, tumours, leprosy, skin diseases, indolent ulcers, cough, bronchitis, inflammations, intermittent fevers, malarial fever, general debility and proctoptosis (Tyler and Robber, 1999). Korpenwar (2012) reported that patients suffering from wounds are cured by local application of leaf paste or juice of *C. infortunatum Linn.* thrice a day for a week. Therefore, this study was designed to evaluate the wound healing potential of *C. infortunatum Linn.* in excisional wound model of rat.

MATERIALS AND METHODS

Experimental animals used

Healthy adult male Wistar rats (140-200g) were procured from Laboratory Animal Resource Section, IVRI, Izatnagar (U.P.), India. The animals, housed in polypropylene cages with free access to fresh water and feed in departmental animal house at a temperature of 22 ± 2 ºC. All the animals were acclimatized for a period of 15 days prior to the commencement of the experiments.

Preparation of ointment

4% ointment of ethanolic extract was prepared in ointment base containing 90% paraffin, 5% lanolin and 5% hard paraffin.

Wound Model

The overnight fasted rats were anesthetised with an intraperitoneal (i.p.) injection of xylazine (10 mg/kg b.w.) and ketamine (50 mg/kg b.w.) and shaved on the interscapular region of dorsal aspect. Approximately 2 x 2 cm^2 (400 mm^2) open excision-type wound was created on the back of the rats to the depth of loose subcutaneous tissue and were housed individually in properly disinfected polypropylene cages.

Wound area was measured on days 0, 3, 7 and 15 post-wounding by tracing its contour using a transparent paper. The area (mm^2) within the boundaries of each tracing was determined planimetrically and expressed as percent wound contraction and calculated by the Wilson's formula:

$$\% \text{ wound contraction} = \frac{\text{0-day wound area-unhealed wound area}}{\text{0-day wound area}} \times 100$$

Wounds were photograph from the same distance above wound on days 0, 3, 7 and 15 post-wounding by digital camera (Sony-cyber-shot 16.1 mega pixels).

Application of ointment

Thirty six healthy rats were taken and divided into two groups consisting of 18 animals and each group was further subdivided into 3 subgroups (i.e., days 3, 7 and 15) with 6 animals in each sub group.

1. Group I (control): Wound was topically treated with ointment base (5% hard paraffin, 90% soft paraffin and 5% lanolin) twice daily.
2. Group II (treated): Wound was topically treated with 4% ointment twice daily.

Tissue harvesting and processing

Six rats from each group were sacrificed by overdose of diethyl ether on days 3, 7 and 15 to collect granulation tissue. The tissue was immediately divided into three parts. One portion was kept for estimation of the level of oxidative stress parameters, second portion was kept for estimation of hydroxyproline by method of Wolssner (1961) (both the parts were stored at -40^{0}c till processing). The third portion was immediately preserved in 10% neutral buffered formalin for histopathological study.

Estimation of oxidative stress parameters

Frozen tissue samples were partially thawed and 1 g of tissue sample was weighed and 10 % homogenate was prepared in ice-cold PBS with Remi homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min in cooling centrifuge. The supernatant of homogenate was used for this experiment.

The oxidative stress parameters were assessed in tissue homogenate as per well known standard methods like for the catalase (CAT) activity by Bergmeyer (1983), superoxide dismutase (SOD) by Madesh and Balasubramanian (1998) and malondialdehyde (MDA) by Stock and Dormandy (1971).

The fixed granulation tissue was embedded in paraffin and subjected to sectioning using microtome and 5 µm thick tissue sections were placed on slide. Then they were stained with hematoxylin and eosin using standard protocol and visualized for histological changes under light microscope (Olympus BX50, Tokyo, Japan).

Statistical analysis

The data was analyzed by applying two-way ANOVA with Bonferroni's multiple comparison test using the GraphPad Prism v4.03 software (San Diego, CA, USA) for the difference at *P<0.05.

RESULTS

Fig. 1 showed gross healing of wound, which was better in *C. infortunatum* ointment treated group evidenced by early formation and shedding of scab. Well-formed and thick red granulation tissue was also distinguishable in treated rats at the time of tissue collection on days 7 and 15 post-wounding, as compared to control rats.

As evident from the Fig. 2, treated wounds revealed significantly higher percent wound contraction as compared to control group. It was revealed that the percent of wound contraction was significantly higher in treated group on days 7 and 15 as compared to control group.

Effects of *C. infortunatum* ointment (4%) on oxidative parameters on days 3, 7 and 15 in granulation tissue

Similarly, the activity of catalase (Fig. 3) in normal treated group was significantly increased on days 7 and 15 as compared to control. However, the activity of catalase was increased but not significantly in normal treated group on day 3 of as compared to control group.

SOD level (Fig. 4) in the treated group was significantly increased on days 3, 7 and 15 in a time dependent manner as compared to control group.

As evidenced from Fig. 5, GSH contents were found to be significantly increased on days 3, 7 and 15 as compared to control in a time dependent manner. *C. infortunatum* ointment (4%) treated group revealed a significantly higher level of GSH in granulation tissue on days 3, 7, 15 as compared to control group.

The lipid peroxidation was measured by estimating of MDA level. Fig. 6 revealed that *C. infortunatum* ointment caused significantly decrease in its level on days 7 and 15 as compared to control. However, on day 3 the level of MDA was not significant in treated group as compared to control group.

The level of hydroxyproline (Fig. 7) was significantly increased on days 7 and 15 in treated wounds, as compared to control.

H & E stained paraffin embedded tissue sections of wound on days 3, 7 and 15 verified gross morphological changes are presented in Fig 8. Microscopic section of wound tissue on day 3 of *C. infortunatum* ointment (4%) treated rats revealed that the presence of blood clot containing fibrin, blood cells, platelets, macrophages and out numbering of neutrophils around the zone of wound gap. However, there was marked and consistent decrease in degree of infiltration of neutrophils, fibrins, blood cells, platelets, and macrophages in case of control group. Formation of large but undifferentiated blood vessels was present in case of treated group than control. In the treated group, the newly formed granulation tissue were rich in fibroblast and capillary buds were progressively invading from the marginal zone towards the central zone replacing the insoluble clot at the site of wound formation, whereas, it was not clearly evident in case of control group after 3 days of wound formation.

On day 7, the neovascularization was maximum, collagen fibrils became more evident, and there were increased fibroblast cells but decreased in inflammatory cells in case of treated rats. Whereas in case of control group there was more cells that were inflammatory until day 7, lesser extent of fibroblast cells and neovascularization was not evident. The process of granulation to control group. Collagen bundles were also

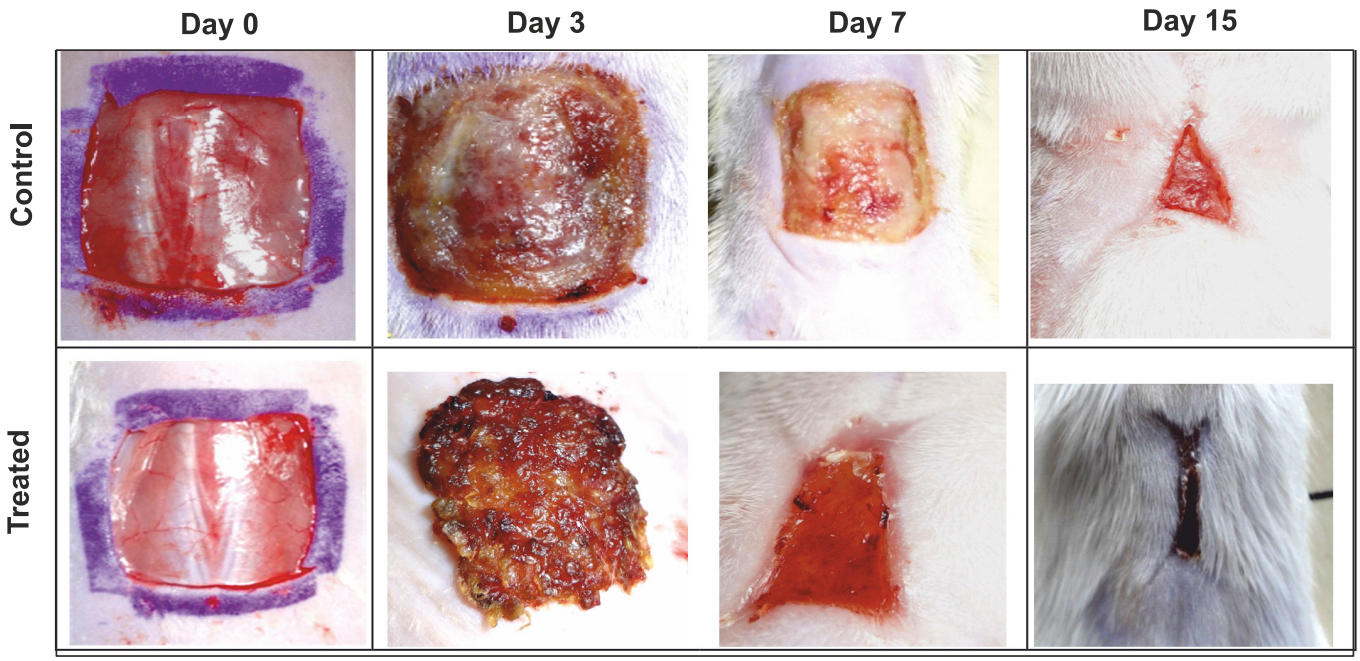

Fig 1.
Photographs showing wound closure in control and treated rats on days 0, 3, 7 and 15.

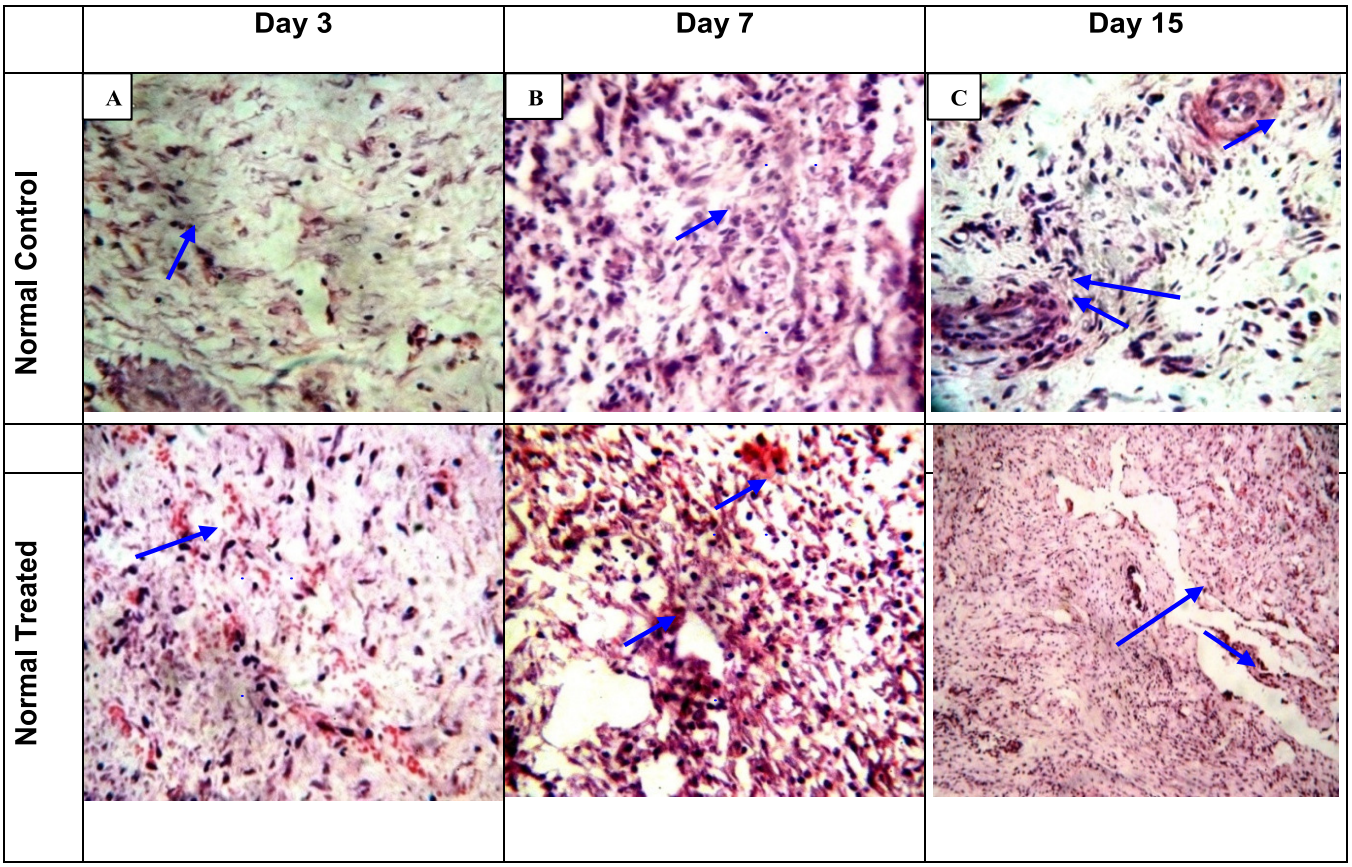

Fig 8.
Histopathological characteristics of tissue section of wound healing of control and treated rats on days 3 (A), 7 (B) and 15 (C). Photographs showing higher accumulation of neutrophils in the wound gap (A), higher sprouting of blood vessels (B) and increased the process of neovascularization (C) in the normal treated group as compared to control group on days 3, 7 and 15 respectively.

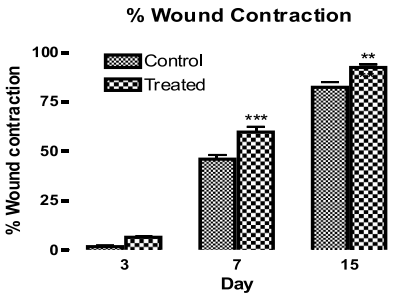

Fig 2.
Effect of C. infortunatum ointment on wound contraction (% contraction) on day 3, 7, and 15 post-wounding in rats. (** P<0.01, *** P<0.001, statistically significant, n=6).

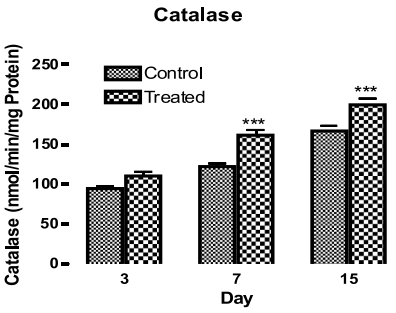

Fig 3.
Effect of topical application of *C. infortunatum* ointment 4% on activities of catalase on days 3, 7 and 15 in granulation/healing tissue in normal rats. (*** P<0.001 statistically significant, n=6).

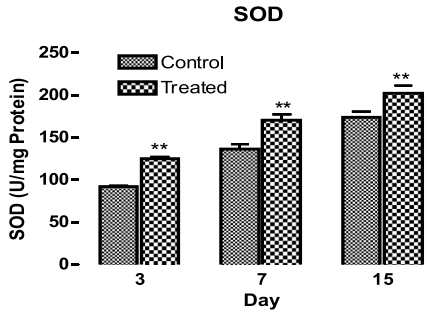

Fig 4.
Effect of topical application of C. *infortunatum* ointment (4%) on activities of SOD on days 3, 7 and 15 in granulation/healing tissue in normal rats. (** P<0.01, statistically significant, n=6).

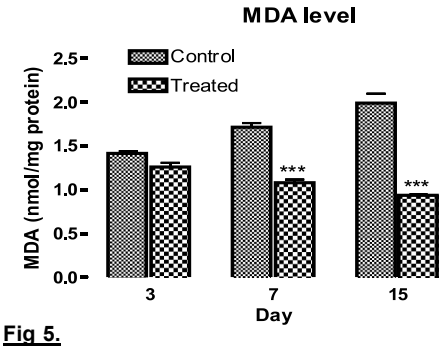

Fig 5.
Effect of topical application of *C. infortunatum* ointment (4%) on activities of GSH on days 3, 7 and 15 in granulation tissue. (*** P<0.001, statistically significant, n=6).

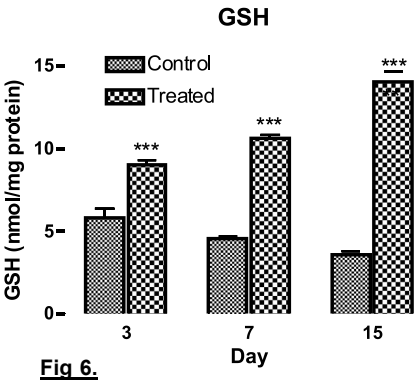

Fig 6.
Effect of topical application of *C. infortunatum* ointment (4%) on level of MDA on days 3, 7 and 15. (*** P<0.001, statistically significant, n=6).

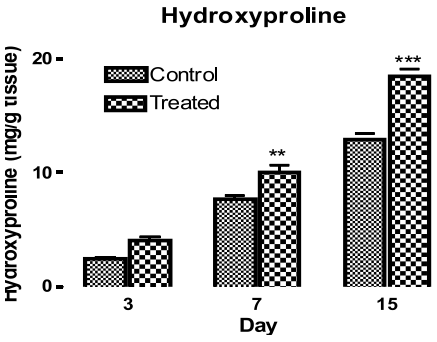

Fig 7.
Effect of topical application of *C. infortunatum* ointment (4%) on activities of Hydroxyproline content on days 3, 7 and 15 in granulation tissue. (**P<0.01, *** P<0.001, statistically significant, n=6).

appeared to more matured and large vessels were also started to differentiated in case of treated group, but it was not clearly evident in case of control group.

On day 15, there was further increase in the number of fibroblast cells, which started to be matured and became more elongated in treated group as compared to control groups. The *C. infortunatum* ointment treated group revealed the evidence of further increased in more number of collagen fibre, more thicken fibres, higher leukocyte infiltrations, and number of matured capillaries as compared to control group.

DISCUSSION

In this experiment, the increased rate of wound contraction in the treated group might be attributed to the antiapoptotic, antiinflammatory, and antioxidative property, which results in conditions conducive for proliferation, transformation of fibroblasts into myofibroblasts and reepithelialisation (Zhang *et al.*, 2003). The presence of myofibroblasts was considered characteristic of tissue undergoing contraction.

The wound healing property of *C. infortunatum* appears to be better due to the presence of its active principles, which accelerates the healing process and confers breaking strength to the healed wound (Nalwaya *et al.,* 2009).

Enzymatic antioxidant mechanisms play an important role in scavenging free radicals. Oxidative stress due to overproduction of various reactive oxygen species (ROS) and reactive nitrogen species (RNS) during prolonged inflammation induces remarkable pro-degradative effects within the wound area leading to impaired after formation of wound (Ram *et al.*, 2014). Wounding also results in the loss either of different enzymatic and non-enzymatic free radical scavengers, which recover partially or completely following healing (Shukla and Mani, 2010).

CAT is a heme containing enzyme causes detoxification of H_2O_2 to water and oxygen. CAT is one of the most efficient enzymes and cannot be saturated by H_2O_2 at any concentration (Liedias *et al.,* 1988). In this study, a significantly increased CAT activity was observed in *C. infortunatum* treated group compared to control. From the results of this study, it can be suggested that

accumulation of excessive H_2O_2 in wound tissues due to increased activity of SOD might be properly neutralised by increased CAT activity.

SOD is considered an inducible key enzyme, and its activity depends on O_2^- concentration in the biological system (Heck *et al.,* 1992). SOD catalyses the dismutation of O_2^- into oxygen and H_2O_2, thus decreases ROS generation and oxidative stress (Ponrasu *et al.*, 2013). Increased activity of SOD has been reported to scavenge the superoxide radicals to protect the tissue damage by free radicals (Gupta *et al.,* 2012). In the present study, SOD activity was increased in the *C. infortunatum* treated group, as compared to control.

Reduced glutathione (GSH) is a major non-protein thiol antioxidant present in the living organisms, which serves as a significant role in antioxidant defence mechanism. In this study, there was significant increase in the level of GSH in *C. infortunatum* treated group, compared to control group. GSH contents were found to be significantly increased on days 3, 7 and 15 in treated group, as compared to control group in a time dependent manner. GSH acts as an effective antioxidant protecting the cellular component from oxidative damage caused by ROS (Pompella *et al.*, 2003).

MDA production is an index of lipid peroxidation. The production of free radicals increases the peroxidation of lipid molecules. In the present study, in *C. infortunatum* treated rats, the lipid peroxidation was significantly lower as compared to control, which indicates that *C. infortunatum* was able to prevent lipid peroxidation by scavenging free radicals in wound. These results were in agreement with Pal *et al.*, (2009) reported that methanolic extract of *C. infortunatum* showed increase amount of percentage inhibition in oxidation with increase in its concentration. The production of oxidants causes microvascular and macrovascular complications and delay wound healing. In this study, the application of *C. infortunatum* ointment scavenges free radicals and ROS and thereby hasten wound healing.

The ethanolic extract of *C. infortunatum* increased cell proliferation and collagen synthesis at the wound site as evidenced by the increase in total protein content and total collagen content reflected by hydroxyproline content of granulation tissue **(**Woessner *et al.*, 1961). In this study, hydroxyproline was significantly increased in *C. infortunatum* treated group as compared with control. These results were in agreement with Udupa *et al.*, (1995) reported that the ethanolic extract of *C. infortunatum* ointment increases the tensile strength of the excision wound by significantly increasing the hydroxyproline content of the granulation tissue in treated group.

Angiogenesis is an important event of the proliferative phase during wound healing, which involves migration and proliferation of endothelial cells and vessel tube formation (Kant *et al.*, 2015). In the present study, the *C. infortunatum* ointment treated group revealed significantly higher angiogenesis as is evident from the histopathological findings in treated group as compared to control. *C. infortunatum* caused significantly increased development of new blood vessels and capillaries in treated group as compared to control group on days 7 and 15. Gouthamchandra *et al.,* (2010) also showed that *C. infortunatum* treatment favours angiogenesis.

In the present study, collagen bundles appeared to be more mature, large vessels were also started to differentiate and the process of granulation was comparatively faster in treated group. In addition to the formation of ECM in healing tissue, its progressive degradation and remodelling in a regulated manner is essential to form mature healed wound tissue. The treated rats in this study showed formation of more fibroblast and collagen bundles in a time-dependent manner as evidenced in histopathological observations (Fig. 8). It is concluded that *C. infortunatum* treatment showed faster and organized healing of cutaneous wounds in rats.

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Research Article

HEPATOPROTECTIVE EFFICACY OF *PICRORHIZA KURROA* AGAINST PARACETAMOL INDUCED HEPATOTOXICITY IN CHICKENS

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ABSTRACT

Hepatoprotective potential of *Picrorhiza kurroa* (PKR) was determined by plasma biochemical and antioxidant enzyme activity against paracetamol (PCM) induced hepatotoxicity in white leg horn (WLH) chickens. Thirty chickens of either sex were divided randomly and equally into five groups. Group I served as control. Birds of groups II, III, IV and V were administered by paracetamol @ 50 mg/kg b wt i/m for 7 days. Chicks of group III were orally administered silymarin (100mg/kg, p/o) for 14 days and groups IV and V chicks were administered with 200 ppm and 400 ppm PKR orally in feed for 14 days. AST, ALT and ALP activities were significantly ($P<0.05$) increased in group II birds as compared to control group birds after 7 days of treatment whereas in groups III and V these values were significantly ($P<0.05$) less than group II. After 14 days of treatment, enzymatic activities of AST, ALT and ALP in groups III, IV and V were decreased significantly ($P<0.05$) as compared group II. There was a significant decline in CAT and SOD activity and decrease in lipid peroxidation in PCM groups and these were normal in groups III, IV and V. Pathological examination revealed congestion, mononuclear infiltration and necrosis in liver of PCM treated chicks. These changes were of mild and moderate intensity in groups IV. The tissue changes were not seen silymarin and PKR (400 ppm) treated groups after 14 days. It is concluded from the above study that PCM @ 50mg/kg, i/m for 7 days produced haemotoxic and hepatotoxic effect and oxidative stress in chicks and *Pikrorhiza kurroa* root powder @ 200ppm and 400ppm in diet revealed ameliorative effects against PCM induced toxicity at par with silymarin @ 100mg/kg, after 14 days in WLH chickens.

Keywords: Hepatoprotection, *Picrorhiza kurroa,* paracetamol, white leg horn

INTRODUCTION

Liver is considered to be one of the most vital organs that functions as a centre of metabolism of nutrients such as carbohydrates, proteins and lipids and excretion of waste metabolites. Additionally, it also carries out the metabolism and excretion of drugs and other xenobiotics from the body thereby providing protection against foreign substances by detoxifying and eliminating them. Liver injury or liver dysfunction is a major health problem that challenges not only health care professionals but also the pharmaceutical industry and drug regulatory agencies. Paracetamol is a drug of choice for hepatoprotective studies. It is a COX inhibitor. Non Steroidal Anti Inflammatory Drugs commonly used as an analgesic and antipyretic drug in man and animals. An overdose or prolonged administration of these drugs can, however, cause severe hepatic damage (Thomas, 1993). Initially, paracetamol is metabolized by cytochrome P-450 including CYP2E1, 1A2, 3A4 and 2A6 (Chen *et al.*, 1998) to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI).This metabolite is toxic to the liver. The available synthetic drugs to treat liver disorders in this condition also cause further damage to the liver. Hence, herbal drugs have become increasingly popular and their use is widespread. Among these *Picrorhiza kurroa* have immense potential which can be harnessed for preparation of hepatoprotective drug. *Picrorhiza kurroa* (of the family *Scrophulariaceae*), also known as Kutki or Katuki, is a perennial herb used in Ayurveda and sometimes used as a substitute for the herb *Gentiana kurroa*; and tends to grow in the Himalaya region on rocky places within 3500-4800m above sea level. It is traditionally used for liver disorders**.** The present study was carried out to evaluate the hepatoprotective activity of PKR roots against PCM induced hepatotoxicity in WLH chickens.

MATERIALS AND METHODS

Experimental design

Thirty white leg horn chickens of either sex, of 2 month old age, weighing between 500 to 600 gm, were procured from Instructional Poultry Farm (IPF), GB Pant University of Agriculture and Technology, Pantnagar. After one week acclimatization, chickens were divided randomly and equally into five groups of 6 chickens each. Group I served as control. Birds of groups II, III, IV, and V were administered PCM @50 mg/kg b wt i/m for 7 days. Group III chickens were administered silymarin (100mg/kg p/o) orally for 14 days and groups IV and V chickens were administered PKR in feed @ 200 ppm and 400 ppm for 14 days. Research work was duly approved by the IEAC before commencement of experimentation.

Biochemical analysis

Blood was collected at 7th and 14th day of the experiment in heparin containing centrifuged tubes. The

tubes were centrifuged to separate plasma and erythrocyte pellet. Enzymes aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) was assessed in plasma by using diagnostics kits (ERBA Mannheim).

Antioxidant parameters

Antioxidant enzymatic activity was assessed in erythrocytes pellet by assessment of lipid peroxidation assay (LPO) as per the method of Rehman (1984). Catalase and super oxide dismutase (SOD) were assessed as per the method of Aebi (1983) and Giannopolitis and Ries (1977), respectively. Pathological studies were done after 14 days of experiment.

Histopathological examination

All the birds from all the groups were sacrificed using humane method and the post mortem was conducted. Gross lesions were duly recorded and the representative samples were liver and kidneys were collected in 10% buffered formalin saline for histopathological studies. The formalin fixed tissue pieces were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax. Tissue sections (4-5 microns thick) were cut and stained in hematoxylin and eosin stain and examined for histopathological changes (Luna, 1968).

Statistical analysis

Data was duly anlysed for statistical significance bt applying analysis of variance at 5% level of significance (Snedecor and Cochran, 1989).

RESULT

The hepatoprotective activity was established under chick model by determining plasma biochemical, antioxidant enzymatic activity and histopathological examination. A reference to Table 1 indicates plasma biochemical enzymatic activity and antioxidant enzymatic activity of various treatment groups at 7th day and 14th days post treatment is shown in Table 1. The values of plasma AST, ALT and ALP enzyme activities, at 7th DPT, significantly (P<0.05) increased in PCM treated group II as compared to control indicating liver damage due to PCM. In group III, IV and V, there was a significant (P<0.05) decrease in the plasma levels of AST, ALT and ALP as compared to PCM treated group II indicating hepatoprotective activity of PKR which was at par with silymarin. But AST, ALT and ALP activity in group V was significantly (P<0.05) lower as compared to group IV which might be due to the dose dependent effect of PKR and after 14th day of treatment, Plasma AST enzymes activity of groups II, III, IV and V further declined, however, were significantly higher than control.

Table 1 indicates that at 7th day, SOD and catalase activities of RBC haemolysate was significantly (P<0.05) decreased in group II as compared to the group I whereas MDA concentration increased indicating an increase in oxidative stress on erythrocytes. SOD and catalase activities of haemolysate significantly (P<0.05) increased in group III and group V. In group IV, there was a decrease in the MDA concentration as compared to group II

Table: 1

Effect of *Picrorhiza kurroa* administration on various biochemical parameters in Paracetamol induced hepatotoxicity in white leg horn chickens at 7th and 14th days post treatment.

Grps Treatment	Group I Control		Group II PCM (50 mg/ kg, IM)		Group III PCM (50 mg/ kg, IM)+Silymarin (100 mg/Kg)		Group IV PCM (50 mg/ kg, IM) +PKR (200 ppm)		Group V PCM (50 mg/ kg, IM)+PKR (400 ppm)	
DPT	7th	14th	7th	14th	7th	14th	7th	14th	7th	14th
AST (IU/L)	48.7± 0.58	51.6± 0.58	96. 7± 0.47^{a}	75.6± 0.40^{aA}	56.5± 0.44^{b}	54.5± 0.75^{b}	78.6± 0.58^{abc}	67.4± 0.34^{abcA}	71.4± 0.61^{abcd}	64.7± 0.64^{abcA}
ALT (IU/L)	19.7± 0.51	20.7± 0.67	32.9± 0.33^{a}	29.4± 0.62^{a}	21.9± 0.42^{b}	21.4± 0.46^{b}	28.7± 0.60^{ac}	26.9± 0.72^{ac}	24.3± 0.63^{abd}	23.3± 0.66^{bd}
ALP (IU/L)	87.5± 0.5	90.7± 0.56	164.8± 0.62^{a}	121.7± 0.59^{aA}	94.7± 0.72^{b}	92.6± 0.50^{b}	152.4± 0.68^{abc}	114.9± 1.04^{abcA}	106.8± 0.64^{abcd}	102.6± 0.44^{abcd}
SOD (U/mg)	3.92± 0.06	4.11± 0.05	2.82± 0.11^{a}	3.74± 0.07^{aA}	3.94± 0.11^{ab}	4.13± 0.04^{b}	2.94± 0.6^{ac}	3.87± 0.13^{acA}	3.72± 0.04^{acd}	3.94± 0.13^{ab}
MDA (nmol/mg)	8.48± 0.21	9.11± 0.77	17.67± 0.64^{a}	14.7± 0.37^{aA}	10.7± 0.46^{b}	10.8± 0.43^{b}	14.9± 0.43^{abc}	12.1± 0.61^{a}	12.7± 0.62^{ab}	11.9± 0.53^{ab}
Catalase (U/mg)	64.2± 0.64	64.7± 0.47	51.4± 1.03^{a}	5.28± 0.59^{a}	62.3± 1.03^{b}	63.2± 1.07^{b}	54.2± 0.64^{ac}	59.7± 0.62^{acA}	59.4± 0.50^{abd}	61.4± 0.95^{ab}

All values represent Mean ± SEM (n=6). PCM administered for 1st to 7th day whereas silymarin and PKR for 1st to 14th day of the study. a=Significant (P<0.05) difference as compared to group I within the same column, b= Significant (P<0.05) difference as compared to group II within the same column., c = Significant (P<0.05) difference as compared to group III within same column., d= Significant (P<0.05) difference as compared to group IV within same column, A = Significant (P<0.05) difference as compared to 7th day within same row.

indicating a reversal of oxidative stress by PKR (400ppm). There was no significant difference in the activities of SOD and catalase activities of birds of groups IV and II. Thus PKR was not effective at low dose level after 7 days of treatment. At 14th DPT, SOD and catalase activities increased significantly ($P<0.05$) in group IV whereas MDA conc decrease in Group IV as compared to its activity at 7th day. This indicated that PKR (200 ppm) also reversed the oxidative stress after 14 days treatment. SOD, catalase activity of group III and group V was restored towards normal after 14 days of treatment.

No gross lesion could be recorded in the liver and kidneys of groups I, III, IV and V birds. Liver and kidneys were pinkish in discoloration in group II birds. No lesions could be recorded in liver of groups I and III birds. Liver of group II birds revealed severe congestion of small and large blood vessel (Plate 1), dilatation of sinusoidal spaces, severe congestion of the sinusoidal spaces throughout the parenchyma (Plate 2), multifocal necrosis at many places with mononuclear cell accumulation. There was severe fibrosis around many of the large blood vessels. At many places, there were focal mononuclear cell accumulations around the large blood vessels also (Plate 2). In liver of group IV birds, these lesions were of mild to moderate intensity and in group V birds, these lesions were of mild intensity. The liver in group IV birds manifested mild congestion of large and small blood vessels, sinusoidal spaces at few places only. Multifocal necroses were also seen at fewer places compared to group II birds. The fibrosis around blood vessels was also evident around few blood vessels. These lesions in group V birds were even milder in nature compared to groups II and IV birds.

No lesions could be recorded in the kidneys of group I and group III birds. Cortex and medulla of kidneys of group II birds exhibited severe congestion of the small and large blood vessels (Plate 3) and severe interstitial haemorrhages at many places in the parenchyma (Plate 4). There was swelling of glomeruli along with congestion of glomerular capillaries at many places in the cortex (Plate 5). In kidneys of group IV birds, these lesions were of mild to moderate intensity while in group V birds, these lesions in kidneys were of mild intensity.

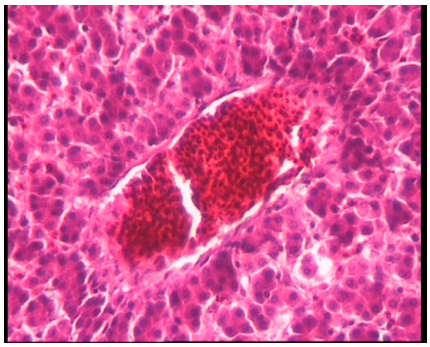

Plate1:
Severe congestion of large blood vessels and sinusoids in group II liver (HE, x 200).

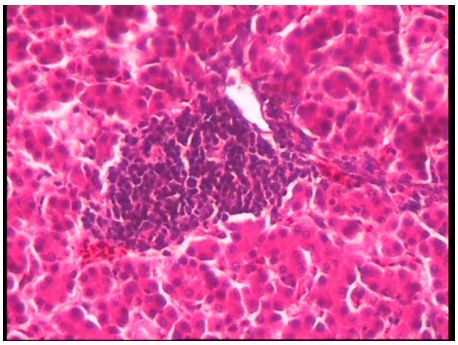

Plate 2:
Mononuclear cell accumulation around blood vessel along with sinusoidal congestion in group II liver (HE, x 200).

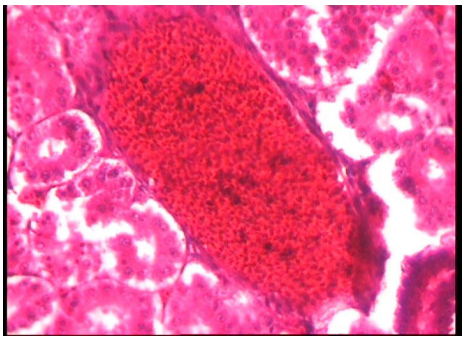

Plate 3:
Severe congestion of large blood vessel in group II kidney (HE, x 200).

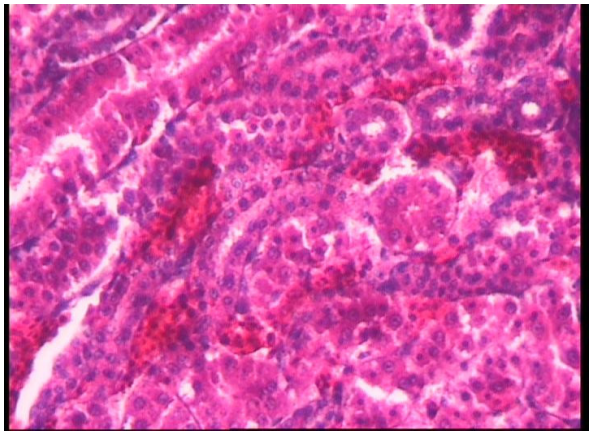

Plate 4:
Interstitial hemorrhages in group II kidney (HE, x 200).

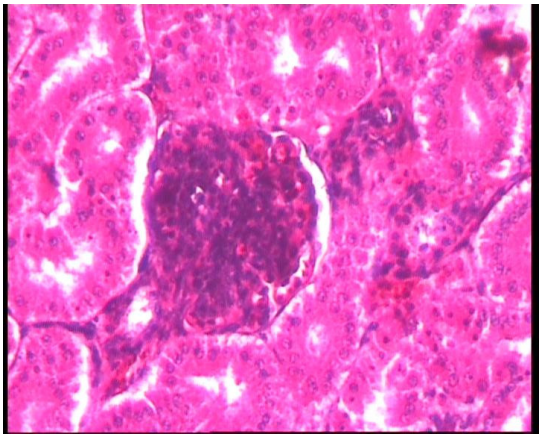

Plate 5: Swelling of the glomerulus and congestion of glomerular capillaries in group II kidney (H & E, x 200).

DISCUSSION

There was no significant difference in the enzymatic activity of groups I and III birds which could be due to complete liver regeneration whereas in groups IV and V enzymatic activity was declined but not as much as of group III showing comparatively slower recovery by PKR in comparing to silymarin.

PKR decreased the levels of all the enzymes as compared to PCM treated group which validates that PKR has a hepatoprotective efficacy. Chander *et al.* (1990) reported that a administration of picroliv, a standardized fraction of alcoholic extent of *Picrorhiza kurroa* (Scrophulariaceae) (3-12 mg/kg/day for two weeks) simultaneously with *P. berghei* infection showed significant decrease in activity of all enzymes in *Mastomys natalensis*. Similar role of *Picroriza kurroa*@0.3 g/KgBW for 30 days against aflatoxin induced hepatic damage in rats was also reported by (Samy and Emad (2011). Rajaprabhu *et al.* (2007) also reported similar effect of *Picrorhiza kurroa* extract @50 mg/Kg for 15 days in andriacin induced cardiopathy in rats.

The hepatoprotective effect of *Picrorhiza kurroa* roots was confirmed by histopathological examination of the liver tissue of control and treated animals as there is severe congestion of small and large blood vessel , dilatation of sinusoidal spaces, severe congestion of the sinusoidal spaces throughout the parenchyma, multifocal necrosis at many places with mononuclear cell accumulation in paracetamol treated group and severity of this got reduced in group IV and group V indicated that *Picrorhiza kurroa* has ameliorative effect on paracetamol toxicity. Similar results of *Picrorhiza kurroa* was obtained by Shetty *et al.* (2010). The nephroprotective effect of *Picrorhiza kurroa* roots was confirmed by histopathological examination of the kidney tissue of control and treated animals as cortex and medulla of kidneys of group II birds exhibited severe congestion of the small and large blood vessels and severe interstitial haemorrhages at many places in the parenchyma. There was swelling of glomeruli along with congestion of glomerular capillaries at many places in the cortex and severity of this effect got reduced in picrorhiza kurroa 400 ppm treated group indicated that *Picrorhiza kurroa* has renoprotective action.

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Research Article

HEPATOPROTECTIVE EFFECT OF *ANNONA SQUAMOSA* ON CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY IN RAT MODEL

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ABSTRACT

The present study was conducted to evaluate hepatoprotective effect of aqueous extracts of *Annona squamosa* following repeated oral administration for 28 days in carbon tetrachloride (CTC) induced hepatotoxic rats. The study was conducted on thirty six male albino Wistar rats dividing them in various groups having six rats in each group. Group I served as vehicle control. Rats of group III, IV, V and VI received 50 % carbon tetrachloride in olive oil at the dose rate of 1 ml/kg b wt, intraperitoneally, twice in a week throughout the study period for induction of hepatotoxicity. Group III received standard drug silymarin @ 50 mg/kg of b wt (p.o.) daily once for 28 days of dosing period. Group IV, V and VI also received aqueous extracts of *A. squamosa* @ 100, 200 and 400 mg/kg, daily once for 28 days of dosing period. Hepatotoxic rats receiving aqueous extracts of *A. squamosa* @ 100, 200 and 400 mg/kg b wt for 28 days showed significant reduction in serum ALT, AST, GGT, ALP, bilirubin, creatinine kinase and serum creatinine and significant increase in serum albumin, globulin and total protein level and preserved normal histoarchitecture as compared to rats of hepatotoxic control group in dose dependent manner except *A. squamosa* (100 mg/kg). The findings of present study suggest that aqueous extracts of *A. squamosa* has hepatoprotective effect at dose dependent manner.

KEY WORDS: *Annona squamosa,* Hepatoprotective effect, Carbon tetrachloride, Rats.

INTRODUCTION

Liver cell injury caused by various toxicants such as certain chemotherapeutic agents, carbon tetrachloride, thioacetamide, chronic alcohol consumption and microbes is well-studied. Enhanced lipid peroxidation during metabolism of ethanol may result in development of hepatitis leading to cirrhosis. Drug-induced liver toxicity is a very common cause of liver injury during medication. Numerous studies noted that CCl_4 is widely used to induce liver damage because it is metabolized in hepatocytes by cytochrome P_{450} generating a highly reactive carbon-centered trichloromethyl free radical, leading to initiating a chain of lipid peroxidation and thereby causing liver fibrosis. Silymarin is most commonly used standard hepatoprotective drug in experimental animals. Silymarin is the bioactive extract from *Silybum marianum* seeds and silymarin presents a pharmacologically effective substance containing four main constituents: silybin (50-60%), isosilybin (5%), silychristin (20%) and silydianin (10%) (Ding *et al.,* 2001). Present study was carried out in albino Wistar rats to explore hepatoprotective effects of aqueous extract of *A. squamosa* leaves in CTC treated rats.

MATERIALS AND METHODS

Experimental Animals

The study was conducted on adult healthy male albino Wistar rats. Rats of 8-12 weeks of age were procured from Zydus Research Centre (ZRC), Moraiya, Ahmedabad, Gujarat. All the protocols as per the CPCSEA guidelines on the care and use of laboratory animals were followed and approved by the Institutional Animal Ethics Committee of Veterinary College, Anand (IAEC/VPT/198/2015). Rats were kept under constant observation during entire period of study.

Preparation of plant extracts

Leaves of *A. squamosa* were collected from the local area of Anand district (Gujarat) the plants was identified and authenticated by Botanist, Department of Genetics and Plant Breeding, B.A. College of Agriculture, AAU, Anand. The dried powder of leaves was extracted in Soxhlet extractor and then reduced to a dark colored molten mass by using rotary evaporator under reduced pressure.

Evaluation of acute oral toxicity

The acute oral toxicity studies of aqueous extracts of *A. squamosa* were carried out as per Organization for Economic Cooperation and Development (OECD) guideline No. 423. Albino Wistar rats were taken for the study and dosed once with 2000 mg/kg, orally. It was observed that the aqueous extract of *A. squamosa* have no toxic effect on rats even at 2000 mg/kg doses, respectively. Therefore, the present study was done @100 mg/kg, 200 mg/kg and 400 mg/kg b wt daily for 28 days.

Experimental Design

Experimentally-induced hepatotoxicity in rats (carbon tetrachloride) was used to study hepatoprotective effects of aqueous extract of *A. squamosa*. After 1 hour fasting, all the animals except vehicle control group were administered 1 ml/kg (i.p.) carbon tetrachloride by dissolving it in olive oil (1:1) twice in a week, throughout

the study period (28 days) and then after 1 hour all those rats were administered test compounds. Silymarin at 50 mg/kg (in NSS) body weight (p.o.) to animals of group III. Aqueous extracts of *A. squamosa* were dispersed in water using the same amount of saline solution and administered to animals of group IV, V and VI at dose of 100, 200 and 400 mg/kg (p.o.), respectively, for 28 days of dosing period. Vehicle control group rats were administered normal saline solution (NSS) orally, once daily for 28 days.

Sample preparation

On 29th day of experimental period, blood samples were collected from the retro-orbital puncture under anesthetic conditions into clean sterilized plain and K_3EDTA added micro-centrifuge tube for serum biochemical and hematological analysis, respectively.

Estimation of Hematobiochemical Parameters

Blood samples collected in test tubes with K_3EDTA were subjected to estimation of various hematological parameters by auto hematology analyzer (Mindray BC-2800 Vet, China). On the day of blood collection, red blood cells (RBC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), total leucocytes count (TLC/WBC) and platelets were estimated. Serum biochemical parameters were estimated in clinical serum biochemistry analyzer (NOVA 2021 Biochemistry analyzer,analytical Technologies Limited, Gujarat, India) including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), serum albumin, serum globulin, total protein, Alkaline phosphatase (ALP), Creatine kinase (CK), billirubin and creatinine.

On 29th day of study, all the rats from each group were sacrificed by cervical dislocation and subjected to post mortem and histopathogical examination.

Statistical analysis

All the data have been presented as mean ± SE. Statistical comparisons of the results were made using one way analysis of variance (ANOVA) by using computer software SPSS (Version 20). Significant differences ($p < 0.05$) between different experimental groups were analyzed by Duncan's test.

RESULTS AND DISCUSSION

There was significant decrease in TEC, Hb, PCV, MCV, MCH, MCHC, TLC and platelets in hepatotoxic control group II as compared to vehicle control group I and There was significant increase in all these parameters in silymarin treated group III and *A. squamosa* extract treated group VI (400 mg/kg) as compared to hepatotoxic control group II (Table-1). There was significant increase in ALT, AST, GGT, ALP, CK, billirubin and creatinine level in hepatotoxic control group II as compared to vehicle control group I and there was significant decrease in these biochemical parameters level in treatment control group III and *A. squamosa* extract treated group IV (100 mg/kg), group V (200 mg/kg) and group VI (400 mg/kg) as compared to hepatotoxic control group II in dose dependent manner. There was significant decrease in serum albumin, globulin, total protein in hepatotoxic control group II as compared to vehicle control group I and there was significant increase in these serum biochemical parameters in treatment control group III and *A. squamosa* extract treated group IV (100 mg/kg), group V (200 mg/kg) and group VI (400 mg/kg) as compared to hepatotoxic control group II (Table 2). Liver from rats of control group was normal whereas hepatotoxic control group II showed paleness and diffuse necrotic foci (Figure 1) Liver sections from the rats treated with CCl_4 alone (Group II) showed massive changes throughout the lobules, with sinusoidal dilatation, cellular vacuolization (Figure 2), necrosis, distortion of the central venules and ballooning of hepatocytes (Figure 3). Sylimarin treated group (Group-III) showed normal hepatocytes without any damage. The treatment of rats with 100 mg/kg of *A. squamosa* extract (Group IV) revealed degeneration of hepatocytes and focal necrosis and more or less intact central vein but still widened sinusoidal spaces. Treatment of rats with 400 mg/kg of *A. squamosa* extract (Group-VI) showed mild sinusoidal dilatation (Figure 4) and preserved normal histoarchitecture of liver section. Microscopic changes in kidneys of rats from group II revealed congestion with degeneration and necrosis of renal tubular epithelium, congestion with cloudy swelling in tubular cells. Kidneys of rats of other groups revealed moderate to mild congestion and degenerative changes in dose dependent manner in histological structure.

There was significant increase in RBCs, Hb and PCV of hepatotoxic rats receiving aqueous extracts of *A. squamosa* @ 200 and 400 mg/kg and silymarin @ 50 mg/kg b wt as compared to rats of hepatotoxic control group. Similarly, Gaikwad *et al.* (2013) found on preclinical evaluation of *A. squamosa* linn for their haematinic activity on rats. Likewise, there was significant decrease in MCV, MCH and MCHC of rats belonging to hepatotoxic control group as compared to vehicle control rats. There was significant increase in MCV and MCHC of hepatotoxic rats receiving aqueous extracts of *A. squamosa* @ 100, 200 and 400 mg/kg and silymarin @ 50 mg/kg b wt as compared to rats of hepatotoxic control group whereas MCH significant increase in *A. squamosa* extract alone treated group VI (400 mg/kg) as compared to hepatotoxic control group. Likewise, there was significant decrease in TLC and platelets of rats belonging to hepatotoxic control group as compared to vehicle control rats. There was significant increase in TLC and platelets of hepatotoxic rats receiving aqueous extracts of *A. squamosa* (200 and 400 mg/kg) and silymarin at dose rate of 50 mg/kg b wt

as compared to rats of hepatotoxic control group.

In the present study, there was significant increase in ALT/SGPT, AST/SGOT and GGT level in carbon tetrachloride induced hepatotoxic control group due to release of these enzymes from hepatic parenchymal cells (Bishayee *et al.,* 1995). In present study all extract treated group and treatment control group showed significant decrease ALT, AST and GGT as compared to hepatotoxic control group. Similarly Rajeshkumar *et al.* (2015) studied phytochemical screening and hepatoprotective efficacy of aqueous extracts of *A. squamosa* leaves against paracetamol decrease in the serum enzymes ALT and AST level when compared to the paracetamol induced hepatotoxic control group. Significant decline in elevated ALT and AST have also been reported by Natarajan *et al.* (2015).

Hepatotoxic rats receiving aqueous extracts of *A. squamosa* at doses of 100, 200 and 400 mg/kg body

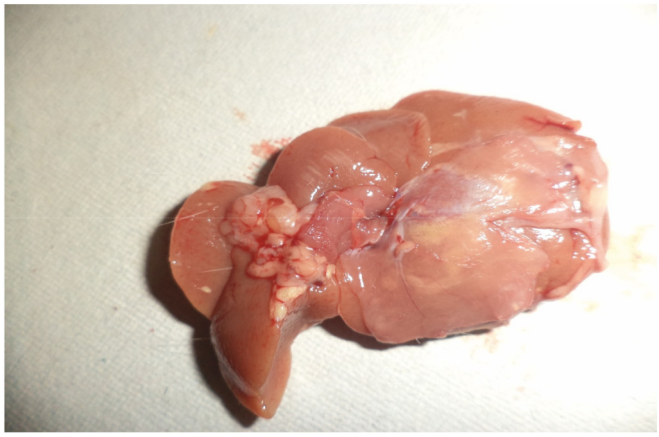

Fig.1:
Photograph showing hepatotoxic rat liver showing paleness and necrotic foci

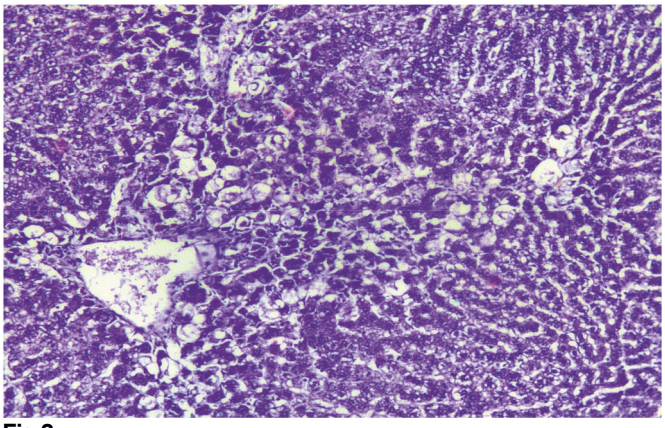

Fig.2:
Section of liver of rat from hepatotoxic control rats (group II) showing vacuolation in hepatocytes (H& E stain X 120)

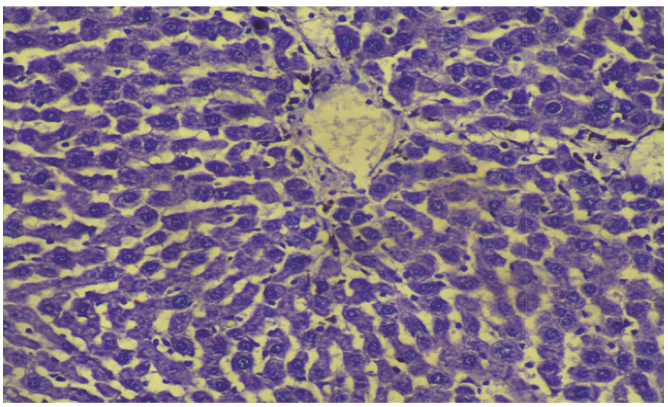

Fig.3:
Section of liver from hepatotoxic control rat (group II) showing dilatation of sinusoidal spaces, distortion of central venules and ballooning of hepatocytes (H & E stain X 240)

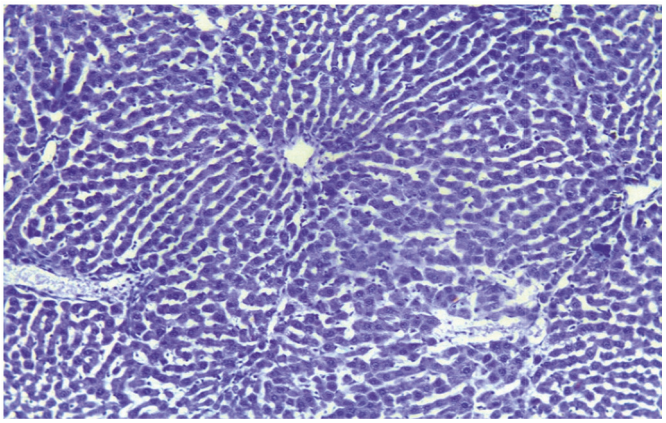

Fig.4:
Section of liver from *A. squamosa* treated group (group VI) showing distention of sinusoidal spaces and almost preserved normal histoarchitecture (H & E stain X 120)

Table-1:
Hematological parameters (Mean ± SE) in different experimental groups (n=6)

Groups	TEC (10^6/µl)	Hb (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	TLC (10^3/µl)	PLT (10^5/µl)
I	8.08 ±0.37^{e}	15.56±0.72^{g}	43.87±1.02^{g}	54.69 ±1.66^{h}	19.28 ± 0.36^{f}	42.35±0.70^{f}	8.88±0.27^{e}	9.30 ± 0.04^{j}
II	6.15 ±0.24^{a}	8.62 ± 0.70^{a}	26.57 ±1.33^{a}	29.77 ±1.02^{a}	12.92 ±0.36^{a}	27.61 ±0.69^{a}	4.62 ±0.41^{a}	5.43 ± 0.05^{a}
III	7.70±0.09^{de}	14.45 ±0.49^{fg}	41.75 ±0.32^{g}	51.77±2.49^{gh}	18.32±0.17^{ef}	41.09 ± 0.69^{f}	8.72 ±0.22^{e}	8.60 ± 0.02^{i}
IV	6.23 ±0.22^{a}	9.80 ± 0.20^{ab}	27.48±0.74^{ab}	44.5 ±2.03^{def}	14.55±0.63^{abc}	29.81 ±0.40^{b}	5.12±0.13^{ab}	6.35 ± 0.05^{b}
V	6.72±0.18^{abc}	10.22±0.24^{bc}	30.02±0.42^{bcd}	44.85±1.22^{ef}	15 ± 0.66^{abc}	31.98 ±0.60^{c}	5.51 ±0.12^{b}	7.40 ± 0.02^{f}
VI	7.13 ±0.28^{bcd}	11.18±0.39^{bcd}	33.8 ± 1.43^{ef}	47.38±0.76^{fg}	17.72 ±1.20^{def}	35.88 ±0.75^{d}	6.20 ±0.11^{c}	7.46 ± 0.02^{f}

Mean value with dissimilar superscript in a column vary significantly at $p<0.05$; Treatment groups: Group II – VI given CCl_4 (1 ml/kg (i.p.) for induction of hepatotoxicity; I: Vehicle Control; II: Hepatotoxic Control; III: Standard Treatment Control; IV: Aqueous Extract *A. squamosa* (100 mg/kg); V: Aqueous Extract *A. squamosa* (200 mg/kg); VI: Aqueous Extract *A. squamosa* (400 mg/kg). TEC: Total Erythrocyte Counts; Hb: Hemoglobin; PCV: Packed Cell Volume; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; TLC: Total Leukocyte Count, PLT: Platelets count.

Table-2:
Serum biochemical parameters (Mean ± SE) in different experimental groups (n=6)

Groups	ALT (U/l)	AST (U/l)	GGT (U/l)	Albumin (g/dl)	Globulin (g/dl)	Total Protein (g/dl)	ALP (U/l)	CK (U/l)	Serum bilirubin (mg/dl)	Serum creatinine (mg/dl)
I	66.47±1.88^{a}	152.17±3.13^{a}	24.76±1.41^{a}	4.47 ±0.10^{b}	3.53 ±0.14^{f}	8.00 ± 0.23^{f}	168.98 ± 5.69^{a}	673.02 ± 6.63^{a}	0.21 ± 0.07^{a}	0.35 ± 0.03^{a}
II	481.6±2.91^{h}	338.83±9.13^{i}	85.91±3.57^{g}	3.58 ±0.11^{a}	2.14 ±0.13^{a}	5.72 ± 0.11^{a}	433.64 ± 4.48^{h}	1036.33 ± 4.19^{g}	1.51 ± 0.14^{e}	1.29 ± 0.08^{f}
III	92.90±1.45^{b}	165.67±1.48^{b}	37.71±1.76^{b}	4.44 ±0.10^{b}	3.14±0.06^{ef}	7.59 ± 0.05^{ef}	190.65 ± 4.83^{b}	676.62 ± 6.58^{a}	0.27 ± 0.03^{ab}	0.51 ± 0.02^{ab}
IV	275.43±2.80^{g}	258.17±3.20^{h}	68.60±0.86^{f}	4.10 ±0.08^{b}	2.20 ±0.13^{ab}	6.31 ± 0.11^{b}	343.36 ± 4.27^{g}	946.25 ± 6.00^{f}	0.86 ± 0.02^{d}	0.85 ± 0.10^{e}
V	220.73±2.38^{f}	241.83±3.73^{fg}	64.92±1.23^{ef}	4.23 ±0.07^{b}	2.72±0.04^{cde}	6.93 ± 0.10^{cd}	292.12 ± 5.66^{e}	866.33 ± 6.04^{e}	0.51 ± 0.08^{abc}	0.74 ± 0.07^{cde}
VI	196.54±3.96^{e}	224.67±3.35^{e}	48.74±2.25^{cd}	4.17 ±0.05^{b}	2.89 ±0.07^{de}	7.06 ± 0.10^{cd}	244.38 ± 4.90^{d}	752.65 ± 3.67^{c}	0.41 ± 0.01^{abc}	0.63 ± 0.06^{bcd}

Mean value with dissimilar superscript in a column vary significantly at p<0.05; Treatment groups: Group II – VI given CCl_4 (1 ml/kg (i.p.) for induction of hepatotoxicity; I: Vehicle Control; II: Hepatotoxic Control; III: Standard Treatment Control; IV: Aqueous Extract *A. squamosa* (100 mg/kg); V: Aqueous Extract *A. squamosa* (200 mg/kg); VI: Aqueous Extract *A. squamosa* (400 mg/kg). ALT: Alanine aminotransferase; AST: Aspartate Aminotransferase; GGT: Gamma-glutamyl transferase; ALP: Alkaline phosphatase; CK: Creatine kinase.

weight for 28 days also showed significant reduction in serum ALT, AST and GGT level as compared to rats of hepatotoxic control group in dose dependent manner. In the present study, there was significant decrease in serum albumin, globulin and total protein level in carbon tetrachloride induced hepatotoxic control group as compared to vehicle control group. Singh *et al.* (2010) found similar results when they studied hepatoprotective screening of polyherbal extract of *A. squamosa* and *Nigella ativa* (dose @ 50, 100 and 150 mg/kg of b. wt. once daily orally for 28 days) on male Wistar albino rats. Uduman *et al.* (2011) studied protective effect of methanolic extract of *A. squamosa* linn (dose @ 250 and 500 mg/kg/d orally for 21 days) in isoniazid-rifampicin (dose @ 100 mg/kg each i.p.) induced hepatotoxicity in rats and results showed that increased AST and ALT were significantly reduced by coadministration of methanolic extract of *A. squamosa* in two different doses (250 and 500 mg/kg).

In the present study, there were significant increase in alkaline phosphatase (ALP) and creatinine kinase (CK) in carbon tetrachloride induced hepatotoxic group as compared to vehicle control group. Result of this study was also showed similarity with other reports by Mohamed Saleem (2008) who studied hepatoprotective activity of *A. squamosa* Linn. (ethanolic extract dose @ 350 mg/kg and aqueous extract dose @ 300 mg/kg of b. wt once daily orally for 21 days) on Wistar rats.

Histological examination of sections of liver of rat from hepatotoxic control group showed massive changes throughout the lobules, with sinusoidal dilatation, cellular vacuolization, necrosis and distortion of the central venules, ballooning of hepatocytes. Shaikh *et al.* (2015) found that liver sections from the rats treated with CCl_4 (2 ml/kg, i/p.). *Annona squamosa* extract treated (ethanolic extract dose @ 350 mg/kg and aqueous extract dose @ 300 mg/kg of b. wt. once daily orally for 21 days) group found minimal inflammation with moderate portal triaditis and their lobular architecture was normal. Singh *et al.* (2010) studied polyherbal extract of *Anonna squamosa* and *Nigella ativa* (dose @ 50, 100 and 150 mg/kg of b. wt. once daily orally for 28 days) treated group showed markedly alleviated the degree of liver fibrosis indicated by decreased collagen deposition and lowed inflammation in male Wistar albino rats.

It is concluded from the study that aqueous extract of *A. squamosa* has hepatoprotective activity in dose dependent manner against drug induced hepatotoxic in rats.

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Research Article

IMMUNE RESPONSE FOLLOWING PROLONGED PESTICIDE EXPOSURE IN COCKERELS

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ABSTRACT

Immunotoxic effects of alphamethrin and monocrotophos pesticides were evaluated studied by lymphocyte stimulation, antibody titers, macrophage function test and delayed type hypersensitivity response. Thirty, eight weeks old, white leghorn chicks were and equally randomly divided in five groups. Group I served as control and rest were as experimental and given medicated ration with alphamethrin (20 & 40 ppm) and monocrotophos (10 and 20 ppm) for 20 weeks. The blood samples were collected at 10 and 20 weeks interval**.** There was a significant ($P<0.01$) reduction of antibody titre, LST, MFT and DTH in all the pesticides treated groups in comparison to control and these reduction indicated pesticide induced immunosuppression in WLH cockerels.

Key Words : Alphamethrin, antibody titer, monocrotophos, LST, MFT, DTH, poultry.

INTRODUCTION

Pesticide hazards are often encountered due to contamination of air and water due to environmental pollutants that are distributed in soil, plants, and food grains. These are subsequently translocated in the body tissues after their consumption either directly or indirectly following short or long term exposure and likely to cause toxic effects on hepato-renal, immunological, respiratory, cardiovascular, nervous and other vital organs. Pesticides are the one of the major environmental pollutants. Insecticides alphamethrin, a synthetic pyrethroid (WHO, 1992), is more effective than the organophosphate pesticides, and is used in many agricultural, commercial and residential applications. Due to lipophilic nature, they are readily translocated across the biological membranes to tissues (Oros *et al.,* 2005). Monocrotophos (MCP) is one of the organophosphorus insecticides extensively used in agriculture and animal husbandry (Rao, 2004). The immunosuppressive effect of insecticides have been reported (Luster *et a*l, 1982; Singh *et a*l, 2007;), The present study was, therefore, designed to evaluate the effect of prolonged exposure of pesticides on immunological parameters in WLH cockerels.

MATERIALS AND MATHODS

Experimental design

Eight weeks old, white leghorn male chicks procured from Instructional Poultry Farm of University were randomly divided into five groups of six birds each. The feed and water was given ad *libitum* during the study. After acclimatization for two weeks, forty two chicks were divided randomly and equally into five groups with six bird each and were treated with alphamethrin @20 and 40 mg/kg b.wt. in group II and III; and monocrotophos @ 10 and 20 mg/kg in group IV and V for 20 weeks. The group I received no chemical treatment and served as control. The dose of pesticides was selected on the basis of median lethal doses. Chicks were kept under constant observation after administration of pesticides for recording behaviour, general response and other clinical manifestations..

Blood collection and lymphocyte isolation

Blood samples were collected from wing vein aseptically at 10 and 20 weeks interval in heparinised test tubes. The heparinised blood (3 ml) was mixed with equal volume of RPMI-1640 medium containing 2mM, of L-glutamate; 50IU/ml, penicillin; and 50 mg/ml, streptomycin. Histopaque-1077(Sigma; 3ml) was taken in another test tube and media mixed blood was carefully overlaid on histopaque-1077. The tubes were then centrifuged at 400g for 15 min in refrigerated centrifuge. The cells at the interface of plasma and histopaque were collected with the help of sterilised Pasture pipette and collected in another sterilised tube. The final pellet was suspended in 1 ml of cold RPMI-1680 medium containing 10% of fetal calf serum (sigma) Cell viability was determined by 0.5% trypan-blue dye exclusion test in which live lymphocytes do not get stained while dead cells are stained black (Boyse *et al,* 1964).Cell suspensions were diluted to a final conc. of $1x10^7$ cells/ml in RPMI-1680 medium and subjected to lymphocyte stimulation test.

Immunological assay

Total and differential leucocytic counts

Total leucocyte count (TLC) (Natt and Herrick, 1952) and differential leucocyte count (Lucas and Jamroj, 1961) were determined from the blood collected at 10 weeks interval. The absolute lymphocyte counts were calculated by multiplying the TLC values with per cent lymphocyte values in each group.

Macrophage function assay

Macrophages were collected from intraperitoneal fluid after 24 h of i.p. injection (3ml) of liquid paraffin**.** The metabolic activity of macrophage was measured employing nitrobluetetrazolium (NBT) reduction after 20 weeks interval by the described methods (Talwar, 1983). NBT positive cells were counted and expressed in percentage (Chauhan, 1995).

Lymphocyte stimulation assay

Lymphocyte stimulation against Con A was evaluated by the method of Rai-el-Balhaa *et al* (1987) with minor modifications (Chauhan, 1995).

Delayed type hypersensitivity response

Delayed type hypersensitivity reaction to dinitro-fluorobenzene (DNFB) was studied (Phanuphak *et al,* 1974) after 20 weeks trial.

Antibody titer estimation

Antibody titers were measured at 7^{th} and 14^{th} day after challenging with the antigen Bovine serum albumin (BSA) after completion of 20 weeks trial by employing ELISA as described by Miers *et al*. (1983) with minor modifications (Chauhan, 1995).

Statistical analysis

Statistical analysis of data was done by using graph pad software one way ANOVA technique. Statistically significant difference was considered at 5% level (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

The mean leucocyte and absolute lymphocyte counts (10^3/µl) of both groups were presented in Table 1. Initially, there were no significant change in TLC and ALC value in insecticides treated groups up to 10 weeks. After 20 weeks the TLC and ALC value suppressed significantly ($p<0.01$) in both high and low dietary groups of pesticides as compared to control. This finding is corroborated with the finding of Singh *et al.* (2007) in chickens. Significant ($P<.05$) reduction of TLC and ALC observed in present study indicated the birds was suffering from leucopenia and that might be due to cytotoxic effect of insecticides. The lymphocytes play vital roles in cell defence mechanism and its suppression indicated directly the immunosuppression (Mandal *et al.*, 1986).

Antibody titers against BSA were depicted in ELISA values in Figure 1. The values were significantly ($P<0.01$) suppressed in cockerels fed on higher dietary level of alphamethrin and monocrotophos on 14^{th} day. This is confirming the immunosuppression in humoral response in these groups. The reduction in the antibody titer could be attributed to hampered proliferation and activation of B-lymphocytes, responsible for biosynthesis of immuniglobulins. These findings are corroborated with the finding of Singh *et a*l. (1999 & 2007).

DTH was evaluated by measuring the skin thickness (mm) in response to DNFB in sensitized chickens. Feeding of insecticide medicated ration for 20 weeks, suppressed cellular response in all the higher dietary groups as revealed by a significant ($P<0.01$) diminution in the thickness of the sensitized skin in response to DNFB in high dietary groups of all the insecticides but was more profound in alphamethrin and monocrotophos Figure 2. These findings suggested the functional impairment of T- lymphocytes (Danneberg, 1991). These findings have further supported by the reduction in TLC and ALC value in alphamethrin and monocrotophos insecticide treated groups in this study.

Microphage function test (MFT) is expressed by % macrophages giving NBT +ve test. A significant ($P<0.01$) reduction in metabolic activity was reported in both the dietary levels of alphamethrin and monocrotophos (Figure3). A reduction in macrophage function contribute to reduction in the phagocytic activity and subsequently the defence mechanism of the body.

LST was employed to study cellular immune response and was represented by "OD. Feeding for 10 weeks did not produce any effect on lymphocyte stimulation but a prolong 20 weeks feeding in all group treated with high doses suppressed significantly ($P<0.05$) lymphocyte stimulation response to Con A. Lymphocyte proliferation assay to Con A mitogen has been suggested as a measurement of T- lymphocyte proliferation capacity (Toivanen and Toivanen, 1983 and Lee, 1977) and indicative of supreesion of cellular immune function of the body.

It is concluded from the present study that the dietary exposure of pesticide at high and low doses in white leghorn cockerels for 20 weeks. There were

Table 1.

Effect of pesticides on total leucocyte counts (TLC) and absolute lymphocyte count (ALC) of blood in chicken(n=6).

Groups	Dietary levels (ppm)	TLC(Mean±SEM)		ALC(Mean±SEM)	
		10 weeks	20 weeks	10 weeks	20 weeks
Control	0	13.61±0.40	13.75±0.36^{abcde}	6.12±0.147	6.14±0.147
Alphamethrin	20	13.01±0.19	11.45±0.30^{ap}	5.63±0.135	4.4±0.105 ^{abcde}
	40	12.23±0.51	10.16±0.21^{bfq}	4.85±0.116^{a}	3.4±0.081^{ap}
Monocrotophos	10	12.56±0.31	11.56±0.30^{dr}	4.68±0.112^{a}	3.96±0.095^{dr}
	20	11.88±0.42^{a}	10.12±0.23^{eg}	4.75±0.114^{ab}	3.48±0.083^{eg}

Mean bearing common superscripts a, b, c, d, e, f, g, h, i or j ($P<0.01$) and p or q ($P<0.05$) differ significantly when compared vertically.

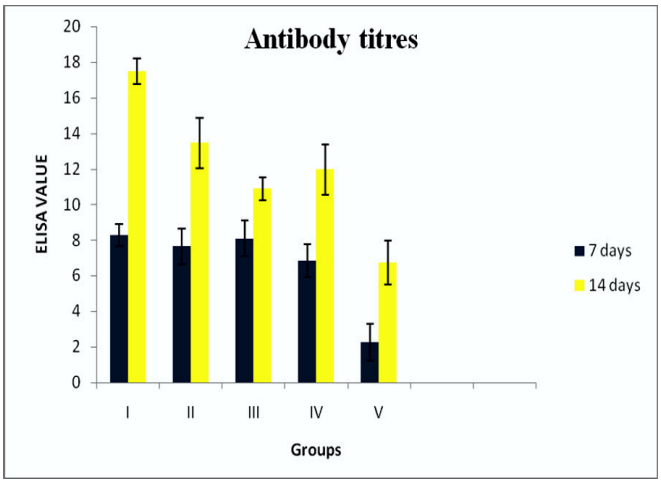

Fig 1:
Effect on antibody titer against bovine serum albumin (BSA) measured by ELISA test in cockerels fed insecticides in diet for 20 weeks (Mean ± SEM; n=6).

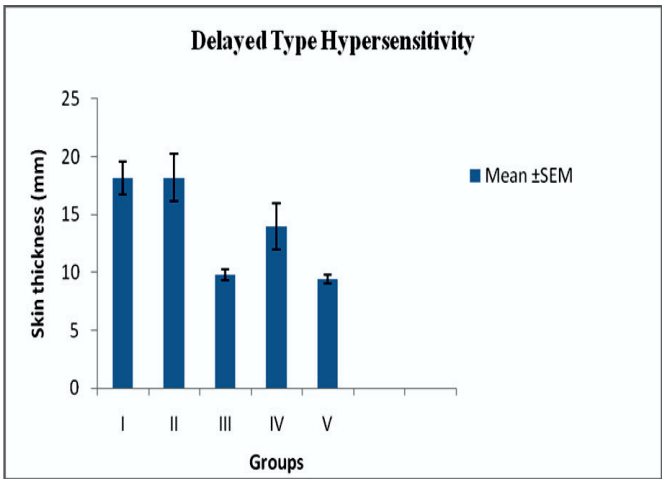

Fig 2.
Effect of chronic oral administration of insecticides to the response of DNFB measured as thickness of skin (mm) in cockerels (Mean ± SEM; n=6).

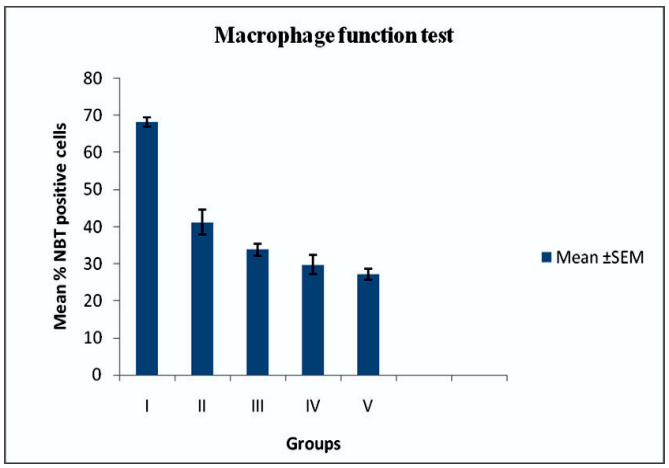

Fig 3.
Effect of insecticides on macrophase function test MFT % in cockerels. Each bar represents mean % NBT positive cells (Mean ± SEM; n=6).

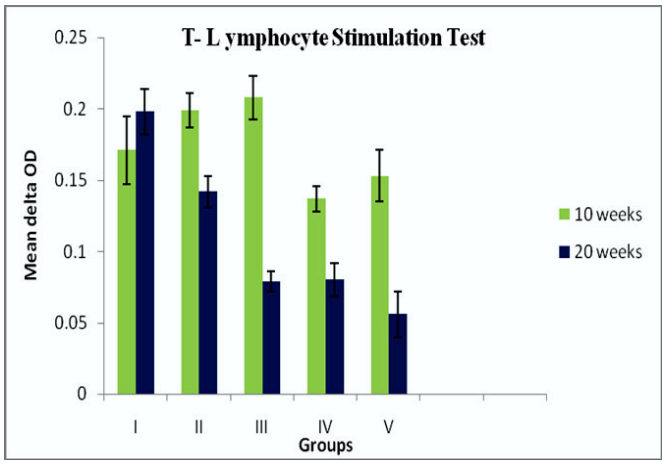

Fig 4.
Effect on lymphocyte stimulation test ("OD) *in vitro* against mitogen Con A in lymphocytes of cockerels fed insecticides in diet for 20 weeks (Mean ± SEM).

significant (P<0.01) reduction of IgG titers, LST, MFT and DTH in all the pesticides treated groups in comparison to control and these reduction indicated that there is immunosuppression in treated groups.

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Short Communication

ANTIPYRETIC EFFECT OF HOT METHANOLIC LEAVES EXTRACT OF *CALOTROPIS GIGANTEA* ON BREWER'S YEAST INDUCED PYREXIA

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ABSTRACT

This investigation was conducted to validate the folklore medicinal claims of use of *Calotropis gigantea* (madar) in fever. The antipyretic activity of the extract was evaluated in lowering the Brewer's yeast-induced pyrexia in comparison to the reference antipyretic aspirin in rats. The per cent reduction in pyrexia among aspirin-treated rats varied between 76.21 and 86.81 per cent. Among the extract-treated rats, the per cent reduction in pyrexia varied between 14 to 17.55 per cent with 100 mg/kg extract, 19.82 to 37.66 with 300 mg/kg extract and 26.66 to 48.66 with 1000 mg/kg extract up to 6 hr of post-treatment. The methanol extract of its leaf powder (100, 300 and 1000 mg/kg, PO) was found to exhibit significant and dose – dependent antipyretic effect in Brewer's yeast induced – pyrexia in Wister rats. However, the extract at 1000 mg/kg oral doses had no effect on the normal body temperature of rats.

Key words: *Calotropis gigantea*, leaves extract, fever, brewer's yeast.

The plant *Calotropis gigantea* (milk weed) belonging to the family Asclepiadaceae locally popular as madar folklore medicine is one of the important indigenous medicinal plants of India. All parts of the plants including the latex have been recognized to possess varied therapeutic potentials, including as a remedy in fever, anthelmintic, antibacterial, corneal opacity, antidiarrhoeal and analgesic properties (Chitme *et al.*, 2005). In view of validating the traditional claims of its use in treating fever the present investigation was undertaken to evaluate the antipyretic activity of *C. gigantea* leaves.

Fresh mature leaves, in bulk, were locally obtained from a single *C. gigantea* plant. The leaves were cleaned and shade-dried under a fan at room temperature. The dried leaves were ground into a fine powder with the help of an electrical grinder. The powder was processed to obtain methanol extract using Soxhlet's extraction.

Antipyretic activity of the methanol extract was determined in male Wister rats against Brewer's yeast induced pyrexia (Loux *et al.*, 1972). Thirty six rats weighing between 130 and 180 gm were randomly assigned to six groups, each comprising of six animals. The Group I rats served as extract control, which were orally administered the extract at the maximum dose level of 1000 mg/kg. The II Group animals served as pyrexia control group, where the rats were administered the pyrogen Brewer's yeast s.c. and at 18 hr thereafter the normal saline. The Group III rats were treated with Brewer's yeast, s.c. and at 18 hr thereafter the reference antipyretic aspirin @ 300mg/kg orally. The Groups IV, V and VI rat were administered with Brewer's yeast s.c. and at 18 hr thereafter the extract at 100, 300 and 1000 mg/kg respectively. Body temperature of each rat was recorded with the help of a property lubricated clinical thermometer by inserting approximately 4 cm into the rectum for 45 sec. immediately before Brewer's yeast s.c. injection, at 18 hr after post Brewer's yeast injection (0 hr) and subsequently at 1, 3 and 6 hr of post drug/ extract administration. The pyrexia was determined from the difference between 0 hr and -18[a] body temperature. Body temperature taken at 0 hr served as the pre- drug pyrexia for each group. The percent inhibition in pyrexia was determined following each treatment at different post-treatment intervals. The results of study were interpreted by using paired t - test (Snedecor and Cochran, 1967).

The observations and results of this experiment are reproduced in Table 1. The mean normal rectal temperature among the rats in all the six groups was statistically similar, which was in the range of 99.35 ± 0.06 to 99.60 ± 0.04 ºF. The rectal temperature in the groups (II to VI) which received the Brewer's yeast was elevated (pyrexia temperature) which ranged between 100.68 ± 0.12 and 101.20 ± 0.06 ºF. The difference in rectal temperature between the respective two intervals in all the groups was significant ($P<0.01$). Subsequent to aspirin administration in Group III the temperature was significantly lowered ($P<0.01$) to 99.98 ± 0.04, 99.86 ± 0.04 and ºF at 1, 3, and 6 hr of post – treatment, respectively. The temperature in Group IV rats, which received the extract at 100 mg/kg at the three post-treatment intervals ranged between 100.57 ± 0.07 to 100.61 ± 0.06 ºF, which was statistically similar to the pyrexia

Table 1.
Antipyretic effect of hot methanolic extract of *Calotropis gigantea* leaves in rats.

Group No.	Treatment	Mean Rectal Temperature (ºF) ± SE				
		Normal	Pyrexia*	Post-Asprin Treatment		
				1 hr	3 hr	6 hr
I	Extract @ 1000 mg/kg	99.45 ± 0.06	99.53± 0.04	99.45 ± 0.03	99.32 ±0.09	99.46 ±0.06
II	Brewer's yeast (BY)	99.60 ± 0.04	101.20 ± 0.06[a]	101.20 ±0.14	101.36 ±0.14	101.18 ± 0.10
III	BY + Aspirin @ 300 mg/kg	99.49 ±0.05	101.20 ± 0.06[a]	99.98 ± 0.04[b](76.21)	99.86 ± 0.04[b](83.00)	99.81 ± 0.03[b](86.81)
IV	Extract @ 100 mg/kg + BY	99.52 ± 0.06	100.80 ± 0.11[a]	100.61 ±0.06(14.00)	100.59 ±0.06(16.03)	100.57 ± 0.07(17.55)
V	Extract @ 300 mg/kg + BY	99.52±0.06	100.68 ± 0.12[a]	100.45 ± 0.10[b](19.82)	100.33 ± 0.11[b](30.17)	100.25 ± 0.11[b](37.06)
VI	Extract @ 1000 mg/kg + BY	99.35 ± 0.06	100.85 ± 0.05[a]	100.45 ± 0.05 [b](26.66)	100.20 ± 0.05[b](43.33)	100.11 ± 0.05[b](48.66)

a : Significantly elevated as compared to normal temperature (P < 0.01). b : Significantly lowered as compared to pyrexic temperature (P < 0.01), * : After 18 hr of injection of Brewer's yeast, Figures in parentheses indicate per cent decrease from pyrexic temperarure
BY: Brewer's yeast.

temperature of 100.80 ± 0.11 ºF. The pyrexic rats in Groups V and VI which received the extract at 300 and 1000 mg/kg respectively, showed the mean temperatures of 100.25 ± 0.11 to 100.45 ± 0.10 ºF and 100.11 ± 0.05 to 100.45 ± 0.05 ºF, respectively, during the post- treatment. The reduction in temperature from that at pyrexic level in both these groups was significant (P< 0.01). Accordingly, the per cent reduction in pyrexia in aspirin treated rats varied between 76.21 and 86.81 in aspirin - treated rats, whereas, the reduction in rectal temperature among the extract treated three groups (IV, V and VI) varied from 14 to 17.55, 19.82 to 37.06 and 26.66 to 48.66 per cent, respectively. The antipyretic effect tended to persist up to or beyond 6 hr of post- treatment at 1000mg/kg dose of the extract similar to aspirin. The pyrexic rats (Group II), which neither received the antipyretic aspirin nor the extract continued to show elevated rectal temperature during the observation period (101.18 ± 0.01 to 101.36 ± 0.14 ºF). Similarly, the Group I rats which received the maximum dose of the extract (1000 mg/kg) continued to show normal temperature, indicating that the methanol extract of C*. gigantea* leaves had no effect on the normal body temperature.

From the results of the present investigation it is evident that the methanol extract of C*. gigantea* leaves did possess dose dependent antipyretic effect.. The antipyretic activity of methanol extract of *C. gigantea* leaves powder is supported by a very recent report (Chitme *et al.,* 2005), who observed antipyretic effect of *C. gigantea* root extracts (at 200 and 400 mg/kg intra-peritoneal doses) against yeast – or typhoid vaccine induced pyrexia in rats and rabbits. The dose dependent effect of ethanolic extract of the flower of *C. procera* on prostaglandin release reported by Mascolo *et al.*, (1998), and they also observed that the plant extract reduced fever in rats by 40 per cent, supports the result of this investigation. The present result of antipyretic efficacy of C. gigantea leaves is also supported by the earlier observations of antipyretic activity of different extracts of *C. procera* experimentally in rats (Larhsini *et al.*, 2002). The ethanolic extract of Calotropis procera plant has been reported (Dewan *et al.*, 2000) to possess antipyretic effect, supports our observation. The traditional use of *Calotropis* plants in fever (Oudhia, 2001) is confirmed by the present observations. It is concluded from this study that methanolic extract of *C. Gigantean* leaf powder in produced antypyretic action in rats.

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Short Communication

OOCYTE RECOVERY AND THEIR QUAILITY IN RELATION TO INFLUENCE OF CORPUS LUTEUM

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ABSTRACT

Experiment was conducted to assess the effect of presence of corpus luteum on ovarian follicular population, oocyte recovery and their quality in buffaloes. A total of 300 ovaries were collected, 165 were bearing corpus luteum (CL) and 135 without CL. A significantly (P<0.05) more number of oocytes were recovered (72.96%) from the ovaries without corpus luteum compared to ovaries bearing CL (67.42%). Usable oocytes (grade A and grade B) could also be recovered from ovaries without corpus luteum (531) as compared to ovaries bearing CL (363).Recovery of usable oocytes per ovary was 3.22±0.1 and 2.69±0.08 from the ovaries without CL and with CL, respectively. Present experiment concluded that presence and absence of corpus luteum (CL) on the ovaries has significant impact on follicular development as well as quality of oocyte recovered, thus this could be use to predetermine the recovery and qualities of oocytes in *invitro* studies using such materials.

Large numbers of good quality oocytes suitable for invitro studies are needed for successful production of buffalo pre-implantation embryos. In the cow and goat ovaries containing corpus luteum (CL), have yielded lower numbers of oocytes than ovaries without CL but such information is scanty in buffaloes. The present study reports the possible impact of CL on recovery of oocytes per ovary and their quality, collected from abattoir.

Buffalo ovaries were collected from local abattoir and transported to laboratory in thermos flask at about 35^{0c}. A total of 300 ovaries were used for this experiment. Out of these ovaries, 165 without CL while 135 were bearing CL.

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The diameter of visible surface follicles was measured, for both type of ovaries, with vernier calipers and the surface follicles were classified into 3 categories i.e. small (1-4 mm), medium (>4-8 mm) and large (>8 mm) according to Abdoon and Kandil (2001). The follicles were aspirated and pooled separately as ovaries were categories in to two; with or corpus luteum. Oocytes collected were graded into different categories (Gupta *et al.,* 2002) under stereo-zoom microscope as follows:

Grade A: Oocytes with 4–5 layers of cumulus cells with homogenous and evenly granular grey ooplasm;

Grade B: Oocytes not having much compaction, with 2–3 layers of cumulus cells surrounding the zona pellucida and having evenly granular ooplasm;

Grade C: Oocytes with 1–2 layers of cumulus cells or partially denuded with irregular dark ooplasm;

Grade D: Oocytes without cumulus cells or with highly expanded or scattered cumulus cells and having irregular dark ooplasm. Recovery and quality of oocytes obtained in different groups were recorded and compared.

Detailed results of present study are given in table 1. It was found that number of follicles differed insignificantly between CL between ovaries with or without CL. The values being 8.89±1.02 and 8.5±1.21 per ovary, when CL was present and absent, respectively. These results are in accordance with Dominguez (1995), who reported that presence of a CL did not affect follicle number between or within cows. In contrast, several researchers have reported that the CL bearing ovary in cows contains more follicles (Pierson and Ginther, 1987). Others also reported that ovaries bearing CL contains less number of follicles than the ovaries, which were not bearing CL (Amer *et al.,*2008; Makwana *et al.,* 2012). This difference may be attributed to the fact that although luteal structures were identified during ovarian examination, their functional status was not confirmed, thus, cows in proestrus contained a regressing CL that may have been defined as a CL if it still showed a relatively large size on the ovarian surface.

In the present study, significantly greater number of oocytes were recovered (72.96%) when corpus luteum was absent as compared to that, when corpus luteum was present on the ovary (67.42%). Similar observations have been made in cattle (Moreno *et al.,* 1993) and in buffaloes (Singh *et al.*, 2001; Ahesh *et al.,* 2014). This is because the follicular development is restricted as, lutein cells occupy the most of the portion of the ovary (Kumar *et al.* (1997). In contrast, Boediono *et al.* (1995). Makwana *et al.* (2012) found no difference between the mean number of the oocytes per ovary between CL bearing and non CL bearing ovaries after aspiration. The present study indicated significantly higher oocyte recovery rate in ovaries, where corpus luteum was absent.

Recovery of usable oocytes per ovary was also significantly higher in ovaries not bearing CL (3.22±0.1) than ovaries bearing CL (2.69±0.08). Similar observations

Table 1.
Effect of presence of corpus luteum on follicular population and oocyte recovery.

S.No.	Attributes		Corpus Luteum absent	Corpus Luteum present
1.	No. of Ovaries		165	135
2.	No. of follicles	Small	1155	1036
		Medium	223	134
		Large	24	18
		TOTAL	1402	1188
3.	No. of follicles per ovaryMean ± SE		8.56±1.21[a]	8.89±1.02[a]
4.	Oocytes recovered	Grade A*(n)	24.34%[a](249)	18.26%[b](146)
		Grade B*(n)	27.57%[a](282)	27.10%[a](217)
		Grade C*(n)	25.7%[b](263)	31.27%[a](254)
		Grade D*(n)	22.39%[a](229)	22.97%[a](184)
		Total	1023	801
		Oocyte recovery (%)	72.96[a]	67.42[b]
5.	Total usable oocytes (grade A + grade B) recovered		531	363
6.	Usable oocytes (grade A + grade B) recovered/ ovary Mean ± SE		3.22±0.1[a]	2.69±0.08[b]

The values bearing different superscripts in the row differ significantly (P<0.05). *Per cent values calculated out of recovered oocytes.

have been made by Moreno *et al.* (1993) and Das *et al.* (1996). This could be explained on the basis of the fact that, the CL may act on the follicles to alter their growth rate to result in atresia (Rexroad and Casida, 1975) and hence poor number and quality of oocytes.

On the basis of our results, it may be concluded that the recovery of per cent total oocyte, total usable oocytes (grade A + grade B) and usable oocytes per ovary were significantly higher in absence of corpus luteum. However, number of follicles per ovary was not affected by corpus luteum.

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Short Communication

ANTIMICROBIAL ACTIVITY OF SOME NEWLY SYNTHESIZED BENZODIAZEPINES

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ABSTRACT

Benzodiazepine compounds are used as a important drug in a pharma chemistry. This study was conducted to evaluate the antibacterial activity of some newly synthesized 1, 5 benzodiazepine derivatives against *Gordonia terrae* and *Bacillus cereus* while *Acinetobactor junii* and *Pseudomonas aeruginosa.* Marked activity has been observed to mostof the benzodiazepine derivatives against *Gordonia terrae* and *Bacillus cereus.* It is concluded from this study that benzodiazepine may also be explored for antimicrobial therapy.

Key Words: Benzodiazepines, antibacterial activity, *Gordonia terrae* and *B. cereus*

Benzodiazepines have been widely used as anticonvulsant, antianxiety, analgesic, sedative, hypnotic and anti-inflammatory agents (Sternback, 1971). In the last decade, the area of biological interest of 1, 5-benzodiazepines has been extended to several diseases such cardiovascular disorders (Di Braccio *et al*., 2001). In particular, 1, 5-benzodiazepines are useful precursors for the synthesis of fused ring benzodiazepine derivatives such as triazolo, oxadiazolo, oxazino, and furano benzodiazepines (Atwal et al., 1987). Due to their wide range of applications, these compounds have received a great deal of attention in connection with their synthesis. Despite their importance from a pharmacological, industrial and synthetic point of view, comparatively few methods for their preparation are reported in the literature. The present study, therefore, was undertaken to synthesize and evaluate the antibacterial activity of some newly synthesized derivatives of 1,5-benzodiazepines.

All the fourmicro-organisms bacterial strains *Bacillus cereus, Pseudomonas aeruginosa, Gordonia terrae* and *Acinetobactor junii* were obtained from the Depatment of Microbiology, CBSH, G.B. Pant university of Agriculture and Technology, Pantnagar, India. Chloramphenicol, Ampicillin, Streptomycin were taken as a standard antibiotics. Nutrient agar (NA) and nutrient broth (NB) were obtained from Hi Media Ltd, Mumbai and stored at 4^0C.

For the preparation of different media, NA and NB were weighed and poured in the distilled water. After proper plugging it was autoclaved at 120^0C at 15-20 lbs pressure for 20 minutes. To prepare agar palates, autoclaved nutrient agar cooled to 45^0C was poured (around 20 ml) into each Petri plate sterlized in laminar flow and kept undisturbed as such till it solidified. After solidification of agar medium, these Petri plates were incubated at 30^0C overnight for sterile testing.

Pure cultures of test bacteria were prepared by emulsifying 5 colonies in 5 mL of sterilized nutrient broth. Tubes with nutrient broth and inoculated bacterial cultures were incubated overnight at 30^0C. Next day cultures showing marked turbidity in the tubes were used.

Disc diffusion method was used (NCCL, 19197) to evaluate the antimicrobial activity**.** Bacterial suspension of 0.1 mL of (10 times diluted) was added to the previously prepared nutrient agar plates and bacterial strain was thoroughly spread on the agar surface, using bent rod. The sterilized Whatman filter paper No. 1 disc (5mm in diameter) was thoroughly soaked with the solution of bdap compounds (50 µL of 30 ppm solution) and placed in the inoculated plates.

The plates were incubated at 30^0C. After incubation, relative susceptibility of each organism was determined by a clear zone of inhibition around the disc impregnated with the compounds as well as the antibiotics. Zone of inhibition (mm) was measured with the help of scale.

Antibacterial screening of all the synthetic compounds was carried out by disc diffusion method (NCCLS, 1997). The results were compared with standard antibiotic streptomycin, chloramphenicol and ampicillin (Table1). Marked activity has been observed to mostly benzodiazepine derivatives against *Gordonia terrae* and *Bacillus cereus* while *Acinetobactor junii* and *Pseudomonas aeruginosa* no significant activity has been recorded. Bdap10 has shown good activity against all bacterial strain. All the compounds except bdap5 and bdap7 exhibited marked activity against *Gordonia terrae.* The compounds bdap1, bdap7, bdap8, bdap9, bdap10 and bdap11 exhibited good activity against *Bacillus Cereus* while compounds bdap2, bdap3, bdap4, bdap5 and bdap6 did not exihibit activity against this becteria. Against *acinetobactor junii,* the compounds bdap1, bdap3, bdap4, bdap5 and bdap10 exhibited good activity while against *Pseudomonas aeruginosa* only bdap8 and bdap10 exhibited good activity. Benzodiazepines are widely used

Table1:
Antimicrobial activities of benzodiazepine derivatives against standard microorganism.

Compounds (30ppm)	Standard Bacterial Strains			
	Bacillus Cereus	*Pseudomonas aeruginosa*	*Gordonia terrae*	*Acinetobactor junii*
Streptomycin	+	+	+	+
Ampicillin	-	-	+	+
Chloramphenicol	+	-	+	+
bdap1	+	-	+	+
bdap2	-	-	+	-
bdap3	-	-	+	+
bdap4	-	-	+	+
bdap5	-	-	-	+
bdap6	-	-	+	-
bdap7	+	-	-	-
bdap8	+	+	+	-
bdap9	+	-	+	-
bdap10	+	+	+	+

+ indicates the inhibitory action wherea – means no inhibitory action.
badp1=[Z] methyl 2, [2,3-dihydro- 2-phenyl -1-H-benzo[b][1,4]-diazepin-4-[5H]-ylidene] acetate;
badp2=[Z]methyl2,[2,3-dihydro-2-[2-nitrophenyl]-1-H-benzo[b][1,4]-diazepin-4-[5H]-ylidene]acetate; badp3=[Z]methyl2,[2,3-dihydro-2-[2-nitrophenyl]-1-H-benzo[b][1,4]-diazepin-4-[5H]-ylidene]acetate; badp4=[Z]methyl,[2,3-dihydro-2-[4-nitrophenyl]-1-H-benzo[b][1,4]-diazepin-4-[5H] yidene]acetate; badp5=[Z]methyl2,[2,3-dihydro-2-[2-hydroxyphenyl]-1-H-benzo[b][1,4]-diazepin-4-[5H]-ylidene]acetate; badp6=[Z]methyl2-[2,3-dihydro-2-[3-hydroxyphenyl]-1-H-benzo[b][1,4]-diazepin-4-[5H]-ylidene] acetate; badp7=[Z]methyl2, [2,3-dihydro-2-[4-methoxyphenyl] -1-H-benzo[b][1,4]-diazepin-4-[5H]-ylidene] acetate; badp8=[Z]methyl 2, [2-[2-chlorophenyl]2,,3-dihydro—1-H-benzo[b][1,4]-diazepin-4-[5H]-ylidene]acetate, badp9=[Z]benzyl,2-[2,3-dihydro-2-phenyl-1-H-benzo [b][1,4]-diazepin-4-[5H]-ylidene] acetate; badp10=[Z]benzyl 2,[2,3-dihydro-[4-methoxyphenyl]-1-H-benzo[b][1,4]-diazepin-4-[5H]-ylidene] acetate

anticonvulsant, antianxiety, analgesic, sedative, hypnotic and anti-inflammatory agents (Brunton *et a*l., 2008). The activity of the compounds could not be correlated with the structure of the compounds or the electronic effects of the substituents on the benzene ring of benzaldehyde. Finding of this study suggests that benzodiazepine may also be explored for antimicrobial therapy.

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