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Review Article

THERAPEUTIC APPLICATIONS OF RIBOZYMES

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ABSTRACT

RNA has been viewed as a passive molecule that only carried information or provided structure to RNA-protein complexes; however, the discovery of ribozymes by Thomas R. Cech in 1982, has changed this view of the function of RNA in chemistry and biology. Ribozymes catalyze highly sequence-specific reactions determined by RNA–RNA interactions between the ribozyme and its substrate RNA molecules. RNA targets for ribozyme-based therapeutics which may encode oncoproteins, growth factors, proinflammatory cytokines and their corresponding cell-surface receptors and signal transduction molecules, viral and microbial mRNAs or genomic RNAs. These RNA targets are also readily cleaved by this approach. These novel activities of RNA now permit the development of enzymatic RNA molecules as therapeutic agents that can suppress the expression of deleterious proteins by catalyzing the trans-cleavage of the corresponding mRNAs or repair of the mutant cellular RNAs by catalyzing RNA trans-splicing. Ribozymes are expected to have excellent therapeutic potential for the treatment of infectious, cancerous, vascular and muscular diseases in man and animals but still there is a need to dedicate more effort in this newer area of therapeutics so that the effect of ribozymes is more efficient and specific.

Key words: Application, cytokines, oncoproteins, ribozymes, RNA, mRNA

INTRODUCTION

Ribozymes are catalytic RNA molecules that can cleave target RNA in a sequence-specific manner. RNA had been viewed as a passive molecule that only carried information or provided structure to RNA-protein complexes. But the discovery of ribozymes has changed our view of the function of RNA in chemistry and biology. It is now clear that RNA can act as an enzyme and is capable of catalyzing several essential biological processes such as RNA splicing, RNA processing, replication of RNA genomes, peptide bond formation during translation and catalyze the cleavage or ligation of the RNA phosphodiester backbone. In contrast to other known ribonucleases, ribozymes catalyze highly sequence-specific reactions determined by RNA–RNA interactions between the ribozyme and its substrate RNA molecules. These specific properties of a ribozyme are determined by two functional units. The first unit consists of complementary flanking sequences that bind to the substrate RNA by base-pairing interactions and provide specific recognition of the substrate. The second unit is represented by the ribozyme's catalytic core which mediates site-specific strand scission.

RNA targets for ribozyme-based therapeutics may encode oncoproteins, growth factors, proinflammatory cytokines and their corresponding cell-surface receptors and signal transduction molecules, viral and microbial mRNAs or genomic RNAs. These RNA targets are also readily cleaved by this approach. These novel activities of RNA now permit the development of enzymatic RNA molecules as therapeutic agents that can suppress the expression of deleterious proteins by catalyzing the *trans*-cleavage of the corresponding mRNAs or repair of the mutant cellular RNAs by catalyzing RNA *trans*-splicing.

HISTORICAL DEVELOPMENT

The ribozymes were discovered by Thomas R. Cech in 1982 who was studying RNA splicing in the ciliated protozoan *Tetrahymena thermophila* and Sidney Altman in 1983 who was working on the bacterial RNase P complex. In 1989, Thomas R. Cech and Sidney Altman won the Nobel Prize in chemistry for their "discovery of catalytic properties of RNA." The term ribozyme was first introduced by Kelly (Kruger *et al.,* 1982).

CLASSIFICATION

Ribozymes are broadly grouped into two classes based on their size and reaction mechanisms:

1) **Large ribozymes :** This group includes the self-splicing group I and group II introns as well as the RNA component of RNase P. These ribozymes consist of several hundreds to 3000 nucleotides and they generate reaction products with a free 3'-hydroxyl and 5'-phosphate group.
2) **Small ribozymes**: This group includes the hammerhead, hairpin, hepatitis delta ribozymes and varkud satellite (VS) RNA as well as artificially selected nucleic acids. These ribozymes are 30 to 150 nucleotides in length and generate products with a 2'-3'-cyclic phosphate and a 5'-hydroxyl group.

DESIGNING OF RIBOZYMES

Naturally occurring ribozymes exist in *cis* form, means they are able to cleave only intramolecular sequences in their natural context. The catalytic center of these naturally self-cleaving ribozymes has been identified

and engineered to develop agents that catalyze intermolecular reactions i.e., ribozymes that cleave external RNA molecules . Many *cis* ribozymes have been modified for intermolecular cleavage and these are known as *trans* ribozymes. These *trans* ribozymes behave like true enzymes i.e. they do not change during the reaction and one ribozyme molecule can process several substrate molecules. *Trans*-acting ribozymes can be engineered to cleave a defined sequence and making possible the inactivation of practically any RNA.

The *cis* form of excised intron can be converted into a *trans* form through *in vitro* RNA selection method. In this method a pool of RNA molecules is subjected to a selection pressure for a desired phenotype. The active molecules are then recovered and amplified. This will yield a new pool with the same molecular organization as the original but showing much reduced heterogeneity. This new RNA *in vitro* is then subjected to new rounds of selection, thus enriching the population with the active variants. Starting from a library of many diverse molecules, the fittest ones for a certain function are selected, amplified, mutagenised and re-selected until the given task is fulfilled with perfection.

PHARMACOKINETICS (PK) OF RIBOZYME

The pharmacokinetic fate of a substance exogenously delivered into an organism results from characteristics like its stability in blood stream and tissues and its interaction with plasma proteins. Synthetic ribozymes if remains unprotected as RNA molecules are almost instantaneously degraded by 3'-exonucleases and pyrimidine-specific endonucleases in serum. Therefore, to stabilise ribozymes, modifications should be done at phosphodiester linkage and the 2'OH group of the ribose.

DELIVERY OF RIBOZYMES

There are two approaches to deliver ribozymes to the target cells for the successful inhibition of gene expression.

a) Exogenous delivery

In exogenous delivery pre-synthesized ribozymes can be administered to an animal or delivered directly into cells. Since ribozymes are charged molecules that cannot cross hydrophobic membranes, ribozymes must be complexed with positively charged phospholipids to enhance their uptake. Thus, cationic lipid reagents assemble into oligonucleotide-containing liposomes which is t adsorbed to cell membranes and subsequently taken up by endocytosis. To facilitate the subsequent release of the nucleic acid from endosomes or lysosomes, helper lipids are often added that interfere with endosomal membranes. Other macromolecular systems for exogenous delivery include highly branched carbohydrates called dendrimers and biodegradable polylactides and lactic acid/glycolic acid copolymers which help in sustained release of the ribozyme over a prolonged period of about one month.

b) Endogenous delivery

Endogenous delivery provides the gene encoding the ribozymes as a part of vector to the cell where transcription generates the ribozymes. These vectors for delivery of ribozyme to the target cell may be bacterium or virus.

i) Delivery using a viral vector

Delivery using a viral vector is known as *transduction*. Many different viruses have been adapted as gene-delivery vectors. The exogenous genetic material or transgene is either added to the complete viral genome or used to replace one or more viral genes. The transgene is therefore delivered as part of a recombinant viral genome, exploiting the virus's natural ability to infect and transfer nucleic acid into animal cells. The preferred viral vectors for *in vivo* delivery of ribozymes are :

1. Retroviral vectors, 2. Adenoviral vectors and 3. Adenoassociated viral vectors

Retroviral vectors are most commonly utilized viral vector because they have high transduction efficiency and they stably integrate the gene into the host cell genome. They have been widely used to deliver ribozymes for the inhibition of expression of genes linked to cancer and viral diseases. Retroviral vectors and Adenoassociated viral vectors are suitable vectors when long term expression of therapeutic gene is preferred. On the other hand adenovirus is suitable vector if transient expression of a therapeutic gene is desired.

ii) Delivery using a bacterial vector

Bacterial vectors, a recent development, relies on bacteria which also invade animal cells. In this case the transgene is delivered on bacterial plasmid called *bactofection*. Invasive bacteria, such as *Salmonella* are capable of transferring genetic material to host cells, leading to efficient expression of the transferred genes.

MECHANISM OF ACTION OF RIBOZYMES

Ribozymes can exert their action by following two mechanisms: 1) catalyzing the *trans*-cleavage of the corresponding mRNAs and 2) repairing the mutant cellular RNAs by catalyzing RNA *trans*-splicing.

1) Property of small ribozymes to carry out cleavage reactions is determined by two functional units. The first unit consists of complementary flanking sequences that bind to the substrate RNA by base-pairing interactions and provide specific recognition of the substrate. The second unit is represented by the ribozyme's catalytic core which mediates site-specific strand scission. Hammer-head ribozyme cleaves the target RNA primarily at the N U H triplet (N is any nucleotide and H is any nucleotide except guanosine) with AUC and GUC sequences being the most effective processing sites (Kore *et al.,* 1998). Another ribozyme often used in therapeutic studies is the hairpin ribozyme. The hairpin ribozyme cleaves the target RNA at the N*GUC sequence (N is any nucleotide). RNA degradation by ribozymes is a 3-step

process: Firstly, Substrate recognition and binding of ribozyme to a complementary sequence by forming classical Watson-Crick base-pairing between the ribozyme's binding-arm sequences and sequences that flank the cleavage site of the target RNA. Secondly, cleavage of substrate RNA at a specific site which involves attack of the 22 -OH that is 52 to the scissile bond in the target, thus destabilizing the target RNA's phosphate backbone, and finally, after cleavage of substrate RNA, the resultant products dissociate from the ribozyme complex and the ribozyme is released and may bind and cleave other targets again. The cleavage event renders the mRNA untranslatable and leads to further degradation of the target by cellular ribonucleases (Schubert and Kurreck, 2004). The ribozyme sequences involved in substrate recognition are not involved in the catalytic step of the reaction. The specificity, ribozymes can easily be changed to target any mRNA of interest.

2) RNA splicing is an essential process in the expression of many genes. In most cases of eukaryotic gene, the coding sequences is interrupted by noncoding sequences. Coding sequences are and non-coding sequences are called introns. Before translation, the introns of pre-RNA must be removed, and this process is called RNA splicing. It consists of the precise excision of the intron and the covalent linkage of the boundary exons. A large number of introns belonging to group I or group II have been shown to catalyze their own splicing *in vitro*. Group I introns carry out two transesterification reactions in order to excise themselves from a precursor transcript through self-splicing reaction pathway (Cech.1993). The 52 splice site is located in helix P1, which is formed by base-pairing between the exon sequence and the internal guide sequence (IGS) of the intron. The 52 splice site, adjacent to a G-U pair in P1, is cleaved using exogenous guanosine in the first step of splicing. In the second step of splicing, the final nucleotide of the intron, also a guanosine, is positioned so that the 32 exon may be ligated to the 52 exon through the reverse of the first chemical step. Pairing between the 52 end of the intron and the 32 exon form P10, which has been implicated in facilitating 32 splice site selection during the second step of splicing.

The IGS of the *Tetrahymena* ribozyme can be modified to base pair with essentially any sequence as long as a G-U wobble pair is maintained at the splice site. If sequences are present downstream of the ribozyme, the ribozyme ligates this portion of the RNA transcript to the cleavage site of the substrate RNA. These 32 exon sequences may be changed to potentially any nucleotide sequence. With the ability to "cut and paste" RNA sequences together, *trans*-splicing ribozymes were quickly recognized to have therapeutic potential in the revision of mutant RNAs.

To use the activity of *trans*-splicing ribozymes to treat genetic disorders, a ribozyme IGS may be modified to base pair with a mutant messenger RNA upstream of the mutation in the target transcript. Therapeutic sequences are incorporated into the ribozyme transcript as a "32 exon" that will be ligated to the cleaved target in the second step of splicing. Because the ribozyme modifies the RNA transcript posttranscriptionally, the natural transcriptional regulation of the gene is maintained. A Group I ribozyme delivers corrective sequences (32 exon) to a mutant transcript. The ribozyme binds upstream of the mutation through base-pairing. Once bound, the ribozyme cleaves the target RNA, releases the downstream cleavage product, and splices the 32 exon sequence onto the upstream cleavage product.

APPLICATIONS OF RIBOZYMES

Ribozymes are expected to have excellent therapeutic applications in human as well as in animals therapy as drugs with a high specificity for their molecular target mRNAs. Ribozymes have wide range of successful therapeutic potential which are being tested in the clinical trials. Through the continuous ongoing researches and experiments, the efficacy of ribozymes therapy has already been recorded by the leading scientists, among which following are the successful achievements:

Viral diseases

Viruses are obligate intracellular parasites carrying either RNA or DNA based genome. Infection with viral pathogens presents an excellent target for therapeutic applications of ribozymes. Riibozymes can block the viral life cycle by 1) blocking the virus entry to the cell 2) inhibiting reverse transcription and proviral integration 3) interfering with viral gene transcription and 4) inhibiting viral -mRNA post-transcriptional modifications or nuclear export. Ribozymes can inhibit viral gene expression to disrupt viral replication and thereby causing a slowing or complete cessation of the viral life cycle. Ribozymes have been targeted against genomes and transcripts of Bovine leukemia virus.

Bovine leukemia virus (BLV)

Bovine leukemia virus (BLV), a retrovirus which causes persistent lymphocytosis and B-lymphocyte lymphoma in cattle and sheep. After initial infection Bovine leukemia virus (BLV) encodes at least two regulatory proteins, Rex and Tax. Tax, the transactivating protein, stimulates the long terminal repeat to promote viral transcription and may be involved in tumorigenesis. Rex is involved in the transition from early expression of regulatory proteins to later expression of viral structural proteins. The ribozymes consist of the hammerhead catalytic motif flanked by antisense sequences that hybridize with the complementary rex/tax mRNA. To evaluate cleavage in a cell-free system, they transcribed portions of rex/tax mRNA and incubated them with synthetic RNA ribozymes. A ribozyme was identified that cleaves >80% of the target RNA. Synthetic DNA encoding this

ribozyme was cloned into the expression vector pRc/RSV and transfected into BLV-infected bat lung cells. Intracellular cleavage of rex/tax mRNA was confirmed by reverse transcriptase PCR. In cells expressing the ribozyme, viral expression was markedly inhibited. Ribozyme inhibition of BLV expression suggests that cattle expressing these sequences may be able to control BLV replication.

Parasitic diseases

Giardia, a most primitive eukaryote, infects several species of animals and it is a major agent of waterborne outbreak of diarrhoea in animals and men. The RNA virus-based transfection system has been developed and used for the genetic manipulation of *Giardia*. In *Giardia* Krr1 protein is responsible for ribosome biosynthesis. So in this study, cDNA encoding hammerhead ribozyme flanked with various lengths of antisense, Krr1 RNA were cloned into a viral vector pGCV634/GFP/GCV2174 derived from the genome of *Giardia canis* virus (GCV). RNA transcripts of the plasmids showed high cleavage activities on Krr1 mRNA in vitro. They were electroporated into GCV-infected *G. canis* trophozoites and Krr1 mRNA level was decreased by 72% with the ribozyme KRzS and 86% with the ribozyme KRzL, while the control ribozyme TRzS showed no effect on the level of Krr1 mRNA. The two hammerhead ribozyme transfected cells grew slowly, their internal structures got blurred and the cells were deformed. These results indicated that GCV could be useful tool for gene manipulation of *G. canis*.

In cancer

A key role in carcinogenesis is played by the change in the expression levels of certain genes i.e. oncogens that are the transcription factors and components of the signal transduction machinery of the cell and their anomalous expression leads to the defective regulation of cell proliferation, apoptosis, differentiation, and invasion. Following genes are targeted by ribozymes, which causes the target mRNA molecule to be destroyed or blocks its translation:

i) EGFR gene in Glioblastoma

The epidermal growth factor receptor (HER1/EGFR) is a 170 kDa single-chain transmembrane glycoprotein that belongs to the HER family of receptors. It consists of three functional domains, *i.e.*, an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase (TK) domain. It's most common ligands are the epidermal growth factor (EGF) and transforming growth factor-a (TGF-a). Once a ligand binds to the extracellular ligand-binding site of HER1/EGFR, activation of the intracellular TK occurs which triggers downstream signaling via the ras-raf-mitogen activated protein kinase (MAPK) or the phosphatidylinositol 3-kinase (PI3-K)/Akt pathways. Subsequently, diverse cellular functions are regulated including cellular proliferation and differentiation. HER1/EGFR was found to be disregulated in various cancers including high-grade glioma. About 50% of glioblastomas were found to overexpress HER1/EGFR (Karpel-Massler *et al.,* 2009).

The gene that encodes HER1/EGFR is located on the short arm of chromosome 7 (p11-p13). It consists of 26 exons and is 110 kb in size. Several mutations of the HER1/EGFR gene have been reported. The most frequent mutant form, arising from rearrangements, is EGFR variant III (EGFRvIII; alternatively termed DEGFR), which accounts for approximately 60% of all HER1/EGFR gene aberrations. Elimination of a DNA fragment containing exons 2-7 of the gene results in an inframe deletion of 801 bp (nucleotide positions 275-1075) of the coding sequence of the extracellular receptor domain and subsequent removal of NH2-terminal amino acid residues 6 to 273 from the extracellular domain of the intact wild-type HER1/EGFR. At the fusion junction, a novel glycine residue is created at position 6 in-between the former amino acid residues 5 and 274. EGFRvIII is characterized by constitutive (ligand-independent) activation and defective receptor down-regulation due to low rates of receptor endocytosis. Moreover, expression of EGFRvIII has been shown *in vivo* to be related to increased proliferation, tumor formation and inhibition of apoptosis, affording enhanced tumorigenicity. These aspects and the additional fact that EGFRvIII is overexpressed by up to 58% of glioblastomas, but not expressed by normal tissues, renders the mutant receptor an interesting target for the treatment of glioblastoma multiforme. The fact that as a result of the deletion mutation, the fusion junction of the mutant gene is created directly upstream of a GTA triplet which is subsequently transcribed into a ribozyme target codon (GUA), gave rise to the development of a ribozyme-mediated therapeutic approach.

ii) Telomerase reverse transcriptase (TERT)

Telomerase is a ribonucleoprotein enzyme that maintains the protective structures of telomeres of eukaryotic chromosomes, thereby extending the life span of replicating cells. Most normal somatic tissues possess undetectable activity of the telomerase. In contrast, the telomerase activity is highly upregulated in a vast majority of cancers and the activity is mainly modulated by TERT expression. Moreover, telomerase upregulation could actively contribute to tumor growth. Therefore, telomerase is an attractive target for the development of anticancer therapeutic ligands.

iii) Pleiotropin (PTN)

Secretary growth factor Pleiotropin (PTN) is developmentally regulated cytokine which is produced in large quantities during the development of the nervous system and "turned off" in adults but it is markedly upregulated in various tumours and tumour cell lines. PTN is an active mitogen for fibroblasts and epithelial cells. Also, it can induce the release of active proteolytic enzymes from endothelial cells and play a crucial role in

angiogenesis. In order to suppress the expression of *PTN* in tumor cells, a ribozyme complementary to the *PTN* mRNA was constructed. It showed considerable antitumor and antimetastatic activity (Czubayko *et al.,* 1996). In order to be effective, the ribozyme was chemically modified in order to protect it from degradation by ubiquitous ribonucleases. In an *in-vivo* experiment the synthetic ribozymes (anti-PTN ribozymes) were injected systemically into mice induced with melanoma (skin cancer) showed that anti-PTN ribozymes caused more than 65% retardation of melanoma growth in mice & inhibited carcinogenesis by 70-85%. This is possible only because the ptn mRNA was cleaved and degraded (Malerczyk *et al.,* 2005). In this way Ribozyme causes retardation of tumor growth or tumor regression and twofold increase in mean survival time for mice (Powers *et al.,* 2002).

Retinitis pigmentosa

Retinitis pigmentosa (RP) describes a heterogeneous group of inherited retinal dystrophies characterized by progressive photoreceptor cell degeneration. It may be inherited as an autosomal dominant, autosomal recessive, X-linked recessive etc. Autosomal-dominant form of retinitis pigmentosa is often caused by gain of function mutation i.e. missence or nonsence mutation in the rod opsin gene that is then transmitted into nonfunctional and nontoxic protein. The effective therapy for autosomal dominant RP should be based on either to prevent the mutation protein from being produced or counter the expression of the protein. Ribozymes catalyze enzymatic reactions that break down RNA. Conceptually it would therefore, be possible to use ribozymes to treat autosomal dominant RP by blocking gene product from the mutant allele thereby halting or slowing the progression of the disease.

Scientists used ribozymes that can cleave a target RNA (mutant rhodopsin messenger RNA) with high specificity. In their approach they used recombinant Adeno-Associated Virus (AAV) to transduce photoreceptor cell of rhodopsin mutent (pro 23 his) in pigs with ribozyme and an opsin promoter. This ribozymes is able to distinguish between the normal and the mutant rod opsin mRNA which is then cleaved and is not able to be translated. The virus is injected under the retina and the ribozyme is transcribed under the control of the rod opsin promoter which increases the specificity of the treatment. They demonstrated quite good results in one pig and several rat models. They showed that a single injection has restored up to 80% of the rod photoreceptors compared to the contra-lateral (control) eye. The effect has not decreased in over two years. This AAV-ribozyme-based strategy holds great promise for the therapy of retinitis pigmentosa, as well as other dominantly inherited diseases.

Muscle diseases

Ribozymes have also been widely used towards the better understanding and therapy of various skeletal and smooth muscle disorders like myotonic dystrophy, myotonia congenita and neuromuscular junction abnormalities. Scientists introduced various strategies for the study or therapy of Muscle diseases by using ribozymes.

i) Myotonic dystrophy

Myotonic Dystrophy type 1 (DM1) is a degenerative neuromuscular disease characterized by a large CTG repeat expansion situated in the 3' UTR (Untranslated Region) of the DMPK gene. The mutant DMPK 3' UTR RNA containing the CUG expansion accumulate to form RNA foci in the nucleus of DM1 cells (Brook *et al.,* 1992). These RNA foci interact with nuclear RNA binding proteins preventing their export from the nucleus (Timchenko *et al.,* 1992). Most of the disease pathomechanisms involve mutant RNA foci and their interactions to various binding proteins and therefore prompted researchers to create specific ribozymes for the destruction of the mutant RNA. Langlois *et al.* (2003) identified most of the accessible ribozyme target sites in the 3' UTR of the DMPK mRNA and designed a hammerhead ribozyme to cut the most accessible site. The use of these hammerhead ribozyme significantly reduced the number of mutant DMPK mRNA-containing nuclear foci in human DM1 myoblasts.

ii) Myotonia congenita

Myotonia Congenita is an inherited disorder. In canines mutation of wild type canine skeletal muscle chloride channel (cClC-1) mRNA leads to the development of mutated form of canine skeletal muscle chloride channel (cClC-1) mRNA and thereby development of the mutant canine skeletal muscle chloride channel (cClC-1) mRNA transcript that causes myotonia congenital. These changes prompted scientists to investigate the feasibility of RNA repair using specific ribozymes. Rogers *et al.* (2002) designed a modified Tetrahymena ribozyme to mediate *trans*-splicing repair of the mutant canine skeletal muscle chloride channel (cClC-1) mRNA transcripts. The ribozyme was able to target the mutant mRNA and replace the mutant containing 3' portion by trans-splicing the corresponding wild type sequence. Furthermore, when the chloride channel function was examined in single cells, a wide range of electrophysiological activity was observed, with 18% of cells exhibiting significant functional restoration and some cells exhibiting complete rescue of the biophysical phenotype.

iii) Myasthenic syndrome

The muscle acetylcholine receptor (AChR) is expressed at the neuromuscular junction and plays the principal role in nerve to muscle signal transmission. A number of mutations have been characterized in the AChR -subunit gene which affect receptor function and give rise to slow channel congenital myasthenic syndrome (Marques, *et al.,* 2005). Abdelgany *et al.* (2005) designed hammerhead ribozymes in order to target RNA transcripts

from four different slow channel congenital myasthenic syndrome mutations. These hammerhead ribozymes were able to efficiently discriminate between mutant and wild type RNA transcripts that differ only by a single nucleotide substitution.

Vascular disease

The vascular smooth muscle cell in mature animals is a highly specialized cell whose principal function is contraction and regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution. However, abnormal environmental signals can lead to adverse phenotypic switching and acquisition of characteristics of smooth muscle cell that can contribute to the development and/or progression of vascular disease (Oostrom *et al.,* 2009). Several attempts have been made by scientists that demonstrate the application and usefulness of ribozymes in smooth muscle cell for the correction of vascular diseases.

i) Hypertension and atherosclerosis

Angiotensin II (Ang II) plays an important role in the development of hypertension and atherosclerosis by inducing vascular smooth muscle cell growth and synthesis of aldosterone. Activation of Leukocyte-type 12-lipoxygenase (12-LO) has been proposed to be an important mechanism for AngII by inducing hypertrophy of vascular smooth muscle cells. This finding prompted scientists to design a chimeric RNA hammerhead ribozyme against the first GUC sequence at nucleotide 7 of porcine leukocyte 12-LO mRNA. The ribozyme was transfected into porcine aortic vascular smooth muscle cells, causing a significant decrease of endogenous porcine leukocyte-type 12-LO mRNA and protein levels. Downregulation of 12-LO levels have the potential to protect vascular smooth muscle cells from hypertrophy (Sasson *et al.,* 2006). The results from this study indicated the feasibility of using new ribozyme technology to study the specific effects of a gene pathway in vascular disease and the potential therapies.

ii) Post-operative occlusion of arteries, or restenosis in coronary arteries

Coronary angioplasty is an effective procedure used in order to open occluded vessels. In spite of a number of technical improvements in the procedure, post-operative occlusion of arteries or restenosis still occurs due to proliferation of injured smooth muscle cells. By preventing the injury-induced activation and proliferation of medial smooth muscle cells after angioplasty, intiminal thickening and restenosis could be prevented (Forrester *et al.,* 1991). In an attempt to inhibit smooth muscle cell proliferation, Jarvis *et al. (1996).* described the activity of several ribozymes targeting c-myb mRNA. Hammerhead ribozymes were capable of cleaving c-myb RNA and as a result inhibit smooth muscle cell proliferation.

Arthritic diseases

Arthritis is an inflammation of one or more joints which results in pain, swelling, stiffness and limited movement. The key mediator of arthritic diseases is matrix metalloproteinase stromelysin. In an *in vivo* study radioactively labeled ribozymes targeting the matrix metalloproteinase stromelysin were employed through intraarticuler route in mice (Flory *et al.,* 1996). Following intraarticular administration radioactively labeled ribozymes were taken up by cells in the synovial lining. The chemically modified ribozymes were stable in the synovium and reduced the interlukin 1-á-induced stromelysin expression and reduced the cartilage invasion by rheumatoid arthritis synovial fibroblasts. This study demonstrate that intracellular expression of ribozymes constitutes a feasible tool to inhibit the production of matrix-degrading enzymes that are involved in arthritic degeneration.

ADVANTAGES OF RIBOZYME THERAPY

Ribozymes as a evolutionary drugs have several advantages over traditional medicines like,

(i) They have very high target selectivity because most ribozymes recognize their substrates through a series of Watson-Crick base pairs, their binding sequences can be modified to target almost any RNA molecule.
(ii) They exhibit turnover (i.e., one ribozyme may successively cleave several substrate molecules) hence they are effective at a low dose.
(iii) They are effective against many untreatable diseases like cancer, viral, genetically inherited and other systemic diseases.
(iv) They have very low toxicity due to metabolism to natural nucleotide components.
(v) They have good potency.
(vi) Their expression levels can be regulated to control the level of expression of the target gene.

LIMITATIONS OF RIBOZYME THERAPY

There are some limitations that need to be overcome in order to make ribozymes as a clinically usefull tool and these include:

(i) Poor intracellular uptake due to the intrinsic negative charge and limited stability in circulating blood.
(ii) Difficulty in identification of catalytic site or section of the substrate RNA molecule to achieve very high specificity inside targeted cell.

CONCLUSIONS

Ribozymes are emerging as a new and broadly useful class of therapeutic agent. Their discovery opens up an exciting new approach for the treatment of various diseases but still it is in a growing stage. The sequence-specific action of ribozyme against target RNA makes them flexible agents for the elimination of pathological gene expression in addition the trans-splicing ribozymes have a unique feature to repair mutant transcripts which is applicable in treatment of many genetically inherited diseases and in cancer. Through the continuous ongoing

researches and experiments, the efficacy of ribozymes therapy has already been recorded by the leading scientists but still there is a need to dedicate more effort in this newer area of therapeutic so that the effect of ribozymes is more efficient and specific.

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Research Article

REGULATORY ROLE OF CALCIUM CHANNEL BLOCKERS AND NITRIC OXIDE MODULATORS ON SPONTANEOUS MUSCULAR ACTIVITY OF RUMEN AMPHISTOME *PARAMPHISTOMUM CERVI*

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ABSTRACT

An important incentive for studying Ca^{2+} and nitric oxide (NO) channels in *Paramphistomum* is to better understand the physiology of excitable cells in these organisms and to obtain clues about the evolution of ion channels. Furthermore, elucidation of the structure and function of these channels might provide targets for new anti-trematodal agents. In the present investigation, Ca^{2+} channel blocking agents and NO modulating agents *viz* verapamil, diltiazem, nifedipine, hydroxylamine and haemoglobin has been elucidated for their role in neuromuscular transmission of *Paramphistomum cervi*. The cumulative addition of verapamil, diltiazem, nifedipine and hydroxylamine produced concentration dependent increase of amplitude, baseline tension and frequency of spontaneous muscular activity of isometrically mounted *P. cervi*. On the other hand, haemoglobin was not responsive in alteration of amplitude, baseline tension and frequency of *P. cervi*.

Key words: Ca^{2+} channel blockers, NO modulators, amplitude, baseline tension, frequency

INTRODUCTION

Neuromuscular system of helminths is an important area for target identification and drug developemet. Voltage-gated calcium channels (VGCCs), are transmembrane proteins involved in the regulation of cellular excitability and Ca^{2+} homeostasis. Major proportion of intracellular Ca^{2+} is achieved through opening of intracellular calcium channels present in the plasma membrane which play an important role in regulating neuromuscular coordination and release of neurotransmetters from nerve terminals. Bathing medium free of Ca^{2+} ions reduces the spontaneous muscular activity of *Schistosoma mansoni* (Wolde mussie *et al.*, 1992). Similarly increasing external Ca^{2+} ions concentration in the medium mimics the inhibitory effect of acetylcholine chloride on spontaneous muscular activity of split preparation of adult *Fasciola hepatica* and *Hymenolepis diminuta* (Sukhdeo *et al.*, 1986; Thompson and Mettrick, 1984). However, calcium channel blockers diltiazem and verapamil cause marked stimulation followed by paralysis of *S. mansoni*, *F. gigantica* and *Gastrothylax crumenifer* (Senft *et al.*, 1986; Kumar and Tripathi, 2000; Verma *et al.*, 2007). Contractions induced by calcium dependent depolarization have been observed in dispersed muscle fibres of *S. mansoni* (Day *et al.*, 1994a) whereas nicardipine a calcium channel blocker, blocks these contractions (Day *et al.*, 1994b).

Calcium currents have been recorded from muscle fibres of *Bdelloura candida*. However, Ca^{2+} currents could not be recorded from muscle fibres of *S. mansoni* (Blair and Anderson, 1994) and *F. gigantica* (Kumar *et al.*, 1995). A number of components vital for Ca^{2+} storage and release involving Ryanodine receptors (R_yR) present in sarcoplasmic reticulum have been demonstrated in the genera of *Schistosomes* (Talla *et al.*, 1998; Silva *et al.*, 1998). Praziquantel an anthelmintic disrupts Ca^{2+} homeostasis in adult *Schistosomes*, and although the exact mechanism is unknown, voltage-gated Ca^{2+} channels may be potential targets for the drug. The molecule nitric oxide (NO) involves in several vital physiological functions of mammals (Lindholm *et al.*, 1998) and has been considered as a neuronal messenger in trematodes (Terenina *et al.*, 1998). Therefore, the present study was carried out in order to investigate the role of different groups of voltage sensitive calcium channel blockers and NO modulating agents on SMA of isometrically mounted parasitic rumen amphistome, *P. cervi*.

MATERIALS AND METHODS

Collection of amphistomes

Mature amphistomes were collected from the rumen of freshly slaughtered cattle at local abattoir in warm (37±1°C) Hank's balanced salt solution (HBSS) in an insulated container and brought to the laboratory. They were kept in the BOD incubator at 37±1°C, until further use. The species of the amphistome was identified as *P. cervi*.

Chemicals

Verapamil hydrochloride (M. wt. 491.1), nifedipine hydrochloride (M. wt. 346.3), diltiazem hydrochloride (M. wt. 451.0), haemoglobin (10µg/ml) and hydroxylamine hydrochloride (M. wt. 69.49) were procured from the Sigma-

aldrich, USA, and were used for the present experiment. Other chemicals used in the study were of analytical grade.

Parasitological and experimental procedures

The mature *P. cervi* was mounted isometrically in HBSS solution at 37±1°C (Ahmed & Nizami, 1990). The amphistome was mounted with the help of two fine hooks. One hook was inserted 1-2 mm caudal to anterior sucker and fixed to the tip of aeration tube and another hook was pierced through the surface of acetabulum and connected to the isometric force transducer (Powerlab, 4/35, 4-Channel Data Acquisition System, Model: ML866/P, AD Instruments, Australia) and a tension of 200 mg was applied to the parasite. After setting up, the preparations were allowed to equilibrate in bath fluid for 30 min and standard time interval of 20 min was allowed between drug exposures. After equilibration, calcium channel blockers viz verapamil hydrochloride, nifedipine, diltiazem hydrochloride and NO modulating agents such as haemoglobin and hydroxylamine hydrochloride were added in a molar concentration of 1 log unit in ascending order (10^{-7} to 10^{-3} M) in the tissue bath to study their effects on spontaneous muscular activity of *P. cervi.* Three parameters *viz* amplitude (average of all peaks per 5 min or average tension), baseline tension (average of all minimum levels of contractions used for measuring amplitude) and frequency (total number of contractions in 10 min) of the isometrically mounted *P. cervi* were measured for a period of 10 min, which was recorded in Chart Window 7 Software programme. Control recordings were made for 15 min before addition of neuropharmacological agents.

Data analysis

Three parameters *viz* frequency, amplitude of rhythmic contractions and baseline tension of the isometrically mounted *P. cervi* were measured and compared with the control to elucidate the effects of various neuropharmacological agents. The results are presented as mean ± standard error. Level of significance was measured by paired "t" test (Snedecor & Cochran, 1989).

RESULTS

Recording of Spontaneous muscular activity of *P. cervi*

The isometrically mounted *P. cervi* exhibited spontaneous muscular activity (SMA) for several hours without any significant change in amplitude, baseline tension and frequency of the rhythmicity. The control amplitude, baseline tension and frequency of SMA were 0.52±0.03 g, 0.19±0.01 g and 55.00 ±5.00 per 5 min respectively. The amplitude (0.48±0.02 g), baseline tension (0.19±0.03 g) and frequency (51.00±4.71 per 5 min) of spontaneous contractions recorded after a period of 2h, were not significantly different from those recorded 15 min after applying the tension to *P. cervi.* Recordings are given in Table 1 and Figure 1.

Effect of calcium channel blockers on SMA of *P. cervi*

Table 1

Effect of verapamil, nifedipine, diltiazem, and hydroxylamine on amplitude, baseline tension and frequency (per 5 min) of SMA of *P. cervi*

Treated Group	Parameters	Concentration					
		Control	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M
Verapa	Amplitude (g)	0.38±0.05	0.41±0.03	0.47±0.04*	0.58±0.05**	0.69±0.05**	0.75±0.07**
mil	Baseline tension(g)	0.13±0.01	0.16±0.03	0.18±0.01	0.22±0.03**	0.26±0.01**	0.33±0.05**
	Frequency(Hz)	48.00±4.34	51.50±4.48	56.50±4.51	54.00±5.36	58.00±4.23*	62.00±4.21**
Nifedi	Amplitude (g)	0.36±0.04	0.39±0.05	0.41±0.04	0.45±0.06*	0.48±0.06**	0.53±0.07**
pine	Baseline tension(g)	0.17±0.02	0.19±0.03	0.20-±0.03	0.22±0.04	0.23±0.03*	0.25±0.05*
	Frequency (Hz)	38.33±2.34	40.66±2.87	42.16±3.12	43.83±3.24	47.00±4.16*	49.66±4.24*
Diltia	Amplitude (g)	0.39±0.07	0.0.15±0.03	0.48±0.06*	0.52±0.07**	0.55±0.07**	0.63±0.06**
zem	Baseline tension(g)	0.44±0.05	0.16±0.04	0.17±0.03	0.19±0.03	0.20±0.04*	0.21±0.04*
	Frequency (Hz)	36.33±3.23	41.33±3.45	49.00±4.12**	52.16±4.35**	0.50±0.07	0.54±0.09
Hydroxy	Amplitude (g)	43.50±4.23*	46.66±4.00	0.57±0.09	0.60±0.07	0.64±0.08**	0.68±0.06**
lamine	Baseline tension(g)	0.28±0.04	0.29±0.05	0.30±0.05	0.32±0.04	0.33±0.06*	0.35±0.03*
	Frequency (Hz)	50.81±5.78	52.85±5.45	64.55±5.67**	66.01±4.89**	72.20±5.00**	78.43±6.12**

Values represented mean ± standard error (n=6) *p < 0.05 and ** p < 0.01, as compared to the control g =gram, Hz = Herz

Table 2

Effect of haemoglobin on amplitude, baseline tension and frequency (per 5 min) of SMA of *P. cervi*

Treated Group	Parameters		Concentration				
		Control	0.001µg	0.01 µg	0.1 µg	1 µg	10 µg
Verapa mil	Amplitude (g)	0.48±0.07	0.47±0.06	0.48±0.08	0.48±0.08	0.47±0.07	0.48±0.08
	Baseline tension(g)	0.18±0.02	0.18±0.03	0.17-±0.01	0.18±0.02	0.17±0.01	0.18±0.02
	Frequency (Hz)	49.00±4.56	48.66±4.33	48.00±4.00	47.16±3.78	48.16±4.12	47.50±3.90

Values represented mean ± standard error (n=6)

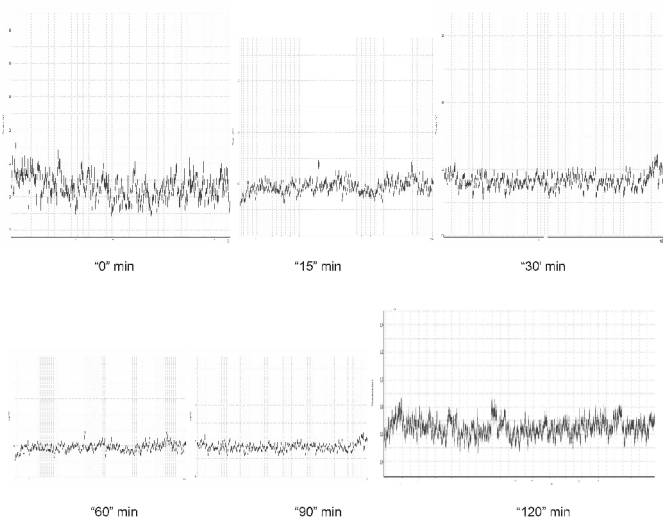

Fig. 1
Time dependent control recording of the spontaneous muscular activity (SMA) of *Paramphistomum cervi*

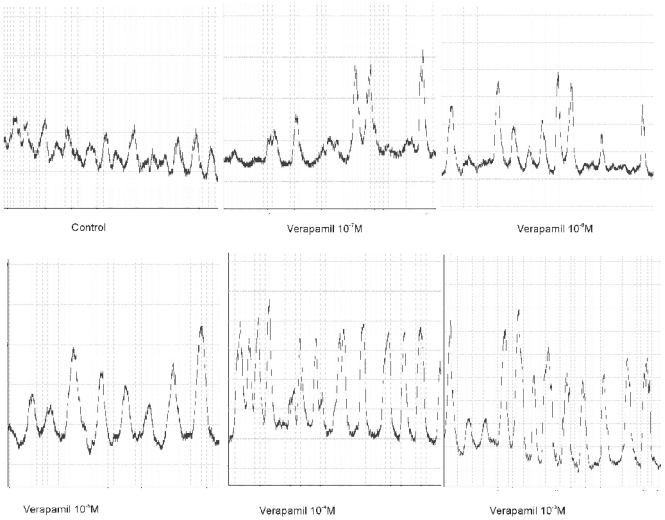

Fig. 2
Time dependent control recording of the spontaneous muscular activity (SMA) of *Paramphistomum cervi*

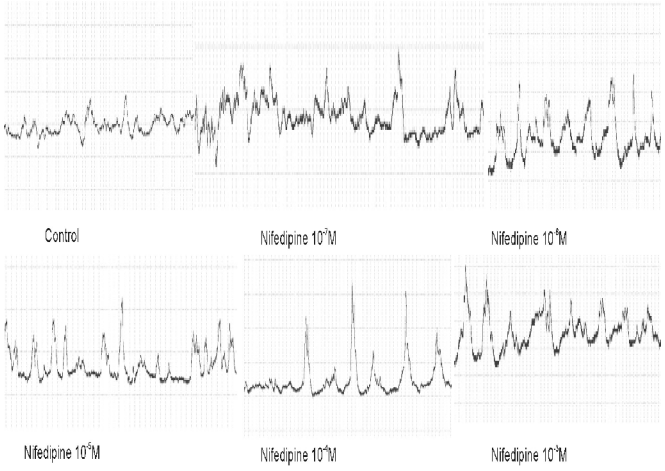

Fig. 3
Effect of different concentrations (10^{-7}M to 10^{-3}M) of nifedipine on spontaneous muscular activity of *Paramphistomum cervi*

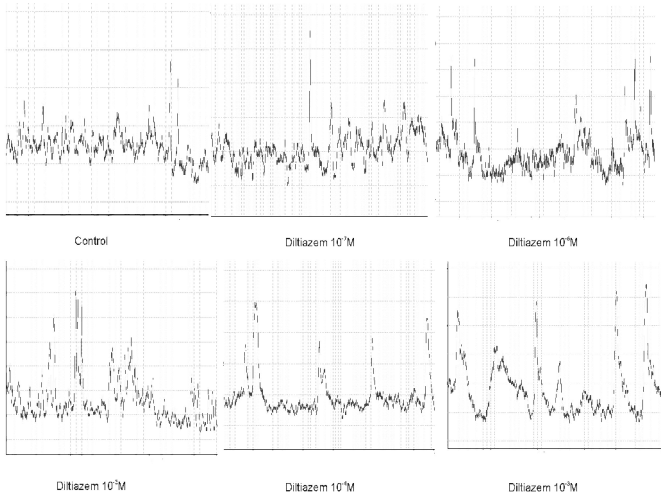

Fig. 4
4 Effect of different concentrations (10^{-7}M to 10^{-3}M) of diltiazem on spontaneous muscular activity of *Paramphistomum cervi*

Following cumulative administration of 1 log unit in ascending order (10^{-7} to 10^{-3} M), calcium channel blocker, verapamil hydrochloride elicited significant ($P < 0.05$; $P < 0.01$) excitation in the amplitude and baseline tension at 10^{-5} to 10^{-3} M, frequency at 10^{-4} to 10^{-3} M concentration as compared to control SMA. The results are summarised in Table 1 and Figure 2.

Similarly, administration of graded (10^{-7} to 10^{-3} M) molar concentration of 1 log unit in ascending order, another calcium channel blocker, nifedipine hydrochloride produced significant ($P < 0.05$; $P < 0.01$) and concentration dependent increase in amplitude at 10^{-5} to 10^{-3} M concentrations and significant ($P < 0.05$) increase in baseline tension and frequency at 10^{-4} to 10^{-3} M concentrations as compared to their respective control values. The results are summarised in Table 1 Figure 3.

Subsequently, diltiazem a benzothiazepine derivative (10^{-7} to 10^{-3} M), (calcium antagonist) exhibited significant ($P < 0.05$; $P < 0.01$) increase in amplitude and baseline tension at 10^{-6} to 10^{-3} M concentrations and frequency at 10^{-4} to 10^{-3} M as compared to those of control values. The results are summarised in Table 1 Figure 4.

Effect of NO modulating agents on SMA of *P. cervi*

Administration of graded (10^{-7} to 10^{-3} M) molar concentration of 1 log unit in ascending order, hydroxylamine hydrochloride produced concentration and significant ($P < 0.05$; $P < 0.01$) dependent increase in amplitude and baseline tension at 10^{-4} to 10^{-3} M concentrations and significant ($P < 0.01$) and dose dependent increase in frequency at 10^{-6} to 10^{-3} M

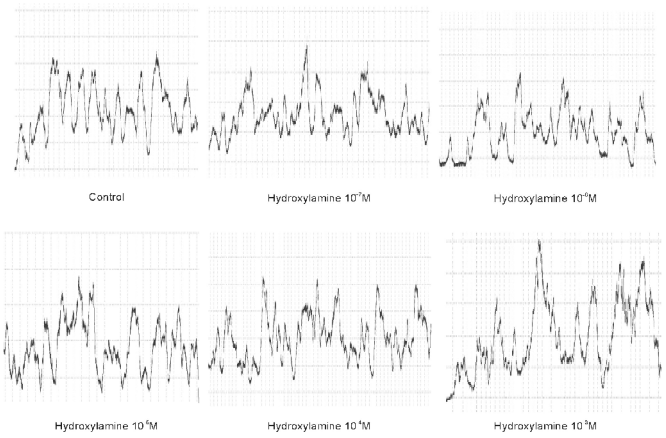

Fig. 5
Effect of different concentrations (10^{-7}M to 10^{-3}M) of hydroxylamine hydrochloride on spontaneous muscular activity of of *Paramphistomum cervi*

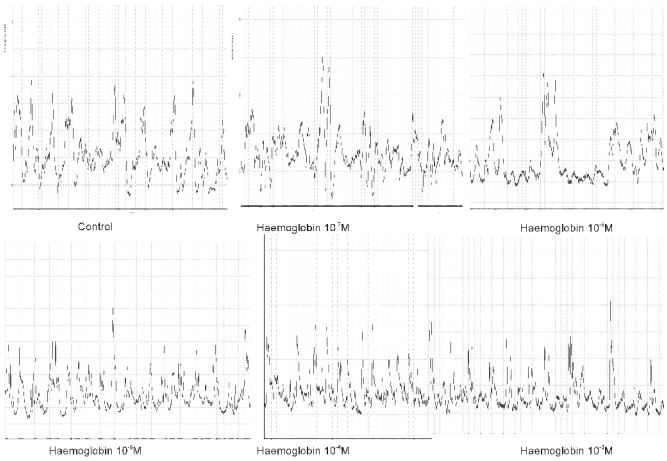

Fig. 6
Effect of different concentrations (10^{-7}M to 10^{-3}M) of haemoglobin on spontaneous muscular activity of *Paramphistomum cervi*

concentrations as compared to their respective control values. The results are presented in Table 1 and Figure 5.

Similarly, haemoglobin, a nitric oxide scavenger did not show any observable effect on amplitude, baseline tension and frequency of spontaneous muscular contractions of isometrically mounted *P. cervi* as compared with their control recordings. The results are presented in Table 2 and Figure 6.

DISCUSSION

Intracellular calcium (Ca^{2+}) is responsible for the muscular contraction and release of neurotransmitters from nerve terminals in mammals. Removal of extracellular Ca^{2+} and/ or blockade of calcium channel adversely affect contractile process and release of neurotransmitters in majority of the neuromuscular preparations *in vitro*. In the present study, however, calcium channel blockers from different groups elicited an excitatory response in amplitude and baseline tension of muscular activity of *P. cervi*. Out of these different groups, dihydropyridine derivatives produced pronounced contractile responses as compared to other blockers. The results are in agreement with the earlier reports on *S. mansoni* (Sneft *et al.*, 1986), *F. gigantica* (Kumar and Tripathi, 2000) and *G. crumenifer* (Verma *et al.*, 2007). However, it has been shown that Ca^{2+} free bathing medium reduced the spontaneous muscular activity of *S. mansoni* (Wolde *et al.*, 1992), while increasing external calcium ion concentration, mimicked the inhibitory effects of Ach on spontaneous muscular activity of spilt-preparation of adult *F. hepatica* (Sukhdeo *et al.*, 1986). These calcium channel blockers might inhibit the release of inhibitory neurotransmitters in the nerve terminals as it is well documented that the release of neurotransmitter at nerve terminals requires Ca^{2+} (Katz and Miledi, 1967) or these calcium channel blockers might produce a direct stimulatory effect on trematode neuromuscular system.

In the present study, nitric oxide donor, hydroxylamine hydrochloride increased the amplitude, baseline tension and frequency of SMA of *P. cervi*. Similar findings have also been reported in *F. gigantica* (Sengottaian, 2000), somatic smooth muscle strips of *A. suum* and *G. crumenifer* (Verma *et al.*, 2007). Contrary to this, NO scavenger, haemoglobin, did not show any observable effect on SMA of *P. cervi*, which is also in agreement with its ineffectiveness in *F. gigantica* (Sengottaian, 2000) and *G. crumenifer* (Verma *et al.*, 2007).

No donor, hydroxylamine hydrochloride significantly increased the amplitude, baseline tension and frequency of contractile activity of *P. cervi* in a concentration dependent manner and in contrast NO scavenger, haemoglobin did not induce any effect on the SMA of *P. cervi*. Further study may reveal the exact role of NO modulators on the SMA of *P. cervi*.

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Research Article

EFFICACY OF INDIGENOUS HERBS *WITHANIA SOMNIFERA* AND *EMBLICA OFFICINALIS* ON EGG YOLK CHOLESTEROL IN BIRDS

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ABSTRACT

Hypocholesteremic activity of *Withania somnifera* and *Emblica officinalis* was elucidated in terms of lipid profile on day 0, 14, 28 and 42 of the experiment in eggs of birds. Group of birds supplemented with *W. somnifera* root powder (2 percent in diet) showed a significant reduction in total lipids, yolk cholesterol and Yolk triglycerides levels showing 20.84 percent, 30.08 percent and 24.30 percent reduction on day 42 respectively. Supplementation of 2 percent *E. officinalis* in diet of birds showed a significant reduction in total lipids, Yolk cholesterol and yolk triglyceride levels showing 21.29 percent, 31.50 percent and 23.11 percent reduction on day 42 respectively.

Key words: Hypocholesteremic, *W. somnifera, E. officinalis*

INTRODUCTION

Egg being most nutritious and unadulterated natural food, has acquired an important place in human diet. Egg is a rich source of all the essential amino acid, minerals and vitamins, however, in addition to these essential dietary components; egg contains about 200-250 mg of cholesterol (Griffin, 1992) which is considered as major source of dietary cholesterol. Because chicken eggs are rich source of cholesterol, anxiety has often been created against their use in human diet. *Withania somnifera,* an indigenous medicinal plant commonly known as Ashwagandha, Indian ginseng and Winter cherry possesses hypocholesteremic, hypoglycemic and diuretic properties (Andallu and Radhika, 2000). *Emblica officinalis* commonly called as Indian gooseberry, Amla and Amlaki, has been reported to possess antioxidant, antibacterial, antiviral and antacid properties. Fruit juice of *E. officinalis* possesses hypolipidemic effect (Mathur *et al.,* 1996).

MATERIALS AND METHODS

The study was conducted on a total of sixty healthy Normal colored birds of thirty-two weeks old. Root of *Withania somnifera* (Ashwagandha) and fruit of *Emblica officinalis* (Amla) were obtained from the Department of Aromatic and Medicinal Plants, Agriculture College, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur. Root of *W. somnifera* and fruit pulp of *E. officinalis* were dried, crushed, powdered and used for supplementation of the diet in birds.

Experimental design

Sixty birds (32 weeks age) were randomly divided into five groups with twelve birds in each group with two replicates of six birds each. The birds of different groups were kept separately in individual cages and maintained under similar hygienic conditions. Group I was kept as control while in group II and III diet was supplemented with dried root powder of *W. somnifera* and in group IV and V diet was supplemented with dried fruit pulp of *E. officinalis* (Table 1).

Egg yolk lipid profile

Eight eggs were collected from each dietary treatment group of birds on day 0, 14, 28 and 42 of the experiment. Yolk was collected from each egg for the extraction of lipids (Folch *et al.,* 1957) and lipids were analyzed for estimation of total lipids (mg/g), (Fringe *et al.,*1972) cholesterol (mg/g) (Roeschlau *et al.,*1974) and triglycerides (mg/g (Mc Gowan *et al.*,1983). The diagnostic reagent kits were used for *in-vitro* determination of cholesterol and triglycerides in egg yolk. The estimation was done using semi auto-analyzer (ERBA CHEM-5).

Statistical analysis

Means were obtained as per standard procedure. The data collected on individual basis of each treatment was analyzed for differences among the replicate group. Analysis of variance did not revealed significant differences among replicates. All the parameters were analyzed by

Table 1.
Design of experiment

Group	Replicates	No. of birds per replicate	No. of birds per treatment	Treatment	Inclusion level (percent)
I	R_1R_2	66	12	Untreated control	-
II	R_1R_2	66	12	*Withania somnifera* root powder	1.0
III	R_1R_2	66	12	*Withania somnifera* root powder	2.0
IV	R_1R_2	66	12	*Emblica officinalis* fruit pulp powder	1.0
V	R_1R_2	66	12	*Emblica officinalis* fruit pulp powder	2.0

using the method for complete randomized design with 5 treatments allotted to group of 12 birds each. The differences within each treatment were tested statistically for their significance (Snedecor and Cochran, 1994).

RESULTS

The findings pertaining to the effect of *W. somnifera* and *E. officinalis* on egg yolk lipid values in laying hens have been summarized in the Table 2. Total lipids were calculated in terms of mg/g of egg yolk on day 0, 14, 28 and 42 of the experimental period in different groups of birds. Control group (Group-I) did not show any significant change in egg yolk lipids and was 306.20 mg/g yolk on day 42, whereas group of birds with dietary supplementation of herbs revealed significant reduction in yolk lipids from day 14 onwards. The mean values of egg yolk lipids in dietary group of birds supplemented with 1 percent *W. somnifera* (Group-II), 2 percent *W. somnifera* (Group-III), 1 percent *E. officinalis* (Group-IV) and 2 percent *E. officinalis* (Group-V) were 254.51, 241.35, 250.39 and 240.54 mg/g yolk, while the percent reduction was calculated to be 16.41, 20.84, 16.81 and 21.29 percent, respectively on day 42 post treatment.

The mean of egg yolk CHO values as influenced by dietary supplementation of *W. somnifera* and *E. officinalis* to laying hens have been presented in the Table 3. Untreated control group did not reveal any significant variation on day 0, 14, 28 and 42 of the experimentation which was calculated to be 17.92, 18.63, 18.92 and 18.97 mg/g yolk, respectively. However, 1 percent *W. somnifera* supplemented group showed a significant reduction on day 28 and 42 and yolk CHO was 14.31 mg/g yolk on day 42 in comparison to 18.44 mg/g yolk on day 0. The percent reduction was calculated to be 22.40 percent on day 42. Group of birds with 2 percent *W. somnifera* supplementation exhibited significant reduction on day 28 and 42 which was 15.01 and 12.88 mg/g yolk, respectively and the reduction in yolk CHO on day 42 was 30.08%.

Group of birds with 1 percent *E. officinalis* supplementation showed a significant reduction on day

Table 2.
Effect of dietary supplementation with *W. somnifera* and *E. officinalis* on egg yolk lipids (mg/g yolk) in birds

Treatment	Inclusion level (percent)	Egg yolk total lipids (mg/g yolk)				Percent reduction			SEM	CD at P<0.05
		Pre treatment	Post treatment							
		Day 0	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42		
Untreated control	-	298.46	303.21	300.75	306.20	-	-	-	4.37	NS
W. somnifera (root powder)	1.0	304.49^{a}	284.58^{b}	268.67^{c}	254.51^{d}	6.54	11.76	16.41	2.98	8.64
W. somnifera (root powder)	2.0	304.88^{a}	276.85^{b}	257.44^{c}	241.35^{d}	9.19	15.56	20.84	4.40	12.76
E. officinalis (fruit pulp powder)	1.0	301.00^{a}	285.45^{b}	265.24^{c}	250.39^{d}	5.17	11.88	16.81	3.54	10.28
E. officinalis (fruit pulp powder)	2.0	305.61^{a}	283.39^{b}	262.43^{c}	240.54^{d}	7.27	14.13	21.29	4.30	12.47

Values are mean of eight observations.,The mean values with different alphabet as superscript differ significantly from each other.,

Table 3.
Effect of dietary supplementation with *W. somnifera* and *E. officinalis* on egg yolk cholesterol (mg/g yolk) in birds

Treatment	Inclusion level (percent)	Egg yolk total lipids (mg/g yolk)				Percent reduction			SEM	CD at P<0.05
		Pre treatment	Post treatment							
		Day 0	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42		
Untreated control	-	17.92	18.63	18.92	18.97	-	-	-	0.53	NS
W. somnifera (root powder)	1.0	18.44^{a}	16.97^{ab}	15.65^{bc}	14.31^{c}	7.97	15.13	22.40	0.71	2.05
W. somnifera (root powder)	2.0	18.42^{a}	16.92^{ab}	15.01^{bc}	12.88^{c}	8.41	18.51	30.08	1.01	2.92
E. officinalis (fruit pulp powder)	1.0	17.88^{a}	17.16^{ab}	15.83^{bc}	14.42^{c}	4.03	11.46	19.35	0.56	1.61
E. officinalis (fruit pulp powder)	2.0	18.22^{a}	16.88^{a}	14.72^{b}	12.48^{c}	7.35	19..21	31.50	0.52	1.52

Values are mean of eight observations.,The mean values with different alphabet as superscript in a row differ significantly from each other.

Table 4.
Effect of dietary supplementation with *W. somnifera* and *E. officinalis* on egg yolk triglycerides (mg/g yolk) in birds

Treatment	Inclusion level (percent)	Egg yolk total lipids (mg/g yolk)				Percent reduction			SEM	CD at P<0.05
		Pre treatment	Post treatment							
		Day 0	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42		
Untreated control	-	182.15	185.00	180.43	187.20	-	-	-	4.73	NS
W. somnifera (root powder)	1.0	192.13^{a}	179.11^{b}	165.14^{c}	153.90^{c}	6.78	14.05	19.90	4.36	12.64
W. somnifera (root powder)	2.0	180.03^{a}	172.46^{a}	153.20^{b}	136.28^{c}	4.20	14.90	24.30	3.30	9.57
E. officinalis (fruit pulp powder)	1.0	185.75^{a}	174.99^{a}	160.14^{b}	152.03^{b}	5.79	13.79	18.15	4.02	11.67
E. officinalis (fruit pulp powder)	2.0	182.74^{a}	170.70^{b}	154.93^{c}	140.50^{d}	6.59	15.22	23.11	4.09	11.86

Values are mean of eight observations., •The mean values with different alphabet as superscript in a row differ significantly from each other., SEM : Standard Error Mean; CD : Critical Difference; NS : Non Significan

28 and 42. The yolk CHO was 14.42 mg/g yolk on day 42 in comparison to 17.88 mg/g yolk on day 0 having 19.35 percent reduction on day 42. Supplementation of 2 percent *E. officinalis* caused a significant reduction in yolk CHO on day 28 and 42 which was 14.72 and 12.48 mg/g yolk, respectively and the percent reduction in yolk CHO was 31.50 percent on day 42.

The mean of yolk triglyceride values in control group was 187.20 mg/g yolk on day 42 and did not show any significant changes, whereas group of birds with dietary supplementation of herbs revealed significant reduction in yolk lipids from day 14 onwards (Table 4). Yolk triglyceride mean values in group of birds having dietary supplementation of 1 percent *W. somnifera,* 2 percent *W. somnifera,* 1 percent *E. officinalis* and 2 percent *E. officinalis* were 153.90, 136.28, 152.03 and 140.50 mg/g yolk, respectively on day 42 post treatment. The percent reduction was highest in 2 percent *W. somnifera* group (24.30 percent), followed by 2 percent *E. officinalis* group (23.11 percent), 1 percent *W. somnifera* group (19.90 percent) and 1 percent *E. officinalis* group (18.15 percent) on day 42 post treatment (Table 4).

DISCUSSION

Total lipids content of egg yolk in the control group was found to be 30 percent of the total egg yolk weight. This value corresponds with that reported by El Bagir *et al.* (2006) and Cunningham and Lee (1978). Various research workers have reported hypocholesteremic activity of *Withania somnifera* and *Emblica officinalis* in mammals such as rats and rabbits; however, scientific literature keeps paucity with regards to their hypocholesteremic effects in birds. The report of Visavadiya and Narasimhacharya (2007) indicated hypocholesteremic effect of root powder of *Withania somnifera* at the dose of 1.5 g/day, added to the diet of rats for 28 consecutive days. They further observed a significant reduction in total lipids by 50.69 percent, cholesterol by 53.01 percent, triglycerides by 44.85 percent, LDL cholesterol by 62.7 percent and VLDL cholesterol by 44.8 percent in plasma. The reports of Visavadiya and Narasimhacharya (2007), are in close conformation to our findings where *Withania somnifera* in dose levels of 1 percent and 2 percent caused a gradual and significant reduction in total lipids, cholesterol, triglycerides, LDL cholesterol and VLDL cholesterol on day 28 and 42 of experimentation. Another medicinal plant; *Emblica officinalis* used in the present study has also been suggested for hypolipidemic activity in rats. The study of Anila and Vijayalakshmi (2002) reported hypolipidemic effect of *Emblica officinalis* in serum and tissues of hyperlipidemic rats. Mathur *et al.* (1996) showed the hypolipidemic effect of fresh juice of *Emblica officinalis* at the dose of 5 ml/kg body weight for 60 consecutive days in hyperlipidemic rabbits which caused significant decrease in serum cholesterol, triglycerides and LDL cholesterol levels by 66, 82, and 90 percent, respectively. The aforementioned reports of various coworkers are in close agreement to our findings.

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Research Article

AMELIORATING EFFECTS OF PIPERINE ON CYPERMETHRIN-INDUCED OXIDATIVE STRESS AND ANTIOXIDANT ALTERATION IN RAT BRAIN

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ABSTRACT

The aim of the present study was to investigate the ability of piperine, a natural alkaloid compound, to attenuate cypermethrin-induced oxidative stress and alteration in antioxidant enzyme activity in the brain of rats. The rats were divided into five groups of six each; the first group served as control and second group was used as vehicle control. While, groups III, IV and V were orally treated with piperine (50 mg/kg body weight), cypermethrin (25 mg/kg body weight) and cypermethrin plus piperine, respectively for 28 days. Cypermethrin administration caused elevated levels of lipid peroxidation in brain tissue. While the activities of non-enzymatic and enzymatic antioxidants levels were decreased except superoxide dismutase.Administration of piperine along with cypermethrin significantly decreased the level of lipid peroxidation and significantly increased the catalse and glutathione peroxidase level. The results indicate that piperine ameliorate the cypermethrin-induced oxidative damage in rats.

Keywords: Piperine, cypermethrin, oxidative stress, rat brain

INTRODUCTION

Cypermethrin is a synthetic pyrethroid insecticide used worldwide in agriculture, home pest control, protection of foodstuff and disease vector control. It is highly hydrophobic compounds and this suggests that their action in biological membranes might be related to association with integral proteins and with phospholipids (Michelangeli *et al.*, 1990). Several studies have shown that cypermethrin toxicity is linked to different mechanisms, including reactive oxygen species generation and oxidative stress (Kale *et al.*, 1999; Gupta *et al.*, 1999). The reactive oxygen species directly react with cellular biomolecules; damage lipids, proteins and DNA in cells ultimately leading to cell death. It is now being realized that one of the reasons for cypermethrin toxicity is imbalance between amount of free radicals generated and antioxidant defences in the brain of the rats (Giray *et al.*, 2001).

Trends on applying plant products in diseases related to oxidative stress have gained immense interest in recent years. Plant products are known to exert their protective effects by scavenging free radicals and modulating antioxidant defence system. Piperine, a main component of *Piper longum* Linn, is a plant alkaloid with a long history of medicinal use in Indian medicine. The compound has many pharmacologic activities such as antioxidant, bioenhancer, anti-inflammatory and hepatoprotective effects (Selvendiran *et al.*, 2004). Recently, we found that simultaneous supplementation of piperine along with cypermethrin lowered lipid peroxidation level and maintained superoxide dismuatase, catalase, glutathione peroxidise and reduced glutathione levels to near those of control rats liver and kidney (Sankar *et al.*, 2011). Therefore, the present study has been undertaken to evaluate the ameliorating effect of piperine on cypermethrin-induced oxidative stress and alteration in antioxidant in brain of rats.

MATERIALS AND METHODS

Chemical and Experimental animals

Cypermethrin (CYP; 96%) was a kind gift from Gharda Chemicals, Mumbai. Piperine was purchased from M/s Sigma Chemicals, USA. All other chemicals were of analytical and molecular grade from different companies. The study was conducted in adult male Wistar rats (6–8 weeks, 100–120 g) procured from the Laboratory Animals Resources Section of the Institute. Animals were maintained under standard management conditions and handled as per the Institute Animal Ethics Guidelines. Rats were given standard rat feed and water ad libitum throughout the experiment. All the animals were quarantined for a period of at least 7 days before beginning of the experiment. The animals were handled and the study was conducted in accordance with the Institute guide lines for the protection of animal welfare.

Experimental design

Rats were divided into five groups containing six animals each. Group I (control), was given normal saline, while Groups II was given once equivalent amount of ground nut oil (1%: Vehicle control). Group III was administered cypermethrin (25 mg/kg, orally) daily for 28 days. Group IV was administered piperine (50 mg/kg, orally) daily for 28 days. Group V was administered piperine (50 mg/kg, orally) and then cypermethrin (25 mg/kg, orally) daily for 28 days. All the rats were observed daily for clinical signs

TABLE 1:
Effect of cypermethrin and piperine and their co-administration on oxidative stress parameters measured in brain of rats

	Control	Groundnut oil	Cypermethrin	Piperine	Cypermethrin+piperine
LPO(nmol MDAformed/g tissue)	20.95±0.53[a]	22.32±0.66[a]	71.72±1.07[a]	21.61±0.73[a]	43.02±1.47[b]
GSH (mmol GSH/g tissue)	0.16±0.01	0.14±0.01	0.07±0.01[a]	0.14±.01	0.11±0.01
SOD (Units/mg protein)	3.56±0.06	3.20±0.02	4.88±0.19[a]	3.31±0.07	4.18±0.07[b]
CAT (mmol H2O2 utilized/min/mgprotein)	113.90±2.66	107.92±1.84	52.11±1.06[a]	104.14±1.17	97.49±1.64[b]
GPx (-mol NADPHoxidized to NADP/min/mg/protein)	244.28±2.01	234.30±0.72	148.25±6.17[a]	235.09±1.19	218.92±1.41[b]

Values given represent the mean ± S.E of 6 animals. Significant differences are indicated by [a] compare to control, [b] compare to cypermethrin in a given row.

of toxicity during the entire period of the study. Rats were sacrificed at the end of the exposure period. Brain was excised, washed with ice cold normal saline and used for the assay of oxidative stress and antioxidant related parameters.

Assessment of oxidative stress and antioxidant enzyme activity

Estimations of different oxidative stress-related biochemical parameters in brain were carried out. A 200 mg of sample was taken in 2 ml of ice-cold phosphate buffer saline. Another 200 mg of sample was separately taken in 2 ml of 0.02 M EDTA for reduced glutathione (GSH) estimation. The homogenate (10%) prepared with homogenizer (IKA, Germany) under ice-cold condition was centrifuged for 10 min at 3000 rpm and the supernatant was stored at "20 æ%C until assay. Lipid peroxidation (LPO) was evaluated in terms of malondialdehyde (MDA) production (Shafiq-u-Rehman *et al.*, 1984). GSH content was evaluated by the method of Sedlak and Lindsay (1968). Catalase (CAT) activity was assayed by the method as described by Aebi (1983). The activity of superoxide dismutase (SOD) was measured as per the method of Madesh and Balasubramanian (1998). Glutathione peroxidase (GPx) activity was determined by the method of Paglia and Valentine (1967). The protein content was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard

RESULTS AND DISCUSSION

Oxidative stress has been implicated to play important role in the pathogenesis of various neurodegenerative and chronic diseases (Irshad and Chaudhuri, 2002). It occurs when generated reactive oxygen species exceeds the capacity of antioxidant defense to counteract reactive oxygen species or the antioxidant capability of the cells deteriorates with xenobiotic insult leading to cellular damage. The measurement of byproducts of lipid peroxidation and the status of antioxidant enzymes like GSH, SOD, CAT and GPx are appropriate indirect ways to assess antioxidant status in tissue and the estimation of MDA, a by-product of lipid peroxidation, continues to be a reliable method to assess the degree of peroxidative damage to cell membrane.

The changes in the LPO level and activities of GSH, GPx, SOD and CAT during exposure to cypermethrin and piperine in the present investigation were depicted in Table 1. In the present study, cypermethrin treatment induced a high degree of lipid peroxidation in the brain tissue of rats. Several studies have indicated that, there is increase in the intracellular levels of reactive oxygen species and oxidative stress in cypermethrin-induced toxicity (Giray *et al.*, 2001). LPO has been shown to cause profound alterations in the structure and functions of the cell membrane, including decreased membrane fluidity and increased membrane permeability (Selvendiran and Sakthisekaran, 2004).

Antioxidant enzymes are considered to be the first line of cellular defense against oxidative damage. The decrease in the activities of GSH and GPx due to the generation of reactive oxygen species leads to enhancement in LPO. A significant reduction in GSH and GPx levels in liver and brain tissues (Giray *et al.*, 2001) and depletion of GSH and GPx in erythrocytes after dermal exposure of cypermethrin in rats have been reported (Raina *et al.*, 2009). SOD is an antioxidant metalloenzyme that reduce superoxide radicals to water and molecular oxygen. The increase in SOD activity in brain of cypermethrin exposed rats may be due to the compensatory adaptive mechanism of the antioxidant system to combat the increased ROS generation by the CYP toxicity. CAT is a haemoprotein, which reduces hydrogen peroxide to molecular oxygen and water. Reduction of CAT activity in cypermethrin treated rats may be due to the enhanced production of hydrogen peroxide.

The present studies suggest that the treatment with piperine and curcumin suppressed the lipid peroxidation and enhanced enzymatic and nonenzymatic antioxidants. Piperine has been shown to inhibit the lipid peroxidation (Rauscher *et al.*, 2000; Selvendiran and Sakthisekaran, 2004). The direct antioxidant activity of piperine against various free radicals, hydroxyl as well as superoxide, was explored under in vitro conditions by Mittal and Gupta (2000). In the present study there was a significant decrease in the lipid peroxidation in cypermethrin plus piperine treated group as compared to cypermethrin alone treated group. The suppressive action of piperine on lipid peroxidation observed in *in vivo*

experiments suggests that piperine may have direct effect on membranes which may decrease the susceptibility of the membranes to lipid peroxides. In the present study, levels of CAT and GPx in brain of cypermethrin plus piperine-treated animals were elevated to near those of control rats and maintained the SOD as that of control group. There is extensive evidence that supplementation of piperine can enhance antioxidant enzymes and other selenoproteins (Dhully *et al.*, 1993). Oral supplementation of piperine increased the enzymatic antioxidants (catalase and glutathione peroxidase) levels to near those of control rats (Selvendiran *et al.*, 2003). Simultaneous supplementation of black pepper or piperine in rats fed high fat diet lowered LPO and conjugated dienes levels and maintained SOD, CAT, GPx and GSH levels close to control rats (Vijayakumar *et al.*, 2004).

In conclusion, the present study shows that piperine treatment mitigates cypermethrin-induced oxidative damage of rats, which could be due its antioxidant nature and free radical scavenging properties.

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Research Article

PHARMACOKINETICS OF CEFPIROME FOLLOWING INTRAMUSCULUR INJECTION IN GOATS

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ABSTRACT

Cefpirome, a fourth generation semi synthetic broad-spectrum cephalosporin has bactericidal activity against against a broad range of gram-negative and gram-positive organisms, including *Pseudomonas aeruginosa* and methicillin susceptible Staphylococcus spp., *Haemophillus influenzae* type B and many members of the Enterobacteriaceae family. Looking to potential for cefpirome in *invitro* studies, pharmacokinetics of cefpirome was determined following intravenous and intramuscular administration (10 mg/kg) in goats. Cefpirome concentration in plasma samples was determined by reverse-phase High Performance Liquid Chromatography. Following intravenous administration of the cefpirome in goats, volume of distribution at steady-state (Vd_{ss}), elimination half-life ($t_{1/2\beta}$) and total body clearance (Cl_B) were reported 0.40 ± 0.01 L/kg, 2.2 ± 0.03 h and 2.5 ± 0.02 ml/min/kg respectively. Following intramuscular administration of the drug, peak plasma concentration (C_{max}), elimination half-life ($t_{½\beta}$), apparent volume of distribution (Vd_{area}), total body clearance (Cl_B), bioavailability (F) were 11.02 ± 0.09 μg/mL, 3.58 ± 0.02 h, 1.07 ± 0.07 L/kg, 3.45 ± 0.01 ml/min/kg and 72 ± 1 %, respectively. Pharmacokinetic – pharmacodynamics integration indicates cefpirome can be useful in goats at dose rate of 10 mg/kg and repeated at interval of 12 h by intramuscular route.

Keywords: cefpirome, pharmacokinetics, intravenous, intramuscular, goat.

INTRODUCTION

Cefpirome is a new investigated cephalosporin in veterinary field. It is broad-spectrum semi synthetic β-lactamase resistant fourth generation cephalosporin. It has increased affinity for PBPs (Pucci *et al.*, 1991), reduce susceptibility to extended-spectrum β-lactamase (Bryskier *et al.*, 1994) and have ability to interfere with PBPs mediated cell wall synthesis ultimately leads to cell lysis (Nikaido *et al.*, 1990). In vitro studies have demonstrated its bactericidal activity against a broad range of gram-negative and gram-positive organisms, including *Pseudomonas aeruginosa* and methicillin susceptible Staphylococcus spp., *Haemophillus influenzae* type B and many members of the Enterobacteriaceae family (Bertram *et al.*, 1994). The disposition kinetics of cefpirome have been investigated in rabbits, dogs, mice, rat, and monkey (Klesel and Seeger, 1983), buffalo calves (Rajput *et al.*, 2007[ab]). However, there is no information available on the pharmacokinetic of cefpirome in goats following intravenous and intramuscular routes of administration. Looking to possibility for species difference, the study was undertaken on pharmacokinetics of cefpirome following intravenous and intramuscular administration in goat.

MATERIALS AND METHODS

Experimental Animals

The experiment was conducted on six healthy adult (1.5 to 2.5 years of age) Surti goat, weighing 22-32 kg. Each animal was housed in a separate pen and provided standard ration with *ad libitum* water. Goats were kept under constant observation for two weeks before the commencement of the experiment and subjected to clinical examination to exclude possibility of any diseases. The experiment was conducted at the Livestock Research Station, Vanbandhu Veterinary College, Navsari Agricultural University. The experimental protocol was approved by Institutional Animal Ethics Committee.

Drugs and Chemicals

Cefpirome technical grade powder was procured from Orchid Pharma Ltd., Chennai. Cefpirome sulphate powder (1 g Ceforth®; Biochem pharmaceutical Industries Ltd., Mumbai) was purchased from local pharmacy. Water, acetonitrile, acetic acid (HPLC grade), sodium acetate and perchloric acid (AR grade) were purchased from S.D. Fine Chem Ltd., Merck India Ltd. and Sisco Research Laboratories Pvt. Limited, Mumbai, India.

Drug administration and sample collection

All six animals were randomly allocated to receive either an intravenous or intramuscular injection of Cefpirome at the dose rate of 10 mg/kg. A washout period of two weeks was observed between treatments. An intravenous injection of Cefpirome was administered in the left jugular vein. Blood samples (3 mL) were collected through an intravenous catheter (Venflon, 22 × 0.9 × 25 mm) fixed in the contra lateral jugular vein in glass test tubes, prior to injection and at 2, 5, 10, 15, 30 min and 1, 2, 4, 8 and 12 h after intravenous administration. Following intramuscular injection of Cefpirome in the left deep gluteal muscle, blood samples (3 mL) were collected before administration and at 5, 10, 15, 30 min and 1, 2, 4, 8, 12, 18 and 24 h. Goats

were monitored for any adverse reactions during the entire study period. Blood samples were centrifuged at 4116 g for 10 minutes at 4°C and plasma transferred to cryo-vials (2 mL) and, stored at – 20°C. Samples were analyzed within 48 h to quantify cefpirome concentration using High Performance Liquid Chromatography.

Analytical assay of cefpirome and pharmacokinetic analysis

Cefpirome concentration in plasma samples was determined by reverse-phase High Performance Liquid Chromatography (HPLC) after extraction, using a reported assay (Nahata, 1991) with minor modifications. The High Performance Liquid Chromatography (HPLC) apparatus of Laballiance (USA) comprised of quaternary gradient delivery pump (model AIS 2000), UV detector (model 500) and C18 column (4.6 × 100 mm ID) were used. Pharmacokinetic data integration was done by software "Clarity" (Version 2.4.0.190). Plasma (500 µL) was deproteinized by addition of perchloric acid (0.8 M) and methanol (50:50) and vortexed for one minute. This was followed by centrifugation at 4116 g for 10 minutes. An aliquot of supernatant was collected in clean vial and 20 µL was injected into the loop of HPLC system. The mobile phase was a mixture of 0.2 M sodium acetate (4%), acetronitrile (20.0 %) and HPLC water (76 %). 0.2M acetic acid was used to adjust pH of 5.1. Mobile phase was filtered by 0.45 µ filters and pumped into column at a flow rate of 1.0 mL/min at ambient temperature. The effluent was monitored at 258 nm wavelength.

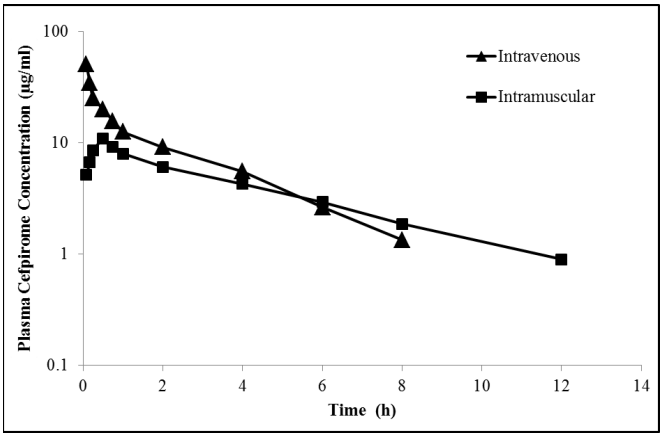
Calibration curve was prepared daily for drug concentration ranging from 0.8 to 200 µg/mL. The assay was sensitive (LLOD: 0.8 µg/mL)and reproducible, and linearity was observed from 0.16 to 20 µg/mL (r^2 = 0.99). Precision and accuracy were determined using quality control (QC) samples at concentrations 1.6, 50, 20 µg/mL (5 replicates each day). Intraday and interday coefficients of variability for five QC samples were satisfactory with the relative deviations (RSD) of less than 5 %. The absolute recovery of cefpirome was measured upto 97.42 %. Various pharmacokinetic parameters were calculated from serum concentration of cefepime using software PK solution (version 2.0). The bioavailability (F) was calculated using following formula:

$$F\ \% = \frac{AUC\ (IM)}{AUC\ (IV)} \times \frac{DOSE\ (IV)}{DOSE\ (IM)}$$

Statistical Analysis

Cefpirome serum concentration and pharmacokinetic parameters of different treatment groups were compared by students' "t" test using SPSS software (version 12.0.1).

Fig. 1:
Semi-logarithmic plot of cefpirome plasma concentration after intravascular and intramuscular administration (10mg/kg) in goats. Each point represents mean of six animals.



RESULTS

The mean plasma concentration-time profile of cefpirome following intravenous and intramuscular administration at 10 mg/kg body weight has been presented graphically in figure 1. Pharmacokinetic parameters (Mean ± SE) calculated after both route of drug administration have been depicted in table 1. Adverse reactions were not observed following single dose intravenous and intramuscular administration of cefpirome in the goats.

The mean peak plasma drug concentration of 50.78 ± 0.20 µg/mL following intravenous administration of cefpirome, was observed at 5 minutes, which rapidly declined to 12.61 ± 0.14 µg/mL at 1 h. Thereafter, gradual decrease in drug concentration was observed in the plasma. Finally drug concentration of 1.35 ± 0.04 µg/mL was detected in plasma up to 8 h. After intravenous administration, therapeutic concentration of cefpirome e" 0.5 µg/mL was maintained in plasma from 5 min to 8 h. Following intramuscular administration of the cefpirome, mean peak plasma drug concentration (C_{max}) of 11.02 ± 0.09 µg/mL was achieved at 0.5 h (T_{max}) which declined rapidly to 6.06 ± 0.06 µg/mL at 2 h. The drug concentration of 0.90 ± 0.01 µg/mL in plasma was detected at 12 h and thereafter drug was not detected in plasma samples collected beyond 12 h post intramuscular administration in goats.

Following intravenous administration of the drug in goats, distribution half-life ($t_{1/2}$) ranged between 0.8 and 0.15 h with a mean of 0.1 ± 0.01 h. The mean values of volume of distribution at steady-state (Vdss) were calculated to be 0.40 ± 0.01 L/kg. The range of elimination rate constant (β) was 0.30 to 0.33 h^{-1} with a mean of 0.30 ± 0.04 h^{-1}. The elimination half-life ($t_{1/2\beta}$) ranged from 2.07 to 2.28 h with a mean of 2.2 ± 0.03 h. Total body clearance (Cl_B) of the drug was 2.49 ± 0.02 ml/min/kg with mean residence time (MRT) of 2.60 ± 0.03 h. Following intramuscular administration of the drug, absorption ($t_{½Ka}$)

Table 1:
Pharmacokinetic parameters of cefpirome (10 mg/kg) following intravenous and intramuscular administration in goats. (Mean ± SE, n=6).

PharmacokineticParameter	Unit	IntravenousMean ± S.E	IntramuscularMean ± S.E
K_a	h^{-1}	-	5.61 ± 1.21
β	h^{-1}	0.31 ± 0.04	0.19 ± 0.01
$t_{½Ka}$	h	-	0.16 ± 0.03
$t_{½\alpha}$	h	0.1 ± 0.01	-
$t_{½\beta}$	h	2.2 ± 0.03	3.58 ± 0.02
C_{max}	μg/mL	-	11.02 ± 0.09
T_{max}	h	-	0.5 ± 0.00
$AUC_{(0-\infty)}$	μg.h/mL	67.30 ± 0.61	48.24 ± 0.17
Vd_{ss}	L/kg	0.4 ± 0.01	-
Vd_{area}	L/kg	-	1.07 ± 0.07
$Cl_{(B)}$	ml/min/kg	2.5 ± 0.02	3.45 ± 0.01
MRT	h	2.6 ± 0.03	2.42 ± 0.03
F	%	-	72.00 ± 1.00

K_a : absorption rate constant, β : elimination rate constant, $t_{1/2\alpha}$: half-life of distribution phases; $t_{1/2\beta}$: elimination half life; $t_{1/2k(a)}$: absorption half-life; $AUC_{(0-\infty)}$: total area under plasma drug concentration-time curve; V_{darea}: volume of distribution based on area; V_{dss}: volume of distribution at steady state; Cl_B : total body clearance; MRT : mean residence time; C_{max} : maximum drug concentration; T_{max} : time of maximum concentration observed in plasma; F : bioavailability.

and elimination half-life (t½β) were 0.16 ± 0.03 and 3.58 ± 0.02 h, respectively. The mean apparent volume of distribution (Vd_{area}), total body clearance (ClB) and mean residence time (MRT) were 1.07 ± 0.07 L/kg, 3.45 ± 0.01 ml/min/kg and 5.05 ± 0.03 h respectively. The bioavailability (F) of the drug following intramuscular administration ranged from 69 to 73 % with an average of 72 ± 1.0 %.

DISCUSSION

Elimination half life ($t_{1/2}$: 2.2 ± 0.03 h) observed in goats following single dose cefpirome intravenous administration, is in agreement to the elimination half life of 2.14 ± 0.02 h reported in buffalo calves (Rajput *et al.*, 2007[a]). However shorter elimination half life of 1.05 h and 0.90 h in dogs, 1.17 h in monkeys and 1.48 h in rabbits (Klesel and Seeger, 1983) have been reported. The calculated mean residence time (2.6 ± 0.03 h) in goats, is in agreement with reported values of 2.89 ± 0.01 h in buffalo calves (Rajput *et al.*, 2007[a]). In addition to this, it was observed that cefpirome principally eliminated by the kidney and 80-90% of the administered drug was recovered as unchanged form in the urine (Bertram *et al.*, 1984; Patani *et al.*, 2008). Faster body clearance (2.5 ± 0.02 ml/min/kg) of cefpirome in goats following intravenous route of administration was obtained in present study. Similarly comparable clearance values of 2.00 ± 0.05 ml/min/kg in buffalo calves (Rajput *et al.*, 2007[a]) and 3.2 ml/min/kg in dogs (Klesel and Seeger, 1983) have been reported following intravenous administration of cefpirome. The mean volume of distribution at steady state (Vd_{ss}: 0.40 ± 0.01 L/kg) in goats following cefpirome intravenous administration indicated the limited distribution of drug into various body fluids and tissues. Similar value of Vd_{ss} (0.40 ± 0.004 L/kg) have been reported in buffalo calves (Rajput *et al.*, 2007[a]). In addition to this limited distribution of other cephalosporins like cefepime was also reported in goats (Patani *et al.*, 2008; Patel *et al.*, 2012[ab]).

The peak plasma concentration (C_{max}: 11.02 ± 0.09 μg/ml) was found at 0.5 h (T_{max}) following intramuscular administration of cefpirome in goats. Similar observation (C_{max} : 9.04 ± 0.5 μg/ml) at 0.5 h was also observed in buffalo calves (Rajput *et al.*, 2007[a]), 11.7 μg/ml at 0.42 h in monkeys and 15.4 μg/ml at 0.70 h in dogs (Klesel and Seeger, 1983). Elimination half-life ($t_{½\beta}$: 3.58 ± 0.02 h) obtained in present study is in agreement to elimination half-life of 2.39 ± 0.05 h reported in buffalo calves (Rajput *et al.*, 2007[b]). However shorter elimination half life of 1.38 h in dogs and 1.23 h in monkeys (Klesel and Seeger, 1983) were also reported. Following intramuscular administration, high clearance (Cl_B : 3.45 ± 0.01 ml/min/kg) of the drug was also observed and similar pattern was observed to intravenous injection of cefpirome. Moreover, value of mean apparent volume of distribution (Vd_{area}: 1.07 ± 0.07 L/kg) indicated good distribution of drugs in body tissues following intramuscular administration of cefpirome in goats. Value of Vd_{area} for goats in present study is higher to the reported values of 0.42 ± 0.01 L/kg in buffalo calves (Rajput *et al.*, 2007[b]). Systemic bioavailability (72 ± 1 %) following intramuscular administration of cefpirome in goats was found higher than 35.3 ± 3.1 % reported in buffalo calves (Rajput *et al.*, 2007[b]). High systemic bioavailability and maintenance of therapeutic concentration up to 12 h following intramuscular injection suggests that cefpirome is suitable for intramuscular administration for the treatment for systemic bacterial infections in goats.

For β-lactam antibiotics, time for which serum drug concentration exceeds the MIC (T>MIC) of pathogens is considered as primary determinant of antibacterial efficacy (Craig, 1995). In addition to this, for β-lactam antibiotics maximum killing was seen when the time above MIC is at least 70 percent of the dosing interval (Hyatt *et al.*, 1995). Minimum inhibitory concentration for a majority of cefpirome sensitive bacteria like *Staphylococcus aureus* and *Escherichia coli* in the range of 0.1 to 0.5 μg/mL (Limbert *et al.*, 1991; Sultana and Arayne, 2007). Integrating cefpirome pharmacokinetic data and the MIC values, cefpirome (10 mg/kg) has to administer intramuscularly at interval of 12 hours.

In conclusions, integration of pharmacokinetics data generated from the present study with MIC range (0.1 to 0.5 μg/mL), cefpirome can be administer following intramuscular route at dose of (10 mg/kg) repeated at 12 h interval to maintain plasma concentration above MIC range.

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Research Article

EFFICACY OF PLANT-DRUGS AGAINST ETHINYL OESTRADIOL ALTERED TRANSAMINASE ACTIVITY

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ABSTRACT

Ethinyl oestradiol (EO), a semisynthetic 17β-oestradiol is a highly potent oestrogen. EO in excess use can cause severe cytotoxicity, including cancer in humans and animals. However, several plant-drugs (herbal drugs) have been reported to act against such untoward effects. One of the plant-drug, ProImmu elicited the immunomodulatory effect and restored the normal histoarchitecture of damaged tissues. In the present study also, the beneficial effects of ProImmu, and its two ingredients, viz., *Tinospora cordifolia* and *Withania somnifera* were noticed against EO altered transaminase activity in the female albino rats. The activities of two transaminases, viz., serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were increased significantly after administration of EO @ 250 ìg/kg, orally, thrice a week for 8 and 12 weeks. After 8 weeks of daily oral administration of ProImmu (@ 150 mg/kg), *T. cordifolia* (@ 250 mg/kg) and *W. somnifera* (@ 250 mg/kg), the activities of SGOT and SGPT could be significantly normalized. However, after 8 weeks of daily oral administration of these plant-drugs at the same dose rate also caused the normalcy of SGOT and SGPT, but to lesser extent than observed after the 8 week.

Key words : Ethinyl oestradiol (EO), ProImmu, *Tinospora cordifolia,* transaminases (SGOT and SGPT), *Withania somnifera.*

INTRODUCTION

Ethinyl oestradiol (EO, a semisynthetic highly potent oestrogen) has been reported to cause severe cytotoxicity, including cancer both in humans and animals. The excessive doses of oestrogen may cause nausea, vomiting, anorexia, migraine, blurring of vision, mental depression, headache, asthma, endometriosis, fibroids, breast engorgement, increased vaginal secretion, oedema, cardiovascular and hepatic diseases, cancer, stroke, Alzheimer's disease, and many others in human beings (Loose and Stancel, 2006; Madhuri, 2008 & 2011; Madhuri *et al.*, 2012).

Many plant-drugs have been used for prevention and treatment of cytotoxicity caused by various toxicants. As per the WHO estimate, more than 80% people depend on herbal drugs for their primary health needs (Sivalokanathan *et al.*, 2005). ProImmu (a herbal formulation, manufactured by Indian Herbs, Saharanpur), containing *Tinospora cordifolia* (Giloe), *Withania somnifera* (Ashwagandha), *Ocimum sanctum* (Tulsi) and *Emblica officinalis* (Amla) possesses immunomodulatory effect and restores the normal histoarchitecture of the damaged tissues. The cytogenic or anticancer effects of ProImmu have been observed against EO induced damage/cancer in the uterus and ovary of rats (Madhuri, 2008 & 2011; Madhuri *et al.*, 2012).

Henceforth, the present study was undertaken to evaluate the efficacy of herbal drugs, viz., *T. cordifolia, W. somnifera* and ProImmu against EO (oestrogen) altered activities of serum transaminases (SGOT and SGPT) in female albino rats.

MATERIALS AND METHODS

To assess the efficacy of plant-drugs, viz., *T. cordifolia, W. somnifera* and ProImmu, fifty four female albino rats were undertaken. The rats were divided into 9 groups, each consisted of six rats. The rats of group 1 were administered normal saline alone to serve as normal group. EO (250 ìg/kg, orally, thrice a week) was administered in groups 2 to 5 for 8 weeks and in groups 6 to 9 for 12 weeks. However, the hydroalcoholic extract (HAE) of *T. cordifolia* (250 mg/kg, orally, daily), HAE of *W. somnifera* (250 mg/kg, orally, daily) and aqueous suspension of ProImmu powder (150 mg/kg, orally, daily) were administered to the rats of groups 3 to 5, respectively for 8 weeks. Similarly, these three plant-drugs at the same dose rate were administered to the rats of groups 7 to 9, respectively for 12 weeks. After the end of experiment, the activities of serum transaminases (SGOT and SGPT) were determined in the blood samples.

The blood was collected from the eye veins (Intraocular method) of rats. The two biochemical parameters, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were estimated as per the methods described by Bradley *et al.* (1972). For statistical analysis, the data of SGOT and SGPT were analyzed and the significance of difference was determined by employing the Duncan's new

multiple range test (Steel and Torrie, 1980) at P = 0.05 (5% level of significance).

RESULTS AND DISCUSSION

The results of SGOT and SGPT are summarized in Table 1. The results indicated the normal value of SGOT to be 72.9±1.4 and 34.0±0.1 IU per L, respectively in group 1 of rats administered with saline alone. The average normal values of SGOT and SGPT in the present investigation corresponds with the value reported by Bhalerao (2006) and Madhuri (2008) in rat, and Pandey (1990) in rabbit.

The SGOT and SGPT activities of normal rat on 1st week (Group 1) significantly (P<0.05) increased on the 9th week (Group 2) and 13th week (Group 6) after administration of EO (Table 1), which indicates that the drug might have damaged the liver tissues. The liver damage has also been observed by Pandey *et al.* (2008) after administration of this drug in the same species of animal. The increased activity of SGOT and SGPT during hepatotoxicity or liver damage can be further correlated with the reports of Benjamin and McKelvie (1978), Bhalerao (2006) and Pandey (1990), who mentioned the increased activities of both these transaminases on exposure to hepatotoxins, including drugs and chemicals. Shar and Kew (1982) have reported the elevated activities of these transaminases during OCs (containing oestrogen) induced cancer in woman.

The increased SGOT and SGPT activities of groups 2 and 6 significantly decreased in groups 3 to 5 and groups 7 to 9, respectively. The results indicated that the SGOT and SGPT activities after treatment with *T. cordifolia*, *W. somnifera* and ProImmu significantly decreased against EO induced damage and returned towards normal level, especially after treatment with ProImmu. Further, the SGPT activity of group 5 exactly returned to normal, as observed in group 1 (normal). The improvement in SGOT and SGPT activities caused by ProImmu was also seen by Das *et al.* (2000) and Madhuri (2008). Similar result of SGPT was also observed by Sultana *et al.* (2005) who reported a significant decrease in carbon tetrachloride (a hepatotoxicant) increased SGPT activity of rat pretreated with *E. officinalis* (an ingredient of ProImmu). The findings of the present study are in accordance with the many investigators. The cytogenic effects of ProImmu have been observed against EO induced tissue damage in the rats (Madhuri, 2008 & 2011; Madhuri *et al.*, 2012). The immunomodulatory effect of *T. cordifolia* was observed by Thatte and Dahanukar (1989). The methanolic extract (200 mg/kg, ip, daily for 5 days) of *T. cordifolia* stem increased the humoral immune response and reduced solid tumour growth in mice (Mathew and Kuttan, 1999). The whole plant extract of *W. somnifera* possesses antioxidant, antiinflammatory, immunomodulating and antistress properties (Mishra *et al.*, 2000).

The present findings can, therefore, be concluded that EO (oestrogen) @ 250 ìg/kg, orally, thrice a week for 12 weeks caused severe cytotoxicity as far as the enhancement of serum transaminase level is concerned. Oestrogen has been reported to cause cellular damage (or cancer) by helping cells to proliferate. After this hormone binds to specific estrogen receptors (ERs) in the cytoplasm

Table 1:
Effects of *T. cordifolia, W. somnifera* and ProImmu on serum transaminases (SGOT and SGPT) in ethinyl oestradiol (EO) induced damage in rat.

Groups*	Treatment	Week of experiment	Mean** ±S.E.	
			SGOT (IU/L)	SGPT (IU/L)
1	Normal saline (Normal group)	1st	72.9^{g}±1.4	34.0^{g}±0.1
2	EO @ 250 ìg/kg, orally, thrice a week for 8 wks	9th	118.1^{b}±1.7 (62.0)#	72.5^{b}±2.2 (113.2)#
3	EO as per group 2 + *T. cordifolia* @ 250 mg/kg, orally, daily for 8 wks from the start of experiment	9th	103.8^{de}±1.0 (42.4)#	54.7^{de}±2.3 (60.9)#
4	EO as per group 2 + *W. somnifera* @ 250 mg/kg, orally, daily for 8 wks from the start of experiment	9th	98.7^{e}±3.9 (35.4)#	50.3^{e}±2.6 (47.9)#
5	EO as per group 2 + ProImmu @ 150 mg/kg, orally, daily for 8 wks from the start of experiment	9th	88.8^{f} ±1.2 (21.8)#	38.8^{fg}±2.6 (14.1)#
6	EO @ 250 ìg/kg, orally, thrice a week for 12 wks	13th	125.5^{a}±1.4 (72.2)#	79.5^{a}±0.8 (133.8)#
7	EO as per group 2 + *T. cordifolia* @ 250 mg/kg, orally, daily for 12 wks from the start of experiment	13th	111.7^{c}±1.7 (53.2)#	65.3^{c}±1.4 (92.0)#
8	EO as per group 2 + *W. somnifera* @ 250 mg/kg, orally, daily for 12 wks from the start of experiment	13th	108.0^{cd}± 1.7 (48.1)#	59.5^{cd}±1.8 (75.0)#
9	EO as per group 2 + ProImmu @ 150 mg/kg, orally, daily for 12 wks from the start of experiment	13th	93.1^{f}±1.1 (27.7)#	44.0^{f}±1.0 (29.4)#

*Number of animals in each group = 6.
**Mean with same superscript does not differ significantly (Duncan's new multiple range test at P = 0.05).
#Figures in parentheses of groups 2 to 9 denote the percent increase in relation to group 1.

of target cells, it turns on the hormone-responsive genes that promote DNA synthesis and cell proliferation. In metabolizing carcinogenic oestrogen, the reactions produce intermediates capable of producing oxygen radicals that can damage the cell's fats, proteins and DNA. Unrepaired DNA damage can turn into a mutation, leading to cancer. Furthermore, ProImmu, *T. cordifolia* and *W. somnifera* possess the cytogenic effect, which is time dependent. The cytogenic effect of ProImmu may be due to its plant-ingredients, which have been reported as potent immunostimulatory and antioxidant plants. The phyto-antioxidants can cure many diseases by protecting the cells from damage caused by 'free radicals'- the highly reactive oxygen compounds. The cytogenic effects and mechanism of actions of ProImmu, *T. cordifolia* and *W. somnifera* may be postulated on the basis of their immunostimulatory, antioxidant, phagocytic and other cytoprotective activities (Madhuri, 2008 & 2011).

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Research Article

GROSS AND HISTOPATHOLOGICAL STUDIES OF INDOXACARB INDUCED TOXICITY IN BROILERS *(GALLUS DOMESTICUS)*

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ABSTRACT

The experiment was undertaken to study the gross and histopathological changes due to indoxacarb induced toxicity in broilers for a period of 28 days. The study was conducted in four groups, each group having (n=30) healthy, day old male broiler chicks of weighing about 50-55 gms. For gross and histopathology broilers were sacrificed on 28th day, organs were collected and fixed in 10% neutral buffered formalin. The effects of indoxacarb were recorded on gross and histopathological examination of liver, kidney, brain and testis. In gross lesions, due to indoxacarb toxicity, liver was slightly enlarged and congested with a few petechial haemorrhages on its surfaces in group-II. Kidneys were congested and swollen in group-II and group-III. The brain and testes did not reveal any appreciable change in all indoxacarb treated groups. Histopathologically liver showed congestion in hepatic parenchyma, vacuolar degeneration, mild fatty changes, hypertrophy of hepatocytes, dilation of central vein, increased sinusoidal spaces and multifocal infiltration of mononuclear cells. Hepatocytes were showing necrobiotic changes. The kidneys showed congestion, vacuolar degeneration of tubular epithelium, mononuclear cell infiltration in parenchyma along with haemorrhagic cystic degeneration. There were hyaline masses inside the renal tubules. Microscopically brain revealed mild congestion, vacuolation and lymphatic aggregation. Histopathologically testes revealed partial to complete arrest of spermatogenesis with vacuolation. Seminiferous tubules showed wrinkled basement membrane along with increased inter tubular space. Severity of these changes was more in the group-II.

Keywords: Gross, Histopathology, Indoxacarb, Broilers

INTRODUCTION

Indoxacarb is a new broad spectrum insecticide that acts via blocking of the sodium channel, a new mechanism of action for insect control. It combines a favorable environmental profile with low mammalian toxicity and excellent crop protection (George *et al.*, 2000). Indoxacarb is a newly introduced carbamate compound in India having broad spectrum activities as pesticide being extensively used on the crops like maize, cotton and different vegetable plants for the protection from various insects and pests (Altuntas *et al.,* 2002).

Besides inhalation from polluted environment, animals are also exposed to pesticides through utilization of treated feeds and fodders and cause serious health hazard to different species of animals (Haas, 1983). Indoxacarb and its metabolites cause to block compound action potential in insect nerves and inhibit sodium currents in cultured insect neurons (Zhao *et al.*, 2003). Lethal dose of Indoxacarb induced toxicity in Birds is 98 mg/kg body weight. The effect of Indoxacarb shows various histopathological alterations in liver, kidneys, brain and testes of broilers.

MATERIALS AND METHODS

The study was conducted in four groups (T1, T2, T3 and T4), each group having (n=30) healthy, day old male broiler chicks of weighing about 50-55 gms. The dose rate was selected on the basis of lethal dose (98 mg/kg b.wt/day) of the insecticide in birds. The indoxacarb was given at 50%, 25% and 15% of lethal dose to group T2, T3 and T4 respectively, but in T1 (Control) group insecticide was not given. The Indoxacarb insecticide was mixed with corn oil and given orally. For gross and histopathology broilers were sacrificed on 28th day, organs were collected and fixed in 10% neutral buffered formalin.

Postmortem examinations of all the birds of toxicity and control groups, which either died or sacrificed, were carried out systemically. After recording the gross lesions the tissues from affected organs like liver, kidney, brain and testis were collected from sacrificed or died birds and subsequently preserved in 10% neutral buffered formalin for at least 24-48 hours. Further these tissues were processed by routine method of dehydration in graded alcohol, clearing in xylene and embedding in paraffin. Sections of 5-6 µ thickness were processed by conventional procedures using routine Haematoxyline and Eosin for histopathological studies (Lillie 1954).

RESULTS AND DISCUSSION

The gross and histopathological studies were

undertaken at the end of experiment on sacrificed experimental broilers of all the experimental groups. The gross examination of all the experimental broilers did not revealed any appreciable pathological changes. The histopathological examination of kidneys in indoxacarb treated broilers revealed cellular swelling, vacuolar degenerative changes, increased sinusoidal spaces, dilation of central vein, minimal to mild fatty change and multifocal mononuclear cell infiltration. The hepatocytes showed mild to moderate necrobiotic changes along with mild focal congestion. The hepatocytes showed mild to moderate necrobiotic changes along with mild focal congestion. The histopathological lesions in group-II and III were characterized by marked congestion and haemorrhages, mononuclear cell infiltration in hepatic parenchyma, mild vacuolar degenerative changes, marked necrobiotic changes in pericentral area. Moderate to severe type of fatty change along with occasional eosinophilic degenerative changes at places was also noted as histopathological change. The liver of broiler in group-IV showed focal areas of moderate congestion, moderate to severe fatty changes, minimal to mild necrobiotic changes at places and dilation of sinusoidal spaces. However, the liver of control group broilers did not showed any appreciable histopathological change.The histopathological findings observed in the liver correlates with the findings of Das and Mukherjee (2000), Benjamin *et al.* (2006), Shit *et al.* (2008) and Bhilegaonkar *et al. (1975).*

The histopathological examination of kidneys in indoxacarb treated broilers revealed, minimal to mild cellular swelling, mild to moderate congestion, vacuolar degeneration and focal mononuclear cell infiltration with occasional lymphoid aggregation at places. Hemorrhagic cysts of variable sizes characterised by presence of RBCs in lumen were also noticed at places. Affected tubules showed presence of hyaline masses in their lumen.

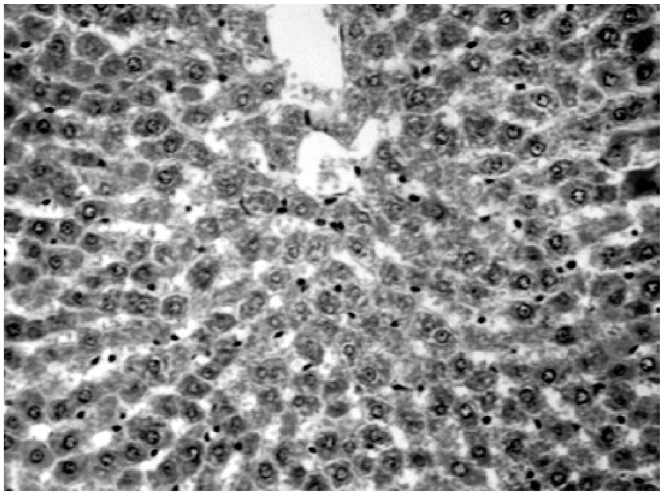

Fig 1.
Photomicrograph of liver showing congestion, dilation of sinusoidal spaces and mononuclear cell infiltration (MNC) with hypertrophy of hepatocytes. H & E 400X

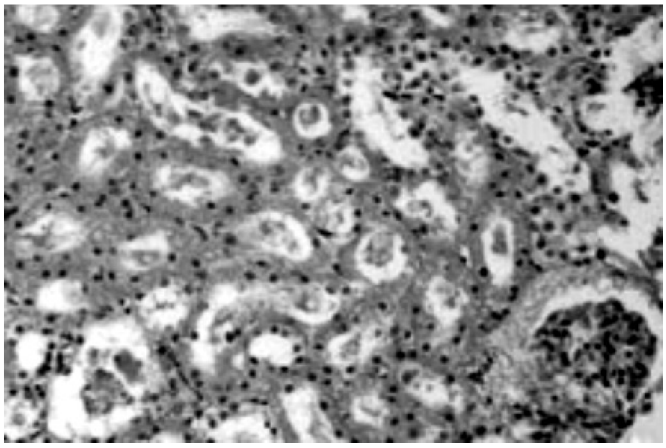

Fig 2.
Photomicrograph of kidney showing necrosis, infiltration of leucocytes and hyaline material in the affected tubules. H & E 400X

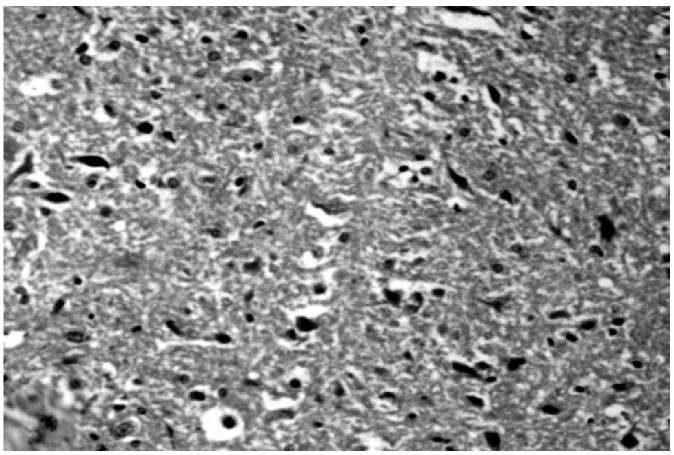

Fig 3.
Photomicrograph of brain showing vacuolar degeneration and mononuclear cell infiltration. H & E 400X

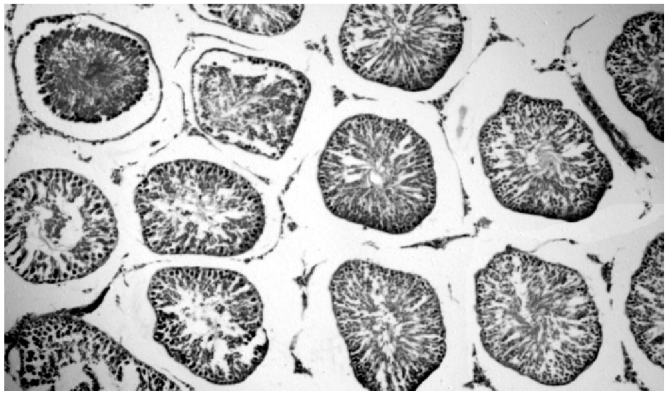

Fig 4.
Photomicrograph of testis showing increased intertubular space, wrinkling of basement membrane and atrophy of seminiferous tubules. H & E 100X

Mononuclear cell infiltration and marked congestion at corticomedullary junction was appeared to be important change in indoxacarb exposed broilers. Tubules showed presence of hyaline masses. The kidney of control group broiler did not showed any histopathological alterations. The histopathological findings observed in the kidney correlates with the findings of Bhilegaonkar *et al.* (1975) observed haemorrhages in kidney due to repeated oral dose of carbamate group insecticide in rats.Similarly, Benjamin *et al.* (2006) investigated the induced effect of orally pesticides on liver and kidney of albino rats. The kidney of pesticide exposed rats showed demarcated tubular necrosis and degeneration parenchyma.

In the Brain sections from group-II broilers there was marked vacuolation, moderate congestion and focal mononuclear cell infiltration. Brain sections from group-III broilers revealed mild multifocal vacuolation along with mononuclear cell infiltration and congestion. While in group-IV broilers similar lesions were also observed but its severity appeared to be low profiled. Brain sections of control group animals did not reveal any noticeable histoarchitectural alterations. The histopathological findings observed in the brain correlates with the findings of Das and Mukherjee (2000).

Microscopic examination of testes sections of group-II revealed increased intertubular space, degenerative changes in seminiferous tubules, marked vacuolation, reduced population of germinal cells, partial to complete arrest of spermatogenesis. Sections of testes from group-III and IV showed wrinkled basement membrane, decrease in spermatogenic cell population and vacuolation. There was increase in intertubular space and partial absence of spermatogenesis in some seminifesous tubules. The histopathological studies of testes from group-I broiler did not revealed any alterations. The histopathological findings observed in the testis correlates with the findings of Kurtz and Hutchinson (1982) observed degenerative changes in testes due to indoxacarb toxicity in ruminants.

Shit *et al.* (2008) used indoxacarb in rats, which is a broad spectrum neurotoxic oxadiazine insecticide used against lepidopteron pests of maize, cotton, fruits and vegetables. Rats were divided randomly in three groups of six animals each, group-I control, group-II dosed with 12 mg/kg and group-III dosed with 24 mg/kg orally daily for a period of 28 days. Necropsy performed on 29th day, showed increased intertubular space in testes, moderate vacuolation of seminiferous tubules in indoxacarb treated rats than control rats.

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Research Article

PHYTOCHEMICAL AND ACUTE TOXICITY STUDY OF *ADHATODA VASICA* AND *BERBERIS ARISTATA*

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ABSTRACT

The per cent extractability of *Adhatoda vasica* and *Berberis aristata* was found to be more in alcohol than water. The consistency of aqueous and alcoholic extracts of *A. vasica* and *B. aristata* was noted as viscous. The colour of the extract was dark green in case of *A. vasica* and dark yellow in case of *B. aristata.* The alkaloid, reducing sugar, glycoside, tannin, saponin, sterol and fixed oil were present in extracts of *A. vasica*. The different constituents of aqueous and alcoholic extracts of *B. aristata* were same as that of *A. vasica* except the sterols, which was not detected. The ALD_{50} of aqueous and alcoholic extracts of *A. vasica* and aqueous extract of *B. aristata* was more than 2000 mg/kg body weight, ip. Thus, these were regarded as safe when compared with alcoholic extract of *B. aristata* (ALD_{50} is more than 250 mg/kg, ip).

Key words: *Adhatoda vasica, Berberis aristata*, Phytochemistry, Acute toxicity.

INTRODUCTION

The plants could be used either in the form of powder, extract and solution, alone or in combination. The leaves of *A. vasica*, commonly known as "Vasaka" are commonly prescribed in Ayurvedic system of medicine for treatment of diseases like rheumatism, chronic bronchitis, cough and asthma (Chopra *et al.*, 1956). The ethnolic extract of leaves of *A. vasica* have also been reported to possess anti-oxidant activity in mice (Singh *et al.*, 2000). The root bark of the *B. aristata*, commonly known as "Daru-haldi" has been found to be effective in ulcers, fever, chorea and acute diarrhoea. It has also been reported to possess anti-inflammatory activity by Akhter *et al.*, 1977. Phytochemical study was aimed in the present work to find out the presence of different active principles in aq. and alc. extracts of *A. vasica* and *B. aristata*. Acute toxicity study was also undertaken in the present study to arrive on a dose to be employed in experimental animals.

MATERIALS AND METHODS

Extractability

Fresh extract was prepared every time for phytochemical study. One gram of plant powder was boiled in 10 ml of distilled water for 5 minutes. The entire content was filtered through Whatman filter paper. Filtered liquid volume was made to 10 ml by adding distilled water.

The plant powder was soaked in distilled water and kept it for 48 hours, then filtered. The filtrate was evaporated on boiling water bath. The per cent extractability was calculated (Shrivastava, 1980) by the following formula:

$$\text{Per cent extractability} = \frac{\text{Total amount of extract obtained}}{\text{Total weight of powder taken for extraction}} \times 100$$

The powdered leaves of *Adhatoda vasica* and root bark of *Berberis aristata* were extracted with absolute alcohol in a Soxhlet apparatus, heated over the Soxhlet extraction heater. The absolute alcohol was then redistilled from the extract. The residue was then transferred to previously weighed petridish and evaporated till it was free from alcohol. The per cent extractability was estimated by the method described earlier.

Phytochemical study

The phytochemical study of aqueous and alcoholic extracts of *Adhatoda vasica* and *Berberis aristata* was done following the methods described by Das *et al.* (1964), Pandey (1980) and Tandale *et al.* (1986). The extracts were analysed qualitatively for the presence of various active principle viz. alkaloid, reducing sugar, glycoside, resin, tannin, sterol, fixed oil etc.

Acute toxicity studies

Apparently healthy Norwegian strain of inbred albino rats of either sex weighing 100-150 gms were used in the present study. The rats were kept in colony cages under identical managemental conditions. The animals were fasted overnight before the experimentation. Only water was given *ad libitum.*

The aqueous and alcoholic extracts of *A. vasica* and aqueous extract of *B. aristata* were administered in graded doses of 500, 1000 and 2000 mg/kg weight, ip and alc. ext. of *B. aristata* was administered in graded doses of 250, 300, 500 and 1000 mg/kg body weight, ip, to determine approximate LD_{50}. Each group consisted of six rats. The observations were made upto 48 hours to record the mortality, if any. The approximate median lethal dose upto 48 hours to record the mortality, if any. The approximate median lethal dose was calculated as per the method of Dhar *et al.* (1968).

During studies, effects on central nervous system

Table 2:
Chemical Constituents in Aqueous and Alcoholic Extracts of *Adhatoda vasica* and *Berberis aristata.*

Active principles	Test applied	*Adhatoda vasica*		*Berberis aristata*	
		Aqueous extract	Alcoholic extract	Aqueous extract	Alcoholic extract
Alkaloids	(A) Dragendroff's reagent	Positive	Positive	Positive	Positive
	(B) Wagner's reagent	Positive	Positive	Positive	Positive
Reducing sugar	(A) Benedict's reagent	Positive	Positive	Positive	Positive
	(B) Fehling's reagent	Positive	Positive	Positive	Positive
Glycosides	(A) Fehling's reagent	Positive	Positive	Positive	Positive
	(B) Benedict's reagent	Negative	Negative	Positive	Positive
Tannin	(A) Lead acetate	Positive	Positive	Positive	Positive
	(B) Ferric chloride	Positive	Positive	Positive	Positive
Resin	Alcohol containing extract in distilled water	Negative	Negative	Positive	Negative
Saponin	Foam test	Positive	Positive	Positive	Positive
Sterol	Salkowski reaction	Positive	Positive	Negative	Positive
Fixed oil	With filter paper	Positive	Positive	Positive	Positive
Protein	(A) Xanthoprotein test	Negative	Negative	Positive	Positive
	(B) Biuret test	Negative	Negative	Negative	Negative
Amino acid	Ninhydrin test	Positive	Positive	Positive	Negative
Anthroquinione	Bontrager's test	Negative	Negative	Negative	Negative
Flavonoid	Extract dissolved in ethanol and heated with HCL and Mg turning	Negative	Positive	Negative	Positive

viz. stimulation, depression, behaviour, respiration and effects on locomotory system were recorded.

RESULTS AND DISCUSSION

The per cent extractability of *A. vasica* and *B. aristata* in water and alcohol as well as the active principles present in both the extracts are shown in table 1 and 2, respectively.

The per cent extractability of aqueous and alcoholic extracts of *A. vasica* was found to be 14.78 and 23.67 per cent, respectively. The consistency was viscous and colour was dark green. Patel (1974) also recorded 16.40 per cent extractability of *A. vasica* in alcohol. The per cent extractability of aqueous and alcoholic extracts of *B. aristata* was found to be 8.33 and 20.01 per cent, respectively, whereas Sharma and Srivastava (1991) recorded 28.00 per cent extractability in water and 8.20 per cent in alcohol. The consistency of both the extracts of *B. aristata* was viscous and colour was dark yellow.

The alkaloid, reducing sugar, glycoside, tannin, saponin, sterol, fixed oil and amino acid was noted to be present in aqueous extract of *A. vasica.* However, resin, protein, anthroquinone and flavonoid could not be detected. Similarly, alc. ext. of *A. vasica* showed the presence of alkaloid, reducing sugar, glycoside, tannin, saponin, sterol, fixed oil, protein, amino acid and flavonoid, but the resin and anthroquinone was not detected in alc. ext. of *A. vasica* (Table-1).

Gupta and Chopra (1954) obtained 0.075 per cent yield of essential oil by steam distillation of the leaves, flowers and roots. Amin and co-workers (1963) isolated vasicinone, an auto oxidative product of vasicine from the extract of leaves of *A. vasica.* The reports of Patel (1974) also confirms the results of the present study.

Similarly, the results of aqueous and alcoholic extracts of *B. aristata* have been shown in Table-2. The alkaloid, reducing sugar, tannin, resin, saponin, fixed oil and amino acid in aqueous extract of *B. aristata* but this extract did not show the presence of glycoside, sterol, protein, anthroquinone and flavonoid. Similarly, alc. ext. of *B. aristata* showed the presence of alkaloid, reducing sugar, glycoside (in traces), tannin, saponin, sterol, fixed oil and flavonoid, whereas, resin, protein, amino acid and anthroquinone could not be detected.

Berberis aristata has been reported to possess alkaloid berberine, as reported by Chopra *et al.* (1958), which are similar to the findings of the present study. Root bark of *B. aristata* has been reported to be rich in alkaloid (Kirtikar and Basu, 1933). Further, Chopra *et al.* (1986) have reported the efficacy of root bark and wood ext. of *B. aristata*. The presence of different active principles observed in aqueous and alcoholic extracts of *B. aristata* also corresponds to the reports of Sharma and Srivastava (1991).

The ALD_{50} of aqueous and alcoholic extracts of *A. vasica* and aqueous extract of *B. aristata* was found to be beyond 2000 mg/kg, ip, in rats. The acute toxic symptoms were initial depression, dullness, increased respiration, huddling and decreased spontaneous motor activity. No behavioral changes were observed in rats with doses of 500 and 1000 mg/kg, ip. A similar observation has also been reported by Sharma and Srivastava (1991) who reported the oral ALD_{50} of Teeburb (a herbal preparation containing *B. aristata* as important constituent) more than 2000 mg/kg body weight.

There was no mortality at the dose of 250 mg/kg,

ip, in 48 hours post-treatment, with alcoholic extract of *B. aristata*. The ALD_{50} of alcoholic extract of *B. aristata* as such was more than 250 mg/kg, ip, in rats. The acute toxic symptoms showed were initial depression, dullness, huddling and decreased spontaneous motor activity. Dhar *et al.* (1968) reported the intraperitoneal LD_{50} of *B. aristata* as 200 mg/kg body weight. Gupta and Chopra (1954) investigated the toxicity of the essential oil of *A. vasica* on albino mice and guinea pigs and found it to have very low toxicity. Patel (1974) also reported that the aqueous and alcoholic extracts of *A. vasica* did not reveal any toxicity up to the dose of 2000 mg/kg, orally and intraperitoneally in rats and mice, respectively. These results therefore, showed that *A. vasica* does not possess any acute toxicity.

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Research Article

EVALUATION OF DETOXIFIED JATROPHA CURCAS SEED CAKE ON REPRODUCTIVE PARAMETERS IN WHITE LEGHORN COCKERELS

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ABSTRACT

The present study was designed for the evaluation of normal as well as detoxified *Jatropha curcas* seed cake as 5% and 10% supplement in normal feed and its effect on reproductive parameters in White Leghorn cockerels. The study was conducted in 35 white leghorn cockerels for 90 days. The birds were divided randomly and equally into five groups with seven birds in each group. Group I served as control, groups II and III were fed untreated *Jatropha* seed cake at 5% and 10%, respectively, and groups IV and V were fed detoxified *Jatropha* seed cake at 5% and 10%, respectively. Reproductive parameters showed prominent effects after 90 days of feeding of 5% detoxified *Jatropha* seed cake whereas 5% normal seed cake and 10% detoxified seed cake produced toxic effect at 60 day feeding.

Key words : *Jatropha curcas* seed cake, reproductive toxicity, white leghorn cockerels.

INTRODUCTION

Jatropha curcas, known as Ratanjyot and is recognized as biodiesel plant**.** *Jatropha* plant have been used since antiquity, as folk medicines for man and human and veterinary ailments, from a long time for having potential anticancerous, hepatoprotective, antiulcer, antifungal, tocolytic, antidiabetic, anti-inflammatory, antioxidant, antimicrobial, wound healing and anthelmintic activity, however, also reported to cause accidental toxicity in animals (Ahmad and Adam, 1979a). The present study was conducted to evaluate the effect of detoxified seed cake on reproductive parameters.

MATERIALS AND METHODS

Experimental design

The present study was designed for evaluation of the detoxified *Jatropha curcas* seed cake as 5% and 10% supplement in normal feed for 90 days in white leg horn cockerels. The study was conducted in 35 white leghorn cockerels for 90 days. Thirty five (6 weeks old) white leghorn (WLH) cockerels, procured from Instructional Dairy Farm of the university and maintained in experimental poultry shed in battery cage system under standard managemental conditions, were divided randomly and equally into five groups with seven birds in each group. Group I served as control, groups II and III were fed untreated *Jatropha* seed cake at 5% and 10%, respectively, and groups IV and V were fed detoxified *Jatropha* seed cake at 5% and 10%, respectively.

Detoxification of *Jatropha* seed cake

Detoxification of toxic principles in *Jatropha* seed cake was done by boiling at 121°C, 15 lbs pressure for 20 minutes, followed by soaking with 0.2N potassium hydroxide solution for 30 minutes and subsequently washing with water to remove potassium hydroxide. Treated seed cake meal was dried at 60ºC in oven for overnight. Dried meal was milled to reduce the particle size for the proper mixing in the normal poultry ration.

Reproductive parameters

Collection of semen

The abdominal cavity of cockerels was cut open after slaughtering. The right and left vas deferens were surgically excised from their point of entry at the cloaca to the inferior edge of kidneys. The contents of the vas deferens were collected in a test tube maintained at 38-40°C with an insulated jacket. A water bath and a slide warmer were used to maintain the samples at this temperature until sperm motility were assessed.

Mass motility of spermatozoa in neat semen

Mass motility in neat semen was estimated by the method of **Wheeler and Andrews (1943)** and graded as per the standards.

Sperm concentration

The concentration of sperms was measured by the method of **Allen and Champion (1955)** using direct cell count method with hemocytometer. The concentration of sperm per volume was found using the formula:

C = 50, 000 x N x D,

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

Percent dead spermatozoa

The percentage of dead spermatozoa was done by differential staining (vital staining) technique **(Clark *et al.,* 1984) and** calculated as:

$$\% \text{ Dead Sperms} = \frac{\text{No. of dead sperms counted}}{\text{Total no. of dead sperms}} \times 100$$

The testicles were preserved in 10% formalin for histopathological examination.

Table 1:
Effect on Reproductive Parameters following 90 days feeding of normal and detoxified *Jatropha curcas* seed cake as 5% and 10% supplement in normal ration in WLH cockerels (Mean±SE, n=7)

Parameters	Groups				
	I	II	III	IV	V
Mass motility	3.6± 0.24	3.2 ±0.37	3.2 ±0.2	3.4 ±0.24	3.2 ±0.2
Dead %	22.6 ±1.66	29.8 ±1.88[a]	34.2 ±1.93[a]	23.2 ±1.56[bc]	6.2±1.66[bc]
Sperm concentration	2.08 ±0.11	1.86 ±0.1	1.32 ±0.06[a]	2.02 ±0.12[c]	2 ±0.16[c]

a= Value differ significantly with 1st coloumn in a rowb= Value differ significantly with 2nd coloumn in a rowc= Value differ significantly with 3rd coloumn in a rowd= Value differ significantly with 4th coloumn in a row

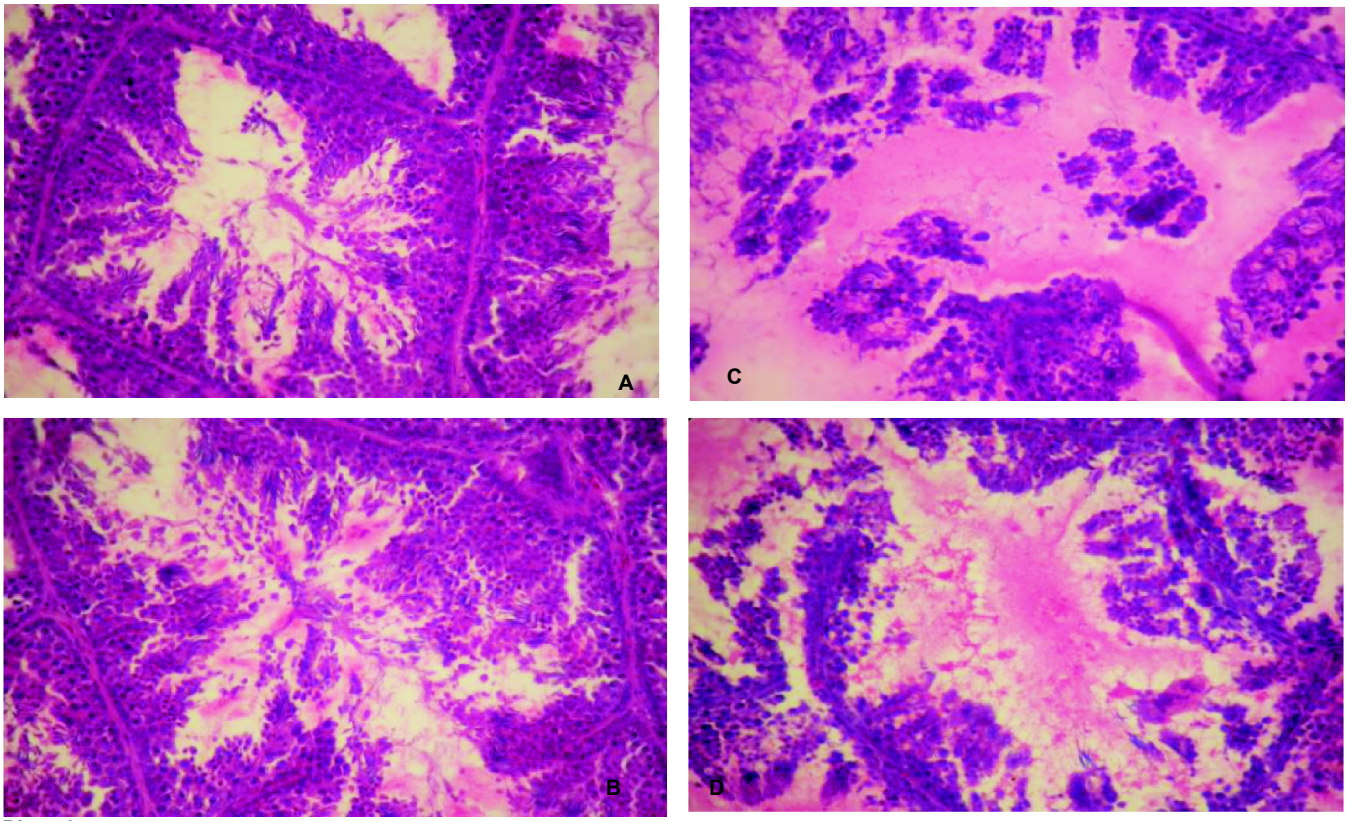

Plate 1:
Photomicrograph of testis showing intact (A), mild degeneration (B), severe degeneration (C)and damaged (D) seminiferous tubules following 5% and 10% detoxified JCS cake for 90 days in cockerels (H&E, 400X).

Statistical analysis

Statistical analysis of 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

The mass motility of the sperms in II, III and IV groups was slightly lower than that of control group but no significant ($P < 0.05$) changes were observed at 90 day of trial in WLH cockerels (Table 1). There was a significant ($P < 0.05$) increase in percentage of dead sperms in 5% and 10% normal seed cake fed groups as compared to control and 5% and 10% detoxified JSC supplement fed groups (Table1).

There was a significant ($P < 0.05$) decline in sperm concentration of WLH cockerels of 10% normal seed cake supplement fed group as compared to control and 5% and 10% detoxified JSC fed groups (Table 1). The decline in sperm concentration and increased dead sperms indicate reproductive toxicity causing potential of *Jatropha curcas*. However, the detoxified groups did not show any change indicating destruction of the antinutritional factors causing reproductive damage during the detoxification.

There was significant increase in dead sperm percentage and decrease in sperm concentration in 10% JSC fed groups as compared to control at the end of the trial. This indicates reproductive toxicity causing potential of *J. curcas*. However, the detoxified groups did not show any change indicating destruction of the antinutritional

factors causing reproductive damage during the detoxification. The results are also supported by damaged testicular parenchyma in toxic group. Similar results were obtained by Akanbi, (1984) in WLH roosters fed with gossipol acetic acid present in cotton seed cake and in rats fed with gossypol (Hadley *et al*., 1981). Ogbuewu *et al*. (2009) reported similar reduction in sperm count and increased dead and abnormal sperms following feeding of neem kernal cake.

Histopathological changes in testis following 5% and 10% detoxified JCS cake for 90 days in cockerels have bee depicted in plate 1 (A to D) which reveals mild to moderate degerative changes and damaged testicular parenchyma in *Jatropha* treated groups. Liver showed hyperemia, vacuolar degeneration of hepatic parenchyma, dilatation of sinusoids and hemorrhage and kidney revealed tubular nephritis with infiltration of inflammatory cells, shrunken glomeruli, degeneration and necrosis of tubular epithelium. The testicles showed mild degeneration and necrotic changes. The degenerative changes in liver, kidney and testicles may be attributed to presence of curcin, toxalbumin of *J. curcas* seed cake. The results of this study are in agreement with the histopatological findings of on toxicity of deoiled *Jatropha* seed cake at 5% and 10% replacement in feed in broiler chicks (Kumar *et al*., 2010). The similar results were found by Ahmad and Adam, (1979a) in calves at 0.025 g/kg seed meal up to 14 days. It is concluded from the 90 days study in white leghorn cockerels that reproductive parameters showed prominent alterations after 90 days of feeding of 5% detoxified *Jatropha* seed cake.

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Research Article

DETOXIFICATION OF THE SEED CAKE OF JATROPHA CURCAS AND ITS TOXICITY EVALUATION IN GOATS

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ABSTRACT

The present study was designed to evaluate effect of various detoxifying treatments of seed cake of *Jatropha curcas* following six week oral feeding in goats in order to develop its utility as a feed supplement in goat ration. The study was conducted on twelve healthy goats, weighing 28-32 kg and 16-18 months old, equally and randomly divided in four groups of three goats in each group for evaluation of the neutralization potential of the different treatments to the seed cake and its toxicity by adding in the diet of the goat @ 10% of the daily diet for 42 days of administration. Group I served as control whereas group II, III and IV served as boiling treatment group, soaking treatment group and soaking plus boiling treatment group, respectively. A significant ($P<0.05$) decrease in Hb, PCV, TEC, TLC, total serum proteins, albumin and globulin values were observed in all treated groups II,III and IV in comparison to the control group I. A significant ($P<0.05$) increase in serum creatinine, serum urea, cholesterol, AST, ALT and ALP was observed in all groups in sub chronic study. Antioxidative parameters as LPO (lipid peroxidation) and GSH (reduced glutathione) increased in the group II, III and IV in comparison to the control. It is concluded from the study that seedcake with different treatments as boiling, soaking and soaking plus boiling have proven safe without any evident toxic effect for 28 days. Boiling of seed cake group was better for neutralization of the antinutritional factors than soaking and soaking plus boiling as it delayed the onset of toxicity.

Key words: Goats, *Jatropha* seed cake, detoxicification, phorbol esters, subchronic toxicity.

INTRODUCTION

Jatropha curcas Linn (Greek:-iatros-doctor, trophe-food), a wonder plant of barren land better known as physic nut, Ratanjyot , a member of Euphorbiaceae family same as rubber tree and has Central and South American origin (Heller, 1996). The plant does not compete with conventional field crops and thus could be an ideal choice to make use of infertile baron land resources that are in majority underutilized till so far**.** The *Jatropha* plant is receiving attention as an alternative to the energy source (Kumar and Sharma, 2008). The major energy carriers for *Jatropha* is raw oil and esters and its use as replacement of energy sources is well documented (Kywe and Oo, 2009).The seed resembles with the castor seed in shape but is smaller in size with dark brown colour. It weighs about 0.75g and contains 30-32% protein and 60-66% lipid (Liberalino *et al.*,1988).

Production of biodiesel by using the seed oil of *Jatropha curcas*, as a raw material, also generates large amount of solid residue, called *Jatropha* seed cake, a co-product generated in the process of oil extraction, which is composed of lignocellulosic compounds, water, proteins and minerals (Achtena *et al.*, 2008). However, it also contains toxic compounds and anti-nutritional factors such as phorbol esters, and may be valuable animal feed after detoxification and can also be used as a fertilizer. The detoxification and recycling of this cake is beneficial to add economic value, and also reduces the chances of toxic manifestations caused due to improper disposal of this by-product. Nevertheless, many products can be obtained from *Jatropha* residues such as fertilizer from the seed cake, pesticides and medical bio-active compounds from *Jatropha* extract (Singh *et al.*, 2008). Several studies have been carried out on the holistic approach to utilize *Jatropha* as a source of multiple energy carriers (Makkar and Beckar, 2009; Gunaseelan, 2009). Therefore, in view of its potential supplementation with normal feed in animals, this investigation was undertaken to detoxify *Jatropha* seed cake and to evaluate its subchronic toxicity in goats.

MATERIALS AND METHODS

The *Jatropha* seed cake used in the experiment were collected from Medicinal Plant Research and Developmental Centre (MRDC)**,** G.B.P.U.A & T, Pantnagar**.** All the chemicals required for this study were procured from Hi Media. ERBA diagnostics kits were used for biochemical analysis of serum total proteins, albumin, serum creatinine, urea, cholesterol and enzymatic activities of serum aspartate aminotransferase, alanine aminotransferase and alkaline phosphatise activity. Experiment was conducted after the approval of the IAEC.

Experimental design

Sub chronic toxicity trial of seed cake was done by giving three types of treatment to the seed cake for neutralization of the toxicants using methods as follows:
1. Boiling in water for 20 min

2. Soaking in 0.1N NaOH for half an hour and dried
3. Soaking for half an hour plus boiling for 20 minutes

After all three treatments the healthy goats of 16-18 months of age were randomly divided into 4 groups of 3 goats each. Group I served as control and group II, III and IV were fed on feed supplemented @ 10 % with detoxified *Jatropha* seed cake by boiling, soaking and soaking + boiling, respectively, for 42 days (Table1). All the animals were kept under constant observation for 42 days and the clinical parameters were recorded daily. Goats were regularly observed for clinical signs such as change in appetite, appearance of dullness, diarrhoea, letharginess etc. After 0, 14th, 28th and 42nd day interval the blood samples were collected for hematobiochemical analysis and anti oxidative analysis..

Hematology

4.0 ml of blood was collected from each goat in clean heparinised microcentrifuge tube and haematological parameters such as packed cell volume, haemoglobin, total erythrocyte count and total leucocytes count were estimated immediately after the collection of blood samples. 0.1N-HCl was used for estimating the blood haemoglobin concentration while, Hayem's RBC diluting fluid and Thomas's WBC diluting fluid were used for TEC and TLC estimation, respectively(Jain, 1986).

Blood biochemical profile

The serum total proteins (Koller, 1984), albumin, globulin (Gendler, 1984), total cholesterol (Warnick *et al.,*1985), serum creatinine and urea were estimated by using ERBA diagnostic kits.

Serum enzyme profile

Serum enzymes viz. aspartate aminotransferase**,** alanine aminotransferase and alkaline phosphatase activity were estimated using standard methods (Murray,1984).

Antioxidative parameters

Parameters for oxidative stress including lipid peroxidation (LPO) by measuring level of malondialdehyde (Rehman, 1984) and reduced glutathione (GSH) (Prins and Loos, 1969) were determined in erythrocytes. Separation of erythrocyte pellet from blood samples was done immediately after collection of the blood sample. Absorbance of all the estimations was read on UV-VIS spectrophotometer.

Statistical analysis

Statistical analysis of data was done by using ANOVA technique. Comparisons among treated and untreated groups were made with help of student's' test. Statistically significant difference was considered at 5% (Snedecor and Cochran, 1969).

RESULTS AND DISCUSSION

There was general weakness, anorexia after third week of feeding of *Jatropha* seed cake mixed diet in group II. No Mortality occurred in any group during the entire course of the study. There was a significant reduction in Hb, PCV, TEC and TLC values in treated groups i.e. II, III and IV ($P < 0.05$) as compared to control might have been due to the haemolytic factor possibly being left in small fractions after neutralization such as phorbol esters, curcin etc. which causes the destruction of erythrocytes and migration of the fluid from extracellular compartment to the intracellular compartment. These finding are in accordance with the findings of Sirsha *et al.* (2008) following feeding of seed cake at 5% level in diet in broiler chicken for 6 weeks. Similar findings were reported following *Jatropha* intoxication in rats at the dose rate of 25% *Jatropha* seed protein on 7th, 14th and 22nd day (Awasthey *et al*, 2011) and in rabbits receiving 5%, 7.5% and 10% *Jatropha* seed meal for 6 weeks (Abdel-safy *et al.*, 2011). A significantly higher value of Hb, PCV, TEC and TLC were observed in IV (soaking plus boiling group) on 14th, 28th and 42th day interval which indicated that the treatment of the seed cake with soaking and boiling was more efficient than boiling and soaking alone in case of haematological parameters. The higher values of the above parameters in with treatment group IV showed that detoxification process has neutralized the toxic principles in the seed cake of *Jatropha curcas* (Table 2).There was significant decrease in total serum protein, albumin and globulin in all treated groups as compared to the control group I that might be attributed to hepatotoxicity and liver insufficiency. The susceptibility of animals to feeding with plant materials is dependent on the type of active constituents and concentration of the content added to the diet as well as the rate of their metabolism in the liver and consequent renal and biliary elimination. Toxic and antinutritional components present in seed meal including Phorbol esters, phytates, saponins and trypsin inhibitors which might be responsible for this effect (Makkar and Becker, 1997). The reduction in the globulin mainly comprising of immunoglobulin is an indicator of the lypmhocytotoxic effect of the seed cake as the destruction of the lymphocytes might be the reason for

Table 1:
Experimental design for sub chronic toxicity of detoxified *Jatropha curcas* seed cake in goats (n=3).

Groups	Treatment	(Dose)	Days of administration
I	Control	Normal feed	42 days
II	Seed cake with boiling treatment	10% of the diet	42 days
III	Seed cake with soaking treatment	10% of the diet	42 days
IV	Seed cake with treatment of soaking +boiling	10% of the diet	42 days

* Treatment comprised of sod. thiosulphate @ 50 mg/kg b wt i.v. route and glutathione @ 0.25 mg/kg b wt via i.m. route.

Table 2 :
Effect on hematological values following daily oral administration of detoxified *Jatropha* seed cake for 42 days in goats (Mean value ± S.E, n=3).

Parameters		Groups			
		Control(I)	Boiling(II)	Soaking(III)	Soaking+boiling (IV)
Haemoglobin (g%)	0 Day	8.25±0.322	8.00±0.361^{a}	7.99±0.153^{a}	8.39±0.231^{a}
	14 days	8.19±0.293^{A}	7.51±0.200^{A}	7.40±0.127^{bA}	8.01±0.036^{b}
	28 days	8.18±0.296^{A}	6.96±0.098^{aA}	6.82±0.014$^{ab\ A}$	6.85±0.146^{abA}
	42 days	8.10±0.228^{A}	6.77±0.174^{aA}	6.62±0.076$^{ab\ A}$	6.73±0.154^{abA}
PCV (%)	0 Day	29.62±0.663	28.94±0.005	28.92±0.512	30.77±0.257^{a}
	14 days	29.73±0.946 ^{A}	26.70±0.763 ^{AB}	25.36±0.407^{AC}	29.90±0.581^{bBC}
	28 days	29.30±0.1.09^{A}	24.56±0.949 ^{AB}	24.33±0.727 ^{AC}	27.84±0.603$^{abc\ BC}$
	42 days	29.28±1.866	24.89±1.000	23.52±1.212	24.89±0.577^{abc}
TEC (X 10^{6}/µl)	0 Day	11.46±0.066	11.45±0.057^{a}	11.33±0.127^{a}	11.44±0.066 ^{a}
	14 days	11.58±0.116	10.22±0.171$^{ab\ A}$	10.08±0.100^{abA}	10.35±0.206^{abA}
	28 days	11.36±0.203^{A}	8.81±0.090$^{abc\ AB}$	8.96±0.038$^{ab\ cAC}$	9.68±0.108$^{ac\ ABC}$
	42 days	11.55±0.184 ^{A}	7.95±0.037^{abcA}	7.66±0.181 ^{abcA}	8.30±0.435^{abcA}
TLC (X 10^{3}/µl)	0 Day	9.44±0.135	9.59±0.194^{a}	9.18±0.303	9.17±0.315^{a}
	14 days	9.36±0.021 ^{A}	9.03±0.101$^{b\ B}$	8.58±0.187^{AB}	8.87±0.165^{bA}
	28 days	9.34±0.053^{A}	8.46±0.372$^{a\ A}$	8.32±0.063 ^{A}	7.93±0.15$^{ab\ A}$
	42 days	9.35±0.052 ^{A}	7.93±0.317 $^{ab\ A}$	8.08±0.363 ^{A}	7.75±0.243 $^{ab\ A}$

Table 3:
Effect on biochemical values following daily oral administration of detoxified *Jatropha* seed cake for 42 days in goats (Mean value ± S.E, n=3).

Parameters		Groups			
		Control(I)	Boiling(II)	Soaking(III)	Soaking+boiling (IV)
	0 Day	7.16±0.145	6.80±0.230^{a}	7.16±0.536^{a}	7.16±0.202 ^{a}
	14 days	7.06±0.073^{A}	6.49±0.029^{bAB}	7.00±0.015^{bBC}	6.75±0.061 ^{abABC}
	28 days	6.88±0.057^{A}	5.94±0.028^{abcAB}	5.54±0.006$^{ab\ AB}$	5.65±0.021 $^{abc\ AB}$
	42 days	6.91±0.066 ^{A}	5.15±0.008 $^{abc\ AB}$	5.04±0.012 ^{abAC}	4.91±0.033 ^{abcABC}
Albumin(g/dl)	0 Day	3.46±0.185	3.40±0.115^{a}	3.63±0.296^{a}	3.50±0.230 ^{a}
	14 days	3.43±0.008 ^{A}	3.21±0.052$^{b\ AB}$	3.64±0.005^{bABC}	3.36±0.006 ^{bBC}
	28 days	3.30±0.057^{A}	2.84±0.066$^{abc\ A}$	2.92±0.009$^{ab\ A}$	2.85±0.010$^{abc\ A}$
	42 days	3.32±0.064 ^{A}	2.35±0.019 $^{abc\ AB}$	2.51±0.003 ^{bAB}	2.41±0.017 ^{abcA}
Globulin(g/dl)	0 Day	3.73±0.028	3.72±0.026^{a}	3.69±0.033^{a}	3.726±0.027 ^{a}
	14 days	3.71±0.023	3.26±0.004$^{ab\ AB}$	3.42±0.017^{abABC}	3.32±0.018 ^{abABC}
	28 days	3.71±0.011^{A}	3.21±0.014$^{ac\ AB}$	2.64±0.017$^{abc\ ABC}$	2.77±0.007$^{abc\ ABC}$
	42 days	3.70±0.030 ^{A}	2.92±0.027 $^{abc\ AB}$	2.55±0.005 $^{abc\ ABC}$	2.47±0.002 $^{abc\ ABC}$
Urea (mg/dl)	0 Day	23.33±0.466	23.32±0.107^{a}	23.55±0.293^{a}	23.28±0.590 ^{a}
	14 days	23.72±0.096^{A}	22.33±0.014$^{ab\ AB}$	21.89±0.055 ^{abABC}	23.58±0.039 ^{bBC}
	28 days	23.95±0.021 ^{A}	25.73±0.012$^{abc\ AB}$	27.58±0.347$^{abc\ ABC}$	24.62±0.192 $^{abc\ ABC}$
	42 days	23.24±0.058 ^{A}	37.07±0.353$^{abc\ AB}$	39.66±0.290^{abcABC}	42.02±0.032^{abcABC}
Creatinine(mg/dl)	0 Day	1.45±0.031	1.446±0.014^{a}	1.46±0.026^{a}	1.45±0.031^{a}
	14 days	1.45±0.037^{A}	1.37±0.018^{abB}	1.94±0.026 ^{abAB}	1.86±0.070 ^{abAB}
	28 days	1.44±0.056 ^{A}	2.53±0.007$^{abc\ AB}$	2.55±0.052$^{abc\ AC}$	2.88±0.005 ^{abcABC}
	42 days	1.46±0.017 ^{A}	3.92±0.027^{abcAB}	4.58±0.015$^{abc\ ABC}$	4.48±0.032 ^{abcABC}
Cholesterol(mg/dl)	0 Day	60.47±0.376	60.156±0.193^{a}	59.77±0.066^{a}	60.43±0.494 ^{a}
	14 days	60.74±0.418 ^{A}	52.63±0.096$^{ab\ AB}$	65.79±0.308^{abABC}	64.36±0.014^{abABC}
	28 days	60.88±0.054^{A}	69.44±0.057$^{abc\ AB}$	79.22±0.222$^{abc\ ABC}$	78.30±0.292$^{abc\ ABC}$
	42 days	60.74±0.387 ^{A}	81.42±0.042$^{abc\ AB}$	98.04±0.017$^{abc\ AB}$	96.59±1.886 ^{abcAB}

low level of gloulin in the serum. There was a significant (P<0.05) increase in serum urea and creatinine levels in all treated groups II , III and IV following daily oral administration of *Jatropha* seed cake after 28 days onwards in goats which might have been due to nephrotoxic effect possibly caused by curcin present in *Jatropha* seed cake. A similar finding was reported by Adeyemi *et al.* (2001) in 4-7 weeks old finishing broiler fed on replacement of 7.8, 15.6, 23.4 and 31.2% of dietary groundnut-cake with using boiled *Jatropha* curcas seed (30.6%). Similar findings were also reported in pigs fed with *J. curcas* meal (JCM) containing 0.8 mg phorbol esters/g being given as soybean meal protein replacer at 6.25, 12.5, 18.75, or 25% levels (Chivandi *et al.*,2006).Within the treated groups boiling group II showed the significant lower levels of urea and creatinine after 28th day of feeding in comparison to the group III and IV. There was significant increase in serum cholesterol level in all treated groups after 28th day of feeding. Damage to liver might have led to disturbances of fat metabolism resulting in increase in total serum cholesterol. Aspartate aminotransferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) values increased in the all treated groups as compared to

Table 4:
Effect on serum enzymatic activities following daily oral administration of detoxified *Jatropha* seed cake for 42 days in goats (Mean value ± S.E, n=3).

Parameters		Groups			
		Control(I)	Boiling(II)	Soaking(III)	Soaking+boiling(IV)
AST (U/L)	0 Day	21.13±1.766	21.33±1.324^{a}	21.84±1.377^{a}	23.84±0.811^{a}
	14 days	21.33±0.01^{A}	20.94±0.14$^{b\ AB}$	22.11±0.177^{bABC}	23.37±0.028^{bABC}
	28 days	21.66±0.063^{A}	35.63±0.641$^{abc\ AB}$	38.55±0.359$^{abc\ ABC}$	37.84±0.095$^{ab\ ABC}$
	42 days	24.42±0.561^{A}	52.40±0.401^{abcAB}	58.58±0.939^{abcAC}	53.38±0.727^{abcABC}
ALT(U/L)	0 Day	17.66±1.20	16.00±0.577^{a}	18.99±0.583^{a}	16.99±0.835^{a}
	14 days	17.61±0.027^{A}	17.14±0.080$^{b\ B}$	18.76±0.665^{bABC}	20.68±0.351^{abABCD}
	28 days	18.18±0.405^{A}	31.77±0.365$^{abc\ AB}$	32.87±0.121$^{abc\ ABC}$	34.52±0.916$^{abc\ ABCD}$
	42 days	18.25±0.376^{A}	41.54±0.907^{abcAB}	40.62±0.323^{abcA}	46.02±0.358^{abcAB}
ALP (U/L)	0 Day	62.92±0.192	61.41±0.820^{a}	62.37±0.488^{a}	62.74±0.472^{a}
	14 days	62.53±0.194^{A}	65.65±0.343^{abAB}	68.62±0.323^{abABC}	67.14±0.018^{abABC}
	28 days	62.85±0.693^{A}	69.73±0.259^{abcAB}	76.33±0.695^{abcABC}	83.50±0.672^{abcABC}
	42 days	63.41±0.326^{A}	80.48±0.255^{abcAB}	95.23±0.052^{abcABC}	97.76±0.461$^{abc\ ABC}$

Table 5:
Effect on antioxidative parameters in RBCs following daily oral administration of detoxified *Jatropha* seed cake for 42 days in goats (Mean value ± S.E, n=3).

Parameters		Groups			
		Control(I)	Boiling(II)	Soaking(III)	Soaking+boiling(IV)
LPO (nM MDA/ml)	0 Day	20.43±0.225	20.98±0.057^{a}	20.70±0.353^{a}	20.47±0.425^{a}
	14 days	20.53±0.338^{A}	25.06±0.367^{abA}	25.39±0.698^{bA}	25.18±0.655^{abA}
	28 days	21.03±0.669^{A}	32.74±0.864^{abcA}	35.62±1.101^{abcA}	34.51±0.870^{abcA}
	42 days	20.45±0.392^{A}	37.73±2.06^{abcA}	39.89±1.154^{abcA}	40.88±1.121^{abcA}
GSH(μM/ml)	0 Day	2.58±0.011	2.56±0.031^{a}	2.42±0.216^{a}	2.58±0.095^{a}
	14 days	2.58±0.014	2.77±0.100^{b}	2.82±0.056^{b}	2.67±0.04^{b}
	28 days	2.73±0.123^{A}	3.56±0.276$^{ab\ A}$	3.55±0.216^{abcA}	3.67±0.124$^{abc\ A}$
	42 days	2.84±0.043^{A}	3.98±0.057^{abA}	4.05±0.056^{abcA}	4.03±0..090$^{abc\ A}$

*Mean values bearing common superscripts with small letters differ significantly (P< 0.05) when compared vertically with in the same column and mean values bearing common superscripts with capital alphabets differ significantly (P<0.05) when compared horizontally in the same row

control. An increase in mean serum AST, ALT, ALP activity might be due to effect of *Jatropha* toxic metabolites which bound cellular macromolecules in the liver that might have resulted in cellular necrosis leading to discharge of intracellular contents into systemic circulation (Table 3 and 4).

Lipid peroxidation (LPO) and reduced glutathione (GSH) level in RBCs increased in seed cake treated groups indicated oxidative damage in erythrocyte which might be attributed to the toxic principles as curcin and phorbol esters present in the cake and that induces the generation of reactive species leading to the cascade of the lipidperoxidation, DNA fragmentation and depletion of GSH, reduction in activity of antioxidant cascade related enzyme like superoxide dismutase (SOD), glutathione peroxidase (GPx) and activation of glutathione reductase (GR) and catalase activities (Kumar *et al.*, 2007)**.** Within the groups boiling group II showed significant reduction in the LPO value after 28th day in comparison to the soaking group III and soaking plus boiling group IV indicated that the boiling treated seed cake is producing the lesser oxidative stress in comparison to the soaking and soaking plus boiling group whereas a non significant increase in GSH value was observed in soaking group III and soaking with boiling group IV in comparison with boiling group II on 42th day interval. (Table 5).

It is concluded from this study that the toxicity develops after 28th day feeding of all treated seed cake. The effect of the soaking plus boiling was better in maintaining the haematological parameters, probably because the soaking plus boiling neutralizes the toxic principles as curcin and phorbolesters which are responsible for the haemolytic effects of the seed cake .The effect of boiling treatment to the seed cake produces better biochemical and antioxidative profile as compared to the soaking and soaking plus boiling treatment.

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Research Article

ROLE OF TRPV4 CHANNEL IN OXYTOCIN-INDUCED UTERINE CONTRACTION

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ABSTRACT

Present study was undertaken in uterine tissues of pregnant and non-pregnant Swiss Albino mice. Oxytocin is a stimulant of myometrial contraction by causing influx of Ca^{2+} by receptor (GPCR) operated Ca^{2+} entry, as well as release from intracellular stores. TRPV4 channel is a crucial gateway for Ca^{2+} entry. Thus, we examined whether myometrial contraction induced by oxytocin is mediated through TRPV4 channel. The basal mean integral tension (MIT) in the uterine strips taken from late pregnant mouse was 26.79 ± 4.65 g min (n=6). The E_{max} of oxytocin-induced contraction in pregnant uterus (343.1 ± 30.51 %; n=6) was significantly ($p<0.001$) attenuated in the presence of HC067047, a selective TRPV4 channel antagonist (149.9 ± 13.70 %; n=6). The pD_2 of oxytocin-induced contractions (6.87 ± 0.44) was not altered by HC067047 (6.16 ± 0.48). The basal MIT in non-pregnant uterus was 7.87 ± 1.79 g min (n=6). Oxytocin did not alter the spontaneous myometrial contraction in non-pregnant mouse. The E_{max} and pD_2 of oxytocin-induced contraction in non-pregnant uterus were 107.3 ± 8.06 % and 8.45 ± 1.06, respectively. We conclude that TRPV4 channels have a role in oxytocin-induced myometrial contractions in pregnant mouse.

Key words: Oxytocin, HC067047, TRPV4 channel, mouse uterus

INTRODUCTION

Oxytocin is one of the most potent contractile agent for myometrial contractions. This substance is used clinically for inducing labor and cervical ripening.External Ca^{2+} entry into myometrial smooth muscle cells is important for uterine contraction in non-pregnant and pregnant uterus. Oxytocin increases $[Ca^{2+}]_i$ by Gáq and PLC signaling pathway (Bernal, 2007) and release from intracellular stores (Sanborn, 2007). Uterine stretch modulates uterine growth and contractility in pregnancy via alterations in calcium signaling (Dalrymple *et al.*, 2007). Role of TRPV4 channels has been implicated in the contraction of vascular smooth muscles by elevating $[Ca^{2+}]_i$ (Watanabe *et al.*, 2003).

Transient receptor potential vanilloid 4 (TRPV4) channels are nonselective cation channels which preferably permeate Ca^{2+}. The physiological functions of TRPV4 channels are central and peripheral thermosensing, mechano-sensing, osmosensing and basal Ca^{2+} homeostasis (Nilius *et al.*, 2003). Reports suggest that these channels cause direct contraction of smooth muscle by affecting Ca^{2+} entry (Sukumaran *et al.*, 2013). But their role in agonist-induced uterine contractions is not known. A recent study shows the role of these channels in 5-HT-induced contraction in mouse pulmonary artery (Xia *et al.*, 2013). Thus the present study was conducted to examine the role of TRPV4 channels in oxytocin-induced myometrial contractions in pregnant and nonpregnant mouse.

MATERIALS AND METHODS

Experimental Animals

Healthy adult female Swiss Albino mice (30-35 g) were procured from the Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh. Late pregnant and non pregnant mice were killed by cervical dislocation and uteri of pregnant and non-pregnant mouse were collected in Modified Krebs Hensleit solution (MKHS) and De Jalon Solution, respectively. Longitudinal uterine strips (4-5 mm×10 mm) were mounted and kept under a resting tension of 0.7 g in a thermostatically controlled (37.0±0.1ºC) organ bath (UGO Basile, Italy) The change in tension was measured by a high-sensitivity isometric force transducer (Model MLT 0202/D, AD Instruments, Australia) and recorded in a PC using the LabChart v6.1.3 pro software programme (Powerlab, AD Instruments, Australia).

Drugs used

HC067047 was purchased from TOCRIS Chemicals (Bristol, UK) and oxytocin was from Novartis (Switzerland). HC067047 was dissolved in dimethyl sulphoxide (DMSO) and oxytocin was dissolved in distilled water.

Measurement of rhythmic contraction

After equilibration for 60 min with MKHS or De Jalon, the normal spontaneous contraction was recorded. The frequency (contractions/min) and amplitude (g tension) were determined and mean integral tension was calculated

by the following formula:

MIT (g min) = $\frac{\text{Integral of selected tracing}}{\text{Selection duration in second}} \times 60$

(Parida *et al.*, 2013

Effect of HC067047 (selective TRPV4 antagonist) on oxytocin-induced contractions in late pregnant and non-pregnant mouse uterus

Concentration-response to oxytocin (10^{-10}-10^{-5}M) was elicited either in the absence or presence of HC067047 (1 ìM) in uterine tissue of late pregnant and non-pregnant mouse. For this purpose, the tissues were incubated with HC067047 (1 μM) for 30 min before constructing concentration-response curve to oxytocin.

Data analysis and Statistics

Contractile responses to oxytocin on uterine tissues were expressed as the percentage of the spontaneous contraction response preceding oxytocin (set at 100%). E_{max} and EC_{50} of oxytocin were determined by nonlinear regression analysis using Graph Pad Prism version 4 (San Diego, California, USA). Sensitivity/potency was expressed as pD_2= -log EC_{50}. Results were expressed as mean±SEM (n= number of animals). Data were analyzed by two-way ANOVA for multiple comparisons followed by Bonferroni post-hoc test.

Table 1:
Effect of TRPV4 channel blocker HC067047 on oxytocin-induced contraction in late pregnant mouse uterus

Oxytocin (Log M)	Control Mean ± SE (n=6) (% Basal MIT)	HC067047 Mean ± SE (n=6) (% Basal MIT)
	146.16 ± 17.94	90.62 ± 6.27
-10	158.25 ± 12.91	83.38 ± 9.71
-9.5	196.10 ± 25.39	98.30 ± 13.37
-9	194.57 ± 30.42	103.85 ± 17.72
-8.5	220.30 ± 44.23	114.95 ± 15.66
-8	235.71 ± 54.37	113.19 ± 7.80
-7.5	247.01 ± 36.74	109.65 ± 12.92
-7	282.15 ± 48.29	116.70 ± 14.42*
-6.5	299.44 ± 51.03	124.61 ± 13.87*
-6	333.97 ± 74.33	139.83 ± 12.18$
-5.5	368.23 ± 67.26	149.29 ± 14.71$
-5		
E_{max}	**343.10 ± 30.51**	**149.9 ± 13.70#**
pD_2	**6.87 ± 0.44**	**6.16 ± 0.48**

*p< 0.05, $p<0.01, #p<0.001

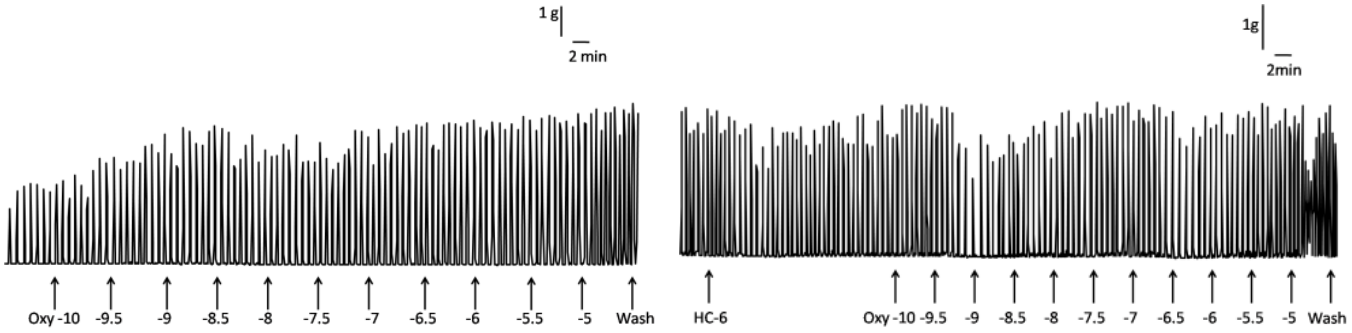
RESULTS AND DISCUSSION

To show whether oxytocin acts as endogenous ligand for TRPV4 channel, the effect of HC067047 on the uterine contractions induced by oxytocin was elicited. Representative tracings in Fig. 1 (A) and (B) illustrate concentration-dependent contractile response to oxytocin and its inhibition by HC067047 (1 μM) in uterine tissue of late pregnant mouse, respectively.

The basal MIT in the uterine strips taken from late pregnant mouse was 26.79±4.65 g min (n=6). Oxytocin caused concentration-dependent increase in basal contractile activity (Fig. 1 A). Incubation with the antagonist HC067047 (1 ìM) for 30 min had no effect on the MIT of basal contractions (25.86±4.08 g min, n=6), which indicates that TRPV4 channel has no significant role in spontaneous myometrial contraction. Line diagram in Fig. 2 shows that the maximal % basal MIT (E_{max}) of oxytocin-induced contractions (343.1±30.51 %; n=6) was significantly (p<0.001) attenuated in the presence of HC067047 (149.9±13.70 %; n=6). However, the pD_2 of oxytocin-induced contractions (6.87±0.44) was not altered by HC067047 (6.16±0.48). The data are shown in Table 1.

The inhibition of oxytocin-induced contractions by HC067047 indicates that TRPV4 channels significantly contribute to uterine contractions induced by this agonist. A recent study in mouse pulmonary artery describes the role of these channels in 5-HT-induced vascular contractions (Xia *et al.*, 2013).These authors showed interdependent role of TRPV4 and voltage-gated Ca2+ channels in mediating this agonist-induced contraction.

Representative tracings in Fig. 3 illustrate



(A) Control **(B) In presence of HC067047**

Fig. 1:
Representative raw tracings showing the effect of oxytocin on spontaneous rhythmic contractions of late pregnant mouse uterus (A) in absence and (B) in presence of HC067047

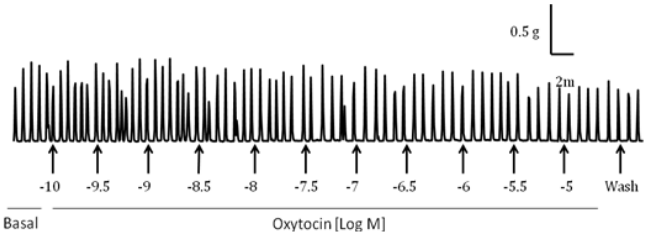


Fig. 2:
Concentration-response curves demonstrating contractile effect of oxytocin alone and after pretreatment with HC067047 (1ìM) on spontaneous uterine contractions of late pregnant mouse

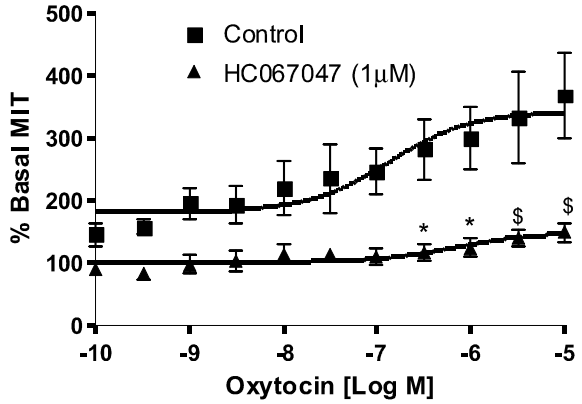


Fig. 3:
Representative raw tracings showing the effect of oxytocin on spontaneous rhythmic contractions of non-pregnant mouse uterus

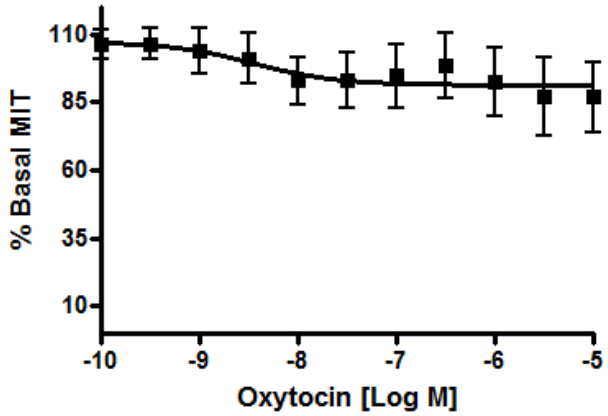


Fig. 4:
Concentration-response curve demonstrating contractile effect of oxytocin on spontaneous uterine contractions of non-pregnant mouse

concentration-dependent contractile response to oxytocin in uterine strips of non-pregnant mouse incubated in De jalon solution. The basal MIT was 7.87±1.79 g min (n=6). Oxytocin showed no significant effect on myometrial contraction. The E_{max} and pD_2 of oxytocin-induced contractions were 107.3±8.06 % and 8.45±1.06, respectively (Fig. 4). Oxytocin did not show any significant effect on spontaneous myometrial contraction in non pregnant mouse uterus, hence we did not perform concentration-response to oxytocin in presence of HC067047.

Thus, our work suggests a possible involvement of TRPV4 channels in oxytocin-induced contraction in pregnant mouse uterus. Nevertheless, further studies are required to elucidate the possible mechanisms of TRPV4 channel activation by this agonist.

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Research Article

ANTIUROLITHIC ACTIVITY OF *TRIBULUS TERRESTRIS* AQUEOUS EXTRACT ON ETHYLENE GLYCOL INDUCED UROLITHIASIS IN RATS

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ABSTRACT

The present study was conducted to determine the effect of aqueous extract of *Tribulus terrestris* on ethylene glycol induced urolithiasis in rats. Urolithiasis was induced in rats by administrating 0.75% ethylene glycol in drinking water for 28 days and was manifested by increased in ALT, Urea, BUN, Creatinine, Uric acid, calcium and phosphorus as compared to vehicle control rats. The aqueous extracts of *Tribulus terrestris* was administered @ 100, 200 and 400 mg/kg body weight orally for 28 days along with 0.75% ethylene glycol in treatment group, respectively. The mean values of serum urea, BUN, creatinine, uric acid, calcium and phosphorus were significantly decreased in rats treated with 200 and 400 mg/kg of aqueous extract of *Tribulus terrestris* as compared to control rats. The reduction was dose dependent. The histopathological studies confirmed the induction of urolithiasis as microcrystal deposition was observed in section of kidney from rats treated with ethylene glycol. Results of the experiment indicate that aqueous extracts of *Tribulus terrestris* could be a potential candidate for phytotherapy against urolithiasis.

Key words: Antiurolithic activity, ethylene glycol, Tribulus terrestris, aqueous extract.

INTRODUCTION

Nephrolithiasis or renal stone is a significant medical and surgical problem which may cause serious complications and it also imposes a great amount of costs to the healthcare system. Plants used in traditional medicine to treat kidney stones are valuable alternative for the control of renal disease. Recently, several plants are being explored for their anti-urolithiatic property on the basis of their usage in the traditional medicine (Verma *et al.*, 2009).

Tribulus terrestris (Zygophyllaceae) is an annual plant native of Mediterranean region, called "Gokhru". In India an important herb commonly used as folk medicine in many countries for different purposes. *Tribulus terrestris* extract contains many compounds such as alkaloids, flavonoids, oil, saponins, resins and nitrates (Li *et al.*, 2002). *Tribulus terestris* is regarded as cooling, diuretic, tonic and aphrodisiac and are used in painful micturition, calculus affections, urinary disorders and impotence (Gupta *et al.,* 1997; Heidari *et al.,* 2007). Hence, the present study was undertaken to investigate renal protective activity of *Tribulus terrestris* aqueous extract on ethylene glycol induced urolithiasis in rats.

MATERIALS AND METHODS

Plant materials and Preparation of the aqueous extract

Tribulus terrestris plants were procured from their natural habitat and were identified and authenticated by a botanist. The fruits of the plant were dried in shade and ground for coarse powder which was used to prepare aquesous extract (Trease and Evans, 2002).

Experimental Animals

Twenty five healthy male wistar rats weighing between 150–250 g were acclimated in the small animal house for 5 days before commencement of experiment. They were kept at 22 ± 2°C with a controlled photo period of 12 hours of light and 12 hours of darkness. Each animal was provided conventional laboratory pellet diet with an unlimited supply of drinking water and followed good management practices during the study. The experiment was conducted following approval by Institutional Animal Ethical Committee (IAEC No. 2010/VPT/79). All rats were randomly divided into five groups as presented in experimental design (Table 1).

Acute toxicity study

The acute oral toxicity study was carried out as per the OECD guideline No. 423. Wistar rats were taken for the study and dosed once with 2000 mg/kg, orally. The treated animals were monitored for 24 hours and up to 14 days for general clinical signs and symptoms like salivation, lacrimation and urination as well as mortality. It was observed that the test extract was not mortal for mice even at 2000 mg/kg dose. Hence, 100, 200 and 400 mg/kg dose of this plant extract were selected and consider safe for further study.

Ethylene glycol induced urolithiasis model

For induction of urolithiasis, 0.75% v/v ethylene glycol (Merck Limited, Mumbai) in drinking water for 28 days was given *ad-libitum*.

Dose preparation and administration

Aqueous extracts of *Tribulus terrestris* were formulated in distilled water for oral administration. Dose were calculated according to body weight of each animal and administrated as per concentration strength of formulation. Aqueous extracts of *Tribulus terrestris* were administered by oral route using sterile 1 ml syringe and oral rat gavage needle.

Collection of blood samples

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

Assesment of anti-urolithiatic activity

Anti-urolithiatic activity of repeated oral administration of aqueous extracts of *Tribulus terrestris* was assessed by studying haematological parameters, serum biochemical parameters and histopathological examination of tissues. Body weights and feed consumption were also recorded weekly interval till day 28 of the experiment period. A careful physical and behavioral examination of all rats was carried out during experimental period.

The data were subjected to statistical analysis (unpaired two tail 't' test), at 5 and 1 percent level of significance.

RESULTS AND DISCUSSION

All rats were observed daily throughout the period of study. All the rats were found active during experimental period and did not reveal any abnormal symptoms attributable to the 28 days oral administration of aqueous extracts of *Tribulus terrestris*. None of the rats died during the study period.

Nephrolithiasis in rats may be induced by ethylene glycol alone, or in combination with other crystal-inducing drugs such as ammonium chloride, Vitamin D3 , gentamicin or a magnesium deficient diet (Jie *et al.*, 1999; Halabea *et al.*, 2003). In the present study, ethylene glycol was used for producing urolithiasis/hyperoxaluria in male Wistar rats. The principal target organ following oral exposure to ethylene glycol is the kidney; moreover, evidence from previous studies indicated that administration of ethylene glycol caused renal stone formation by increasing hyperoxaluria (Green *et al.*, 2005). In the present study, male rats were selected to induce urolithiasis because the urinary system of male rats resembles that of humans and previous studies have shown that the amount of stone deposition in female rats was significantly less. Urinary super saturation with respect to stone-forming constituents is generally considered to be one of the causative factors in kidney stones. Previous studies have shown that renal calculi composed mainly of calcium oxalate form in response to 14 day period of ethylene glycol (0.75%, v/v) administration in young male albino rats (Selvam *et al.*, 2001).

Body weights and feed intake were recorded weekly interval till day 28 of the experiment period. There was non-significant change in body weight as well as feed consumption of rats of treatment groups as compared to lithiatic control group are presented in Table 2 and 3.

Values of hematological parameters of treatment and control groups are presented in Table 4. The mean values of Hb and MCHC were significantly decreased and platelet count was significantly increased in lithiatic control group as compared to corresponding vehicle control group.

TABLE 1:
Experimental Design

Sr. No.	Group No.	Substance	Dose (mg/kg)	No. of Rats
1	I	Lithiatic Control (Ethylene glycol)	0.75 % V/V in drinking water	5
2	II	Vehicle control (Distilled water)	Same volume	5
3	III	Aqueous Extract of *Tribulus terrestris*	100 mg/kg	5
4	IV	Aqueous Extract of *Tribulus terrestris*	200 mg/kg	5
5	V	Aqueous Extract of *Tribulus terrestris*	400 mg/kg	5

All groups received 0.75% v/v Ethylene glycol in drinking water for 28 days except vehicle control group.

Table-2:
Effect of *Tribulus terrestris* aqueous extract on body weight of Wistar rats (n=5)

Group		Day 1	Day 7	Day 14	Day 21	Day 28
Vehicle Control	Mean	235.00	238.33	236.67	238.33	243.33
	SEM	2.89	1.67	3.33	6.01	3.33
Lithiatic Control	Mean	226.67	226.67	243.33	241.67	238.33
	SEM	12.02	16.67	8.82	13.02	8.33
TT Aqueous 100mg/kg	Mean	210.00	223.33	256.67	256.67	266.67
	SEM	10.41	3.33	14.53	13.33	13.33
TT Aqueous 200mg/kg	Mean	253.33	276.67	276.67	300.00	286.67
	SEM	8.82	8.82	14.53	20.82	24.04
TT Aqueous 400mg/kg	Mean	260.00	266.67	265.57	267.00	273.33
	SEM	13.23	8.82	8.82	20.82	20.88

Table-3:
Mean feed consumption per day per rat in *Tribulus terrestris* extract treated groups (n=5)

Group	Week 1	Week 2	Week 3	Week 4
Vehicle Control	12.50	13.07	11.43	13.86
Lithiatic Control	11.90	13.10	11.86	11.95
TT Aqueous 100mg/kg	12.52	14.76	13.81	12.38
TT Aqueous t 200mg/kg	11.62	12.86	13.14	13.33
TT Aqueous 400mg/kg	10.24	14.52	14.48	15.29

The hematological parameters of rats treated with 100, 200 and 400 mg/kg aqueous extracts for 28 days did not differ significantly ($P < 0.05$) from the corresponding values observed in lithiatic control rats.

Serum biochemical parameters were analyzed using automatic biochemical analyzer (Junior Selectra Vital Scientific NV). Serum biochemical parameters like alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), urea, blood urea nitrogen (BUN), creatinine, uric acid, calcium, phosphorus and magnesium were quantified by using standard assay kits (Merck Specialities Pvt. Ltd., Goa, India). As per the results, in urolithiasis, the glomerular filtration rate (GFR) decreases due to the obstruction of the outflow of urine by stones in urinary system. Due to this, the waste products, particularly nitrogenous substances such as urea, creatinine and uric acid get accumulated in blood. Values of serum biochemical parameters of treatment and control groups are presented in Table 5.

The results indicated that the mean values of ALT, urea, BUN, creatinine, uric acid, calcium and phosphorus significantly increased in lithiatic control group as compared to vehicle control rats. This elevated serum levels are indicative of the marked renal damage in rats. Whereas, mean values of urea, BUN, creatinine, uric acid, calcium and phosphorus significantly decreased in rats treated with 200 and 400 mg/kg of aqueous extract of *Tribulus terrestris.*

Increased urinary calcium is a factor favoring the nucleation and precipitation of calcium oxalate or calcium phosphate from urine and subsequent crystal growth results in stone formation (Robertson and Peacock, 1980). Increased urinary phosphate excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which induces calcium oxalate deposition (Gitelman, 1967). But treatment with aqueous extract of *T. terrestris* restored the phosphate level, thus, reducing the risk of stone formation. Urolithiasis also decreases the glomerular filtration rate due to obstruction in outflow of urine by stones in the urinary system. Due to this, waste products, particularly nitrogenous substances such as urea,

Table 4:
Effect of *Tribulus terrestris* aqueous extract on hematological parameters of rats (n=5).

Groups	Unit	RBC 10^6/µl	Hb g/dl	PCV %	MCV Fl	MCH Pg	MCHC g/dl	WBC 10^3/µl	Lympho %	Neutro %	Platelets 10^5/µl
Vehicle control	Mean	7.71	14.60	41.88	54.00	18.95	34.80	7.58	79.40	14.05	4.03
	SEM	0.02	0.17	0.33	0.58	0.26	0.12	0.27	1.04	0.55	0.18
Lithiatic control	Mean	6.71	12.47*	36.14	57.33	17.80	32.97*	9.13	79.10	16.30	5.34*
	SEM	0.75	0.63	3.63	3.93	0.53	0.44	1.17	3.76	3.25	0.31
TT Aqueous 100mg/kg	Mean	7.29	12.23	40.20	55.00	16.33	33.40	7.45	69.37	20.03	5.66
	SEM	0.11	0.90	0.58	1.15	0.94	0.87	0.62	5.24	3.79	0.21
TT Aqueous 200mg/kg	Mean	8.37	13.57	41.98	50.00	16.70	33.60	8.87	66.80	15.20	5.64
	SEM	0.21	0.64	0.76	0.58	0.15	0.50	0.45	6.74	2.29	0.38
TT Aqueous 400mg/kg	Mean	5.81	15.07	30.56	58.33	16.00	33.93	6.36	73.37	20.93	5.96
	SEM	0.47	1.42	1.69	6.01	2.04	0.55	0.60	7.83	5.18	0.72

* Significant difference ($p< 0.05$), RBC: Red blood cells; Hb: Haemoglobin; PCV: Packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; WBC: White blood cells.

Table 5:
Effect of *Tribulus terrestris* aqueous extract on serum biochemical parameters of rats (n=5)

Groups	Unit	SGPT/ALT IU/L	SGOT/AST IU/L	AKP IU/L	TP g/dl	UREA mg/dl	BUN mg/dl	Creatinine mg/dl	Uric Acid mg/dl	Ca mg/dl	P mg/dl	Mg mg/dl
Vehicle control	Mean	43.78	84.68	168.35	6.68	51.78	24.18	0.53	1.82	9.52	5.06	3.11
	SEM	1.98	3.29	15.33	0.44	2.08	0.97	0.07	0.28	0.04	0.07	0.01
Lithiatic control	Mean	71.33*	84.10	170.27	6.92	97.60**	45.58**	0.97*	3.63**	13.01**	8.56	3.71
	SEM	5.55	7.00	31.51	0.29	1.00	0.47	0.12	0.08	0.41	0.40	0.65
TT Aqueous 100mg/kg	Mean	71.90	84.57	185.13	7.12	83.37	38.94	0.67	2.27	10.70	8.33	3.27
	SEM	4.72	2.06	7.08	0.31	7.80	3.64	0.13	0.50	0.81	0.24	0.01
TT Aqueous 200mg/kg	Mean	73.70	68.58	169.77	6.05	76.48*	35.72*	0.53*	1.92*	10.03*	7.02*	3.18
	SEM	4.93	1.01	11.65	0.13	2.17	1.01	0.03	0.20	0.70	0.37	0.03
TT Aqueous 400mg/kg	Mean	73.30	74.27	210.10	6.01	73.92*	34.52*	0.52*	1.80*	10.06*	6.78*	3.19
	SEM	4.77	3.38	13.41	0.48	5.33	2.49	0.09	0.22	0.49	0.28	0.07

* Significant difference ($p< 0.05$), ** highly significant difference ($p< 0.01$), ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AKP: Alkaline phosphatase; TP: Total protein; BUN: Blood urea nitrogen; Ca: Calcium, P: Phosphorus; Mg: Magnasium

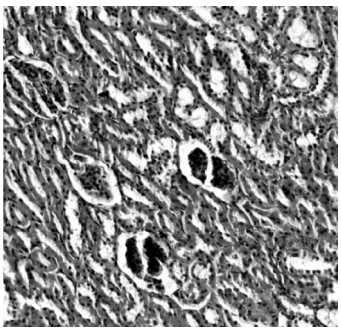

Plate 1:
Vehicle control group showing no pathological microscopic lesion in kidney

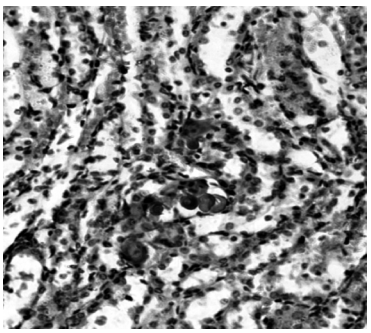

Plate 2:
Lithiatic control group showing crystals accumulated inside renal tubules

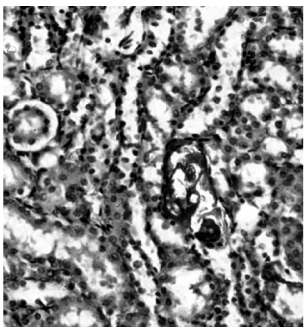

Plate 3:
Lithiatic control group showing crystals in renal tubules accompanied by cast, severe degeneration and necrosis of renal tubular epithelium

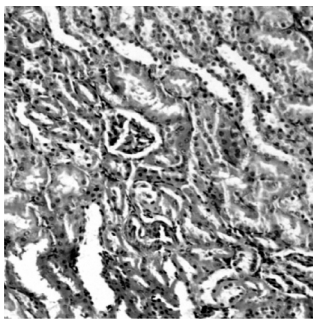

Plate 4:
Tribulus terrestris aqueous extract treated (400mg/kg) group showing mild degeneration and necrosis of renal tubular epithelium

creatinine, and uric acid are accumulated in the blood (Ghodkar, 1994). Uric acid is also known to promote calcium oxalate crystal growth because of predominance of uric acid crystals in calcium oxalate stones (Roger *et al*, 1997). The curative and prophylactic treatment with *Tribulus terrestris* aqueous extract causes diuresis which hastens the process of dissolving the preformed stones, increase excretion of urea, creatinine and uric acid as well as normalized the kidney functions and prevents the formation of new stone in the urinary system (Singh *et al.*, 1991).

All the rats were subjected to postmortem examination on day 29 in the confined disinfected laboratory to determine the presence or absence of gross and histopathological lesions. For gross lesions liver, kidney, heart and spleen were collected and examined after opening the body of sacrificed experimental rats. Gross pathological examination did not reveal any abnormal lesions attributable to the 28 days administration of aqueous extracts of *Tribulus terrestris* in all three treatment groups.

For histopathological examinations, tissues from liver, kidney, heart and spleen were collected and processed as per standard methods and sections were stained with haematoxylin and eosin (H & E) stains and examined for microscopic lesions. Ethylene glycol induced urolithiasis group revealed presence of polymorphic irregular crystals in renal tubules accompanied by cast formation which causes dilation of proximal tubules. Severe degeneration and necrosis of renal tubular epithelium were also observed (Plates:1-3). These histological observations support the presence and growth of renal calculi in kidney as observed in urolithiasis.

Sections of liver, kidney, heart and spleen were studied from all rats. Aqueous (200 and 400 mg/kg) extract of *Tribulus terrestris* treated group revealed moderate to few crystals along with mild degeneration and necrosis of renal tubules. Presence of few crystals in the renal tubules indicates the ability of aqueous (200 and 400 mg/kg) extract of *Tribulus terrestris* to dissolve the pre-formed stones (Plate-4). There were no changes in cellular structures and no other abnormal microscopic lesions in liver, heart and spleen. Histopathological examination also supported by the biochemical parameters observed from moderate and higher dose of aqueous extract of *Tribulus terrestris* decrease the development of nephrotoxicity. Overall, the data presented in this current paper indicates that the administration of aqueous extracts of *Tribulus terrestris* to experimentally ethylene glycol induced urolithiasis rats reduced the deposition of crystals into kidneys, confirming its antilithiatic effect. Further studies are necessary to clarify the mechanism underlying this effect.

Our study was well supported by the report of the anticalcifying proper-ties of *Tribulus terrestris* on calcium oxalate crystal nucleation and growth *in vitro* as well as fur-ther examining the potency of *Tribulus terrestris* on oxalate induced injury in NRK 52E (rat renal tubular epithelial) cells (Aggarwal *et al.*, 2010). In addition to report of aqueous extracts of *Tribulus terrestris* inhibit the growth of urinary calcium hydrogen phosphate dihydrate crystals *in vitro* (Joshi *et al.*, 2005). Kavitha and Jagadeesan (2006) also reported that the oral administration of methanolic fraction of *Tribulus terrestris* fruit extract at dose 6 mg/kg body weight provided protection against the mercuric chloride induced toxicity in the mice (Kavitha and Jagadeesan, 2006).

In conclusion, oral administrations of aqueous extracts of *Tribulus terrestris* @ 200 and 400 mg/kg at every 24 hours were effective for prophylaxis against ethylene-glycol induced urolithiasis in rats. Further characterization of its active compounds could lead to a new candidate drug for phytotherapy against urolithiasis.

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Research Article

PHARMACOKINETICS OF OFLOXACIN AFTER INTRAVENOUS ADMINISTRATION IN BUFFALO (*BUBALUS BUBALIS*) CALVES

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ABSTRACT

Pharmacokinetics of ofloxacin was investigated in buffalo calves (n=5) following an intravenous dose of 5 mg.kg^{-1}. Plasma concentrations of ofloxacin were determined by microbial assay method having the minimum detection limit of 0.08 µg.ml^{-1}and disposition kinetics determined by three-compartment open model. Ofloxacin was very rapidly distributed from the central to peripheral compartments as evident from very rapid distribution half-life (0.13±0.02 h), high K_{12} (1.62±0.35 h^{-1}) and K_{13} (2.69±0.58 35 h^{-1}). Ofloxacin was slowly eliminated from plasma as it could be detected in plasma for up to 24 h and the biological half life was 19.82±2.32 h. The large volume of distribution (5.67±0.46 L.kg^{-1}) indicated excellent distribution of ofloxacin in body tissues and fluids of buffalo calves. Area under plasma drug concentration–time curve and total body clearance were 24.99±1.08 µg.ml^{-1}.h and 201.46±8.88 ml.kg^{-1}.h^{-1}, respectively. Based on integration of PK/PD parameters (AUC/MIC and C_{max}/MIC ratios) obtained in the present study, it may be suggested that ofloxacin be administered intravenously to buffalo calves at 24 h interval at 5 mg/kg body weight for treatment of bacterial infections requiring the MIC values of d"0.25 ug/ml, however, it may be administered either at higher dose levels or repeated at 12 h interval, against the microorganisms having MIC values of >0.25 ug/ml.

Key words: Buffalo calves, Ofloxacin, Microbial assay, Pharmacokinetics, Dosage regimens.

INTRODUCTION

Fluoroquinolones are highly effective broad-spectrum antimicrobial agents and have maintained high antibacterial activity against susceptible pathogens over the years (Karlowsky *et al.*, 2002). These are widely distributed in body and their concentrations in target tissues are significantly higher than those achieved in blood (Giles *et al.*, 1991, Intorre *et al.*, 1997). Microbial resistance to their action does not develop rapidly (Hooper, 2000), therefore, these have revolutionized the therapeutic armamentaria against bacterial pathogens, especially those which are resistant to traditionally used antibacterial agents, including beta-lactam antibiotics, aminoglycosides, third generation cephalosporins, tetracyclines, macrolides, sulfonamides etc. (Vancutsem *et al.*, 1990; Brown 1996, Mckellar 1996). Ofloxacin, a fluorinated quinolone carboxylic acid derivative, is effective against gram–positive and gram-negative bacteria, *Mycoplasma* and *Rickettsiae* and is required in very low concentrations (0.03-0.50 ìg/ml) against common pathogens of animals (Greene and Budsberg, 1993). It is generally safe and thus can be employed for treating the gastrointestinal-, respiratory-, urino-genital, skin and other systemic inflections of animals.

Disposition kinetics data of ofloxacin in human beings (Orlando *et al.*, 1992), rabbits (Marangos *et al.*, 1997; Ahmad *et al.*, 2008), dogs (Yoshida *et al.*, 1998), pigs (Son *et al.*, 2000), neonatal calves (Gaur *et al.*, 2004, 2005), goats (Baruah *et al.*, 2004), chickens (Liu and Fung, 1997; Kalaiselvi *et al.*, 2006) and buffalo calves (Kumar *et al.*, 2009) have revealed extensive species-dependent, age-dependent and healthy-versus diseased models-dependent differences in pharmacokinetic behavior of this antimicrobial. Apparent paucity of sufficient data on disposition kinetics of ofloxacin in buffalo calves prompted us to take up the present study to generate pharmacokinetic data following a single intravenous (IV) bolus dose and suggest the rational dosing schedule of ofloxacin for buffalo calves.

MATERIALS AND METHODS

Six to eight months old healthy female Murrah buffalo (*Bubalus bubalis*) calves (n=5) weighing between 48 and 60 kg were procured from Dairy Farm of the Institute and maintained under standard managemental conditions. The calves were offered *ad libitum* seasonal green fodder and wheat straw. Concentrate was also provided as per requirement and animals had free access to drinking water.

Ofloxacin (Technical grade, 99.6% purity; Ranbaxy Research Laboratories, India) was initially dissolved in 0.1 N hydrochloric acid to prepare 5.0 per cent stock solution (w/v) of the drug. The stock solution was later diluted to 3. 0 per cent strength (W/V) using sterile water for injection

immediately before administration. Freshly prepared drug solution was injected into the jugular vein of buffalo calves at the dose rate of 5 mg/kg body weight. Blood samples were collected from catheterized contra-lateral jugular vein into the heparinized test tubes before injecting ofloxacin (0 h) and at 0.04, 0.08, 0.17, 0.25, 0.33, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48, 72, 96 and 120 h after drug administration. Plasma was separated and stored at -20°C until assayed.

Plasma ofloxacin concentrations were determined employing the microbiological assay method of Arret *et al.* (1971) using *E. coli* (ATCC 25922) as the test organism. All the standards and test samples were assayed in triplicate and the mean of these replicates was determined. Minimum quantification limit of the assay procedure employed was 0.08 ìg/ml. Consistency, validation and variations in the assay method were ensured as reported earlier (Gaur *et al.*, 2005).

Based on apparent visual curve fitting of plasma ofloxacin concentration versus time data of individual animal on semi-logarithmic plot, pharmacokinetic behaviour of ofloxacin in buffalo calves was determined employing the tri-exponential equation (Baggot, 1977):

$Cp = Pe^{\pi t} + Ae^{át} + Be^{\beta t}$

Where Cp is the ofloxacin plasma concentration at time t after IV administration of drug; π, á and β are the rate constants of each of the first order processes, namely- initial rapid distribution, distribution and elimination phases, respectively for three- compartment open model, and P, A and B are the Y intercepts of the corresponding phases. The rate constants so derived were used to calculate the respective half life values. Other pharmacokinetic parameters were computed according to the standard formulae (Gibaldi and Perrier, 1982). Values of all the pharmacokinetic parameters have been expressed as the mean ± SE.

RESULTS

Mean (± SEM) plasma ofloxacin concentrations after IV injection (5.0 mg/kg) in buffalo calves are shown in Fig. 1. At 2.5 min, plasma ofloxacin level was 13.60±0.43 ìg/ml which initially declined very rapidly to 6.67 ± 0.22 ìg/ml at 0.25 h, almost half of the initial value. After 15 min, plasma levels exhibited a comparatively less rapid rate of decline and the concentration of ofloxacin decreased to 1.02±0.07 ìg/ml at 4 h. From 6 h onward, the decline in plasma ofloxacin level was very slow and its level was 0.22±0.02 ìg/ml at 24 h as shown in Fig. 1. Although ofloxacin could be consistently detected in blood of buffalo calves up to 24 h but plasma concentration above the minimum inhibitory concentration (MIC) level of >0.25 ìg/ml were maintained up to 12 h only.

Following IV administration, initial rapid biphasic decline in plasma concentrations suggested distribution of the drug in two phases- very rapid followed by rapid with the very rapid distribution phase half life ($t_{1/2\pi}$) of 0.13 h and distribution half life ($t_{1/2á}$) of 1.93 h. Different disposition kinetic parameters have been summarized in Table 1. Elimination half life of ofloxacin in buffalo calves in the present study was 19.82±2.32 h. Mean values of apparent volume of distribution (Vd_{area}), area under the plasma distribution time curve (AUC) and total body clearance (Cl_B) in the present study were found to be 5.67±0.46 L/kg, 24.99±1.08 ìg.h/ml and 201.46±8.88 ml/kg/h, respectively. The transfer rate constants of drug from the central to peripheral compartment and vice versa were very high as revealed by the values of K_{12}/K_{21} (10.30±3.77 and K_{13}/K_{31} (3.70±0.50) as summarized in Table 1.

DISCUSSION

Plasma ofloxacin concentration versus time data in buffalo calves after IV administration was best described by three compartment model where as it has been described by two-compartment open model in rabbits (Marangos *et al.*, 1997), chickens (Liu and Fung, 1997) and neonatal cow calves (Gaur *et al.*, 2004) and model-independent methods in dogs (Yoshida *et al.*, 1998), pigs (Son *et al.*, 2000) and chickens (Kalaiselvi *et al.*, 2006).

Distribution phase half life values of ofloxacin in buffalo calves ($t_{1/2\pi}$ - 0.13h and $t_{1/2á}$ of 1.93 h) suggest its instant and more fast distribution into body tissues and fluids of buffalo calves compared to that in neonatal cow calves (Gaur *et al.*, 2004). This observation also suggests

Table 1:
Pharmacokinetic parameters (mean±SE) of ofloxacin following single intravenous dose administration @5 mg/kg body weight in buffalo calves (n=5)

Pharmacokinetic parameters (Units)	Mean±SE
P ($\mu g.ml^{-1}$)	14.38±2.17
A ($\mu g.ml^{-1}$)	3.28±0.63
B ($\mu g.ml^{-1}$)	0.51±0.02
Cp_0 ($\mu g.ml^{-1}$)	18.18±2.58
π (h^{-1})	6.01±1.08
α (h^{-1})	0.40±0.05
β (h^{-1})	0.036±0.004
$t_{1/2\pi}$ (h)	0.13±0.02
$t_{1/2\alpha}$ (h)	1.93±0.35
$t_{1/2ß}$ (h)	19.82±2.32
AUC ($\mu gml^{-1}.h$)	24.99±1.08
AUMC ($\mu gml^{-1}.h^2$)	452.85±83.87
MRT (h)	17.78±2.64
Vc (Lkg^{-1})	0.29±0.03
K_{21} (h^{-1})	0.11±0.029
K_{31} (h^{-1})	0.74±0.12
K_{10} (h^{-1})	1.29±0.28
K_{12} (h^{-1})	1.62±0.35
K_{13} (h^{-1})	2.69±0.58
K_{12}/K_{21}	10.30±3.77
K_{13}/K_{31}	3.70±0.50
$Vd_{(area)}$ (Lkg^{-1})	5.67±0.46
Cl_B (ml/kg/h)	201.46±8.88

Data presented are mean±SE of five animals.

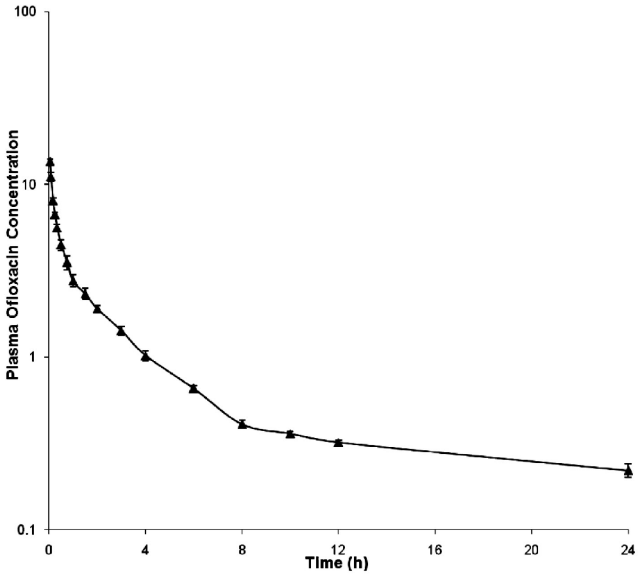

Fig.1:
Semilogarithmic plot of the mean plasma concentration-time profile following a single intravenous injection @ 5 mg/kg body weight in buffalo calves.Values presented are mean±SE of five animals.

its usefulness by intravenous route in the treatment of systemic infections where therapeutic concentrations are required to be achieved immediately. Characterization of the π phase was very conspicuous in buffalo calves and it should not be attributed to just enough blood sampling shortly after IV administration as we had collected blood samples at the same time intervals in neonatal calves too (Gaur *et al.*, 2004); therefore, it seems to be species-specific in buffalo calves.

Elimination half life value of ofloxacin in buffalo calves in the present study (19.82 h) was almost four folds higher compared to 4.82 h in chickens (Liu and Fung, 1997; Kalaiselvi *et al.*, 2006) and 4.9 h in human beings (Orlando *et al.*, 1992) and many folds greater compared to that of 1.59 h in rabbits (Marangos *et al.*, 1997) and 1.96 h in dogs (Yoshida *et al.*, 1998) following intravenous administration but shorter than that of 26.27 h in neonatal calves (Gaur *et al.*, 2004).

High value of volume of distribution (5.67±0.46 L/kg) suggests very good distribution and tissue penetration ability of ofloxacin in buffalo calves as the values of Vd_{area}, Vd_{ss} and V_2 (volume of peripheral compartment) of >1.0 L/kg generally imply wide distribution, extensive tissue binding or both as has been reported in human beings (Orlando *et al.*, 1992), pigs (Son *et al.*, 2000), rabbits (Marangos *et al.*, 1997), chickens (Liu and Fung, 1997; Kalaiselvi *et al.*, 2006) and neonatal calves (Gaur *et al.*, 2004).

Wide distribution and penetration of ofloxacin into body tissues and fluids of buffalo calves after intravenous administration is further supported by the value of ratio of transfer rate constants of drug from the central to peripheral compartment and vice versa (K_{12}/K_{21}-10.30±3.77; K_{13}/K_{31}-3.70±0.50). Value of AUC in buffalo calves (24.99±1.08 ìg.h/ml) was almost similar to that in human beings (Orlando *et al.*, 1992), rabbits (Marangos *et al.*, 1997), chickens (Liu and Fung, 1997) and neonatal calves (Gaur *et al.*, 2004). Total body clearance value of ofloxacin in buffalo calves (201.46±8.88 ml/kg/h) was almost comparable to that of 189.96+19.82 ml/kg/h in neonatal calves (Gaur *et al.*, 2004) but was much lower compared to that in humans (Orlando *et al.*, 1992) or chickens (Liu and Fung, 1997) which possibly may be due to species-dependent differences in elimination rates of drug.

It has been unequivocally established for concentration-dependent antibacterial agents including fluoroquinolones that plasma C_{max} to MIC ratio and AUC to MIC ratio are the most important efficacy predictors (Dudley, 1991; Toutain *et al.*, 2002) and high C_{max}/MIC value of 8-12 and AUC/MIC value of >100 are necessary for avoiding emergence of bacterial resistance (Dudley, 1991; Walker 2000). We did not determine MIC values of ofloxacin against any specific clinical isolates from buffaloes. Considering ofloxacin MIC value as 0.25 μg/ml against most of the sensitive pathogens and the observed plasma ofloxacin concentration of 13.60 µg/ml at 2.5 min as C_{max}, value of C_{max}/MIC was found to be 54.40 and AUC/MIC value was almost 100 (99.96). Therefore, based on the integration of PK/PD parameters (AUC/MIC and C_{max}/MIC ratios) obtained in the present study, it may be suggested that ofloxacin be administered intravenously to buffalo calves at 24 h interval at 5 mg/kg body weight for treatment of bacterial infections requiring the MIC values of d”0.25 ug/ml, however, ofloxacin be used at higher dose level or used more frequently, may be at 12 h interval, against the microorganisms having MIC values of >0.25 ug/ml.

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Research Article

IN VITRO EVALUATION OF ANTHELMINTIC ACTIVITY OF *TAGETES PATULA* AGAINST *HAEMONCHUS CONTORTUS*

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ABSTRACT

The present study was carried out to investigate the efficacy of the methanolic flower extract of *Tagetes patula* against *Haemonchus contortus* worms. The extract was found to possess excellent *in vitro* anthelmintic activity. The crude extract was subjected to partial purification of bioactive constituent(s) using silica gel column chromatography. The maximum adulticidal activity (71.66 ± 11.66 %) was found in fraction F5 (chloroform:methanol, 50:50) at 2 mg/ml concentration. Further rechromatography of the bioactive fraction revealed that fraction R4 (chloroform: methanol 75:25) possessed maximal adulticidal action against the test parasite. In TLC profile a probable bioactive constituent with Rf value 0.782 in chloroform:methanol:ethyl acetate (75:25:1) solvent system was detected. The probable bioactive phytochemical(s) on further purification, can lead to the development of an effective green anthelmintic.

Key words: *Tagetes patula*, *Haemonchus contortus*, goat, anthelmintic activity

INTRODUCTION

The concept of Green medicine is currently receiving world wide attention owing to lesser or no side effects, eco-friendly attributes and easy access. India with plant biodiversity of about 15,000-20,000 medicinal plants (Kumar, 1996) has enormous potential of developing plant based anthelmintics. *Tagetes patula* (Gainda) is grown abundantly as ornamental plant in the Indian subcontinent. Its oil possesses larvicidal, fly repellent activities (The Wealth of India, 1976), besides, other biological activities including nematicidal action from volatile and non volatile components (Kyo *et al*, 1990; Reynolds *et al*, 2000). In the present paper, we report the *in vitro* adulticidal action of methanolic extract of *Tagetes patula* (Gainda) against *Haemonchus contortus* (Rudolphi, 1982).

MATERIALS AND METHODS

Preparation of methanolic extract

Fresh flowers of *Tagetes patula* were collected from the campus of CSK Himachal Pradesh Krishi Vishvavidyalaya, Palampur during the month of November. Methanolic extract of *T. patula* was made by cold extraction procedure and the filtered extract was dried using rotary vacuum evaporator at 40ºC. The residue was used for *in vitro* trials on *Haemonchus contortus* worms and for the chromatographic separation of nematicidal constituents. The residue for *in vitro* trials was reconstituted in Ringer Locke* solution.

In vitro trials on *H. contortus* worms

The *in vitro* trials of the methanolic extract of *T. patula* (flowers) were conducted on active and motile mature *Haemonchus contortus* (female) worms of goats by petridish method (Sharma *et al*, 1971). The worms were collected in Ringer Locke (RL) solution (In mM: Sodium chloride 154.0; Potassium chloride 5.6; Calcium chloride 2.2; Sodium bicarbonate 6.0; Glucose 11.1.) from the abomasums of freshly slaughtered naturally infected goats. The efficacy of the extract was evaluated at different concentrations i.e. 1.0, 2.0, 4.0 and 8.0 mg/ml at 38.5°C. To prepare homogenous suspension of the extract in aqueous medium, methanol (10 µl/ml) was used. RL solution with methanol at the concentration same as in test set served as negative control. Ten ml of homogenous suspension was distributed in three petridishes (one set). Five active and motile adult worms were added to each petridish. Each set was taken in triplicates. The inhibition of the motility of the worms was used as the main criteria for judging the anthelmintic activity. The motility of the worms was observed at hourly interval up to five hours by gross examination and motility scoring was done. To ascertain the viability of the motionless worms, pinch technique was used. The per cent mortality was calculated and anthelmintic activity was scored.

Chromatographic detection/separation of nematicidal constituents

Column chromatography

A glass column (3x45 cm) was used for column chromatography (CC). Silica gel (60-120 mesh, 80 g) was activated at 110°C for 1h and dry-packed in the column. The residue left after removal of solvent from methanolic extract of *Tagetes patula* flowers was dissolved in 10 ml methanol and adsorbed on activated silica gel (30 g). Solvent was removed *in vacuo*. The sample adsorbed on silica gel was loaded on previously packed column. The elution was done with chloroform, chloroform:methanol (95:5), chloroform: methanol (90:10), chloroform:methanol

(75:25), chloroform:methanol (50:50) and finally with methanol. Fractions of 500 ml each were collected and dried and were taken for assay for anthelmintic activity. The efficacy of the fractions was evaluated at 2 mg/ml concentration. Following the testing of the anthelmintic activity of fractions eluted, the active elutes were identified and pooled. Rechromatography of the pooled fractions was attempted for the further bioactivity-guided purification. Activated silica gel (60-120 mesh, 40 g) was packed in a glass column (3x45 cm) for rechromatography. The residue left after removal of solvent from the fractions collected from main chromatography was dissolved in methanol and adsorbed on activated silica gel (20 g). The solvent was removed *in vacuo* and the sample adsorbed on silica gel was loaded on the previously packed column. The elution was done with chloroform (500 ml), chloroform:methanol (95:5) (250 ml), chloroform:methanol (90:10) (250 ml), chloroform:methanol (75 : 25) (2 fractions of 500 ml each), chloroform:methanol (65:35) (500 ml), chloroform:methanol (50:50) (500 ml) and finally with methanol (300 ml). The fractions were collected and solvent was removed *in vacuo*. The residues were taken for assay of adulticidal activity and thin layer chromatography.

Thin layer chromatography

Thin layer chromatographic analysis of the crude extract and the chromatographic fractions was done on glass plates using silica gel G in choloroform:methanol: ethyl acetate; 75:25:1 using iodine as indicator (Stahl, 1969).

RESULTS

In vitro trials on *H. contortus* worms

The anthelmintic activity of the methanolic extract of *T. patula* is presented in Table 1. The adult untreated worms in control group showed high motility with no mortality till 5 h of incubation. In the test groups, worms exhibited high to moderate motility with no mortality in all the concentrations up to 3 h of incubation. The same pattern was observed after 4 h of incubation at 1.0 mg/ml concentration. However, at 2.0, 4.0 and 8.0 mg/ml, the motility of worms was moderate to low and mortality was observed. The extract exhibited dose and time dependent increase in mortality after 5 hours of incubation, with mortality of above 80 per cent at 4.0 and 8.0 mg/ml (Table 1). An increase in concentration from 4 mg/ml to 8 mg/ml, however, did not show any significant change in adulticidal activity. The studies on bioactive constituents collected following column chromatography and rechromatography were done at 2 mg/ml.

Chromatographic separation of nematicidal constituents

Column chromatography

Seven fractions (F_1-F_7) of 500 ml each were collected after elution with different solvent systems as mentioned earlier. The efficacy of the residue of various fractions was evaluated by conducting *in vitro* trials at 2 mg/ml concentration. The results of these *in vitro* trials are presented in Table 2. Fractions F_1, F_2 F_3 and F_7 were found to be ineffective, five hours post incubation. Most of the bioactive constituent(s) was eluted in fraction (F_5) (chloroform:methanol; 50:50) as evident from the per cent mortality of 71.66 ± 11.66 which was reduced to 50.00 ± 0.0 in fraction F6.

Rechromatography

Eight fractions (R_1-R_8) were collected using different solvent systems from the bioactive fraction 5 (F_5). *In vitro* trials using the residue of various fractions of rechromatography revealed that the fraction R_4 eluted with the solvent system chloroform: methanol (75:25) possessed high anthelmintic activity. The other fractions were found to be ineffective (Table 3). Anthelmintic activity for the fractions R_1, R-$_2$ & R_3 could not be determined for the want of sufficient sample.

Thin layer chromatography

TLC profile using the solvent system Chloroform: methanol: acetyl acetate; 75:25:1, on exposure to iodine vapours revealed appearance of a distinct band with Rf value 0.782 in the crude extract and bioactive fraction R_4 having putative anthelmintic activity. This band was absent in inactive fractions.

DISCUSSION

Several herbal products and plant based medicaments have been documented for the adulticidal

Table 1:
Anthelmintic activity of the methanolic extract of *T. patula* against *H. contortus* at different concentrations

Conc.(mg/ml)	Motility score		Percent Mortality	Anthelmintic activity
	Pre-incubation	5 h Post-incubation		
Control	++++	++++	0.0 ± 0.0[a]	Nil
1.0	++++	+++	0.0 ± 0.0[a]	Nil
2.0	++++	++	44.0 ± 0.0[b]	Moderate
4.0	++++	0	81.0 ± 4.50[c]	Excellent
8.0	++++	0	83.33 ± 9.62[c]	Excellent

Values are Mean ± SEM; Means with different superscripts vary significantly ($p<0.05$) with each other
Motility score: 0=Immotile, +=Dull, ++=Low motility, +++=Moderate motility, ++++=High motility; Anthelmintic activity (On the basis of Percent mortality): Nil=0%, Very low=1-20%, Low=20-40%, Moderate=40-60%, Excellent= >80% mortality (Upper Limits are exclusive)

Table 2:
In vitro anthelmintic activity of the chromatography fractions of the methanolic extract of *T. patula* against *H. contortus* at 2mg/ml concentration

Fraction No.	Motility score		Percent Mortality	Anthelmintic activity
	Pre-incubation	5 h Post-incubation		
F1	++++	++++	0.0 ± 0.0	Nil
F2	++++	+++	0.0 ± 0.0	Nil
F3	++++	++++	0.0 ± 0.0	Nil
F4	++++	+	30.0 ± 10.0	Low
F5	++++	0	71.66 ± 11.66	High
F6	++++	+	50.0 ± 0.0	Moderate
F7	++++	++++	0.0 ± 0.0	Nil

Values are Mean ± SEM; Motility score: 0=Immotile, +=Dull, ++=Low motility, +++=Moderate motility, ++++=High motility; Anthelmintic activity (On the basis of Percent mortality): Nil=0%, Very low=1-20%, Low=20-40%, Moderate=40-60%, Excellent= >80% mortality (Upper Limits are exclusive)

Table 3:
In vitro Anthelmintic activity of the rechromatography fractions of the methanolic extract of *T. patula* against *H. contortus* at 2mg/ml concentration

Fraction No.	Motility score		Percent mortality	Adulticidal Activity
	Pre-incubation	5 h Post-incubation		
R4	++++	0	68.33 ± 9.27	Moderate
R5	++++	+	13.33 ± 6.67	Very low
R6	++++	++	0.0 ± 0.0	Nil
R7	++++	+++	0.0 ± 0.0	Nil
R8	++++	++++	0.0 ± 0.0	Nil

Values are Mean ± SEM; Motility score: 0=Immotile, +=Dull, ++=Low motility, +++=Moderate motility, ++++=High motility; Anthelmintic activity (On the basis of Percent mortality): Nil=0%, Very low=1-20%, Low=20-40%, Moderate=40-60%, Excellent= >80% mortality (Upper Limits are exclusive), *Anthelmintic activity for the fractions R1, R2 & R3 could not be determined for the want of sufficient sample.

activity against *Haemonchus contortus* with limited degree of success (Garg and Mehta, 1958; Gilthori *et al.*, 2002). The methanolic extract of dried flowers of *T. patula,* however, exhibited excellent anthelmintic activity at 4.0 mg/ml concentration at 5 hours post incubation with 81.0 ± 4.5 % mortality in adult worms. The adulticidal action was dose and time dependent up to the concentration of 4 mg/ml but further increase in concentration did not show any increase in activity. The nematicidal action of *T. patula* has been widely documented against plant nematodes (Supratoyo, 1993; Khanna *et al*, 1998; Mateeva *et al*, 1973). However, there is apparently no report on the nematicidal action against *Haemonchus contortus* and other important blood sucking nematodal parasites of the ruminants. Partial purification of the methanolic extract yielded fraction F_5 with maximum adulticidal activity (71 .66 ±11.66% mortality), which was eluted with chloroform:methanol (50:50). On rechromatography of F_5, eight fractions (R_1-R_8) were collected and were subjected to *in vitro* trials. Fraction R_4 eluted with chloroform:methanol (75:25) exhibited maximum anthelmintic activity with per cent mortality of 68.33 ± 9.27 at five hours of incubation. The TLC analysis of crude extract as well as different fractions was done using solvent systems of different polarity. The solvent system chloroform:methanol:ethyl acetate (75:25:1) provided the best resolution and a distinct band with Rf value 0.782 was found in the crude extract and bioactive fraction R_4. As this band was absent in inactive fractions, thus the constituent(s) corresponding to the band seemed to be responsible for the anthelmintic activity. Several other chromatographic constituents of varying polarity (Gommers, 1973; Hatakeda *et al*, 1985; Ragvanshi *et al*, 1985) have been isolated from *T. patula* flowers, leaves and roots. Our findings corroborate with the findings of earlier workers who observed maximal nematicidal action in the methanolic extract of *Tagetes spp* (Ray *et al.*, 2002). The findings of the present studies indicate that certain nematicidal constituent(s) is present in the methanolic flower extract of *Tagetes patula* which is effective against adult *Haemonchus contortus* worms under *in vitro* conditions and therefore there is a need of further purification of the probable bioactive phytochemical(s) which can lead to the development of an effective green anthelmintic.

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Research Article

STUDIES ON TISSUE DISTRIBUTION OF HALQUINOL FOLLOWING DIETARY INCLUSION IN CHICKEN AND FIXATION OF MEAT WITHDRAWAL PERIOD

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ABSTRACT

The present study was carried out in broiler chickens to determine tissue distribution and to establish withdrawal period following dietary inclusion of halquinol. Four hundred day-old broiler chicks (Vencobb®) were randomly assigned to four groups of 100 each. Group I birds received only normal poultry diet, while birds in group II (T_1), III (T_2), IV (T_3) received poultry diet containing halquinol at 60, 120 and 240 ppm respectively for a period of 40 days. There was significant (pd"0.05) increase in concentration of 5,7-dichloroquinolin-8-ol in liver and kidney in T_1, T_2 and T_3 groups of birds between each successive sampling days of halquinol exposure. The concentration of 5,7-dichloroquinolin-8-ol in breast muscles was significantly more (pd"0.05) in T_3 group (240 ppm) between each successive samling days while its concentration in T_1 & T_2 groups were below the limit of quantification. Consequent to dietary withdrawal of halquinol, there was significant decrease (pd"0.05) in concentration of 5, 7-dichloroquinolin-8-ol in liver and kidney tissues and reached to levels below limit of quantification on day 4 in all the treatment groups. The depletion of 5, 7-dichloroquinolin-8-ol from breast muscles of birds exposed to halquinol @ 240 ppm was relatively rapid and the residues were below the level of quantification on day 3 following its withdrawal from diet. Irrespective of halquinol exposure levels, the depletion trend for 5,7-dichloroquinolin-8-ol from edible tissues was in the order of breast muscles > liver $\geq$ kidney. Hence, it is suggested to adopt three days pre-slaughter withdrawal period in broiler chickens.

Key words: Halquinol, tissue distribution, broiler chicken, meat, withdrawal period

INTRODUCTION

In the early 1960s, several antibiotics were used as growth promoters at low doses, apart from being used to treat bacterial infections in man and animals. But soon there was a concern that the practice of using as growth promoters, though helpful in achieving higher meat production, may turn out to be a public health problem. This is because continued use of any antibiotic at sub-therapeutic doses over a period of time may lead to local bacterial populations acquiring resistant to the antibiotic.

In considering phasing out or banning antibiotic growth promoters, other alternatives are considered along with better security and hygiene on the farm. A non antibiotic growth promoter, Halquinol is in use from 1960s and is still being used in India and other Asian countries as well as Latin American countries to overcome common challenges of modern poultry and swine farming. Halquinol is a broad spectrum antimicrobial having weak antibacterial, antifungal and antiprotozoal activity (Ellenrieder and Sensch, 1972; Fiedler and Kaben, 1966; Heseltine and Campbell, 1960; Lamy, 1964; Cosgrove and Baines, 1978; Forster and Duggan, 1974).

There is paucity of scientific data in poultry with respect to tissue distribution and residue kinetics of halquinol in edible tissues. Also, the pre-slaughter withdrawal time in poultry has not been specified. Keeping the above points in view, the present study was undertaken in broiler chickens to determine the distribution pattern to edible tissues and to establish withdrawal period for meat following dietary inclusion of halquinol.

MATERIALS AND METHODS

Experimental birds

The study was conducted in unsexed Vencobb 400® broiler birds. Day old chicks were procured from reputed breeder (M/s Venkateshwara Hatcheries, Bangalore, India) and were housed under deep litter system. Necessary approval from the Institutional Animal Ethics Committee (IAEC), Veterinary College, Hebbal, Bangalore, India was obtained (No.38/LPM/IAEC/2009) before conducting the present experimental study.

Drugs and chemicals

Halquinol is a mixture obtained by chlorinating quinolin-8-ol. It contains not less than 57 per cent and not more than 74 per cent of 5,7-dichloriquinolin-8-ol, not less than 23 per cent and not more than 40 per cent of 5-choloroquinolin-8-ol and not more than 4 per cent of 7-chloroquinolin-8-ol and the total content of the three components is not less than 95 per cent and not more than 105 per cent (Anon., 1980). The commercial product

of halquinol, HALQUINOL BP 80, composed of 71.39 % of 5,7-dichloroquinolin-8-ol, 26.61 % of 5-chloroquinolin-8-ol and 0 % of 7-chloroquinolin-8-ol, the total content of the two components being 98 % w/w, manufactured and supplied by M/s. Provimi Animal Nutrition India Pvt. Ltd., Bangalore was used in the present experimental study. A study by Pavithra *et al.* (2011) has determined the identity and purity of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol in above said commercial product of halquinol. For standardization of extraction procedures of halquinol from tissues, pure reference standards of 5,7-dichloroquninolin-8-ol and 5-chloroquinolin-8-ol, the two constituent molecules of halquinol were obtained from Sigma, St. Louis, USA. Halquinol and its constituent molecules were kept free from metal contact and were protected from light to avoid chelating and photo degradability respectively.

Experimental design and halquinol administration

Four hundred day-old broiler chicks were divided into a control group and three treatment groups comprising of 100 chicks (Vencobb®, M/s Venkateshwara hatcheries Pvt, Ltd., Bangalore). The experimental design is presented in Table 1. The required amount of standard starter broiler feed and finisher broiler feed were manufactured to an identical formulation, containing no antimicrobial agents and/or growth promoters. Halquinol was added to feed at different levels of 60, 120 and 240 ppm and mixed thoroughly using commercial poultry feed mixer. Representative feed samples containing halquinol at 60, 120 and 240 ppm were assayed using high performance liquid chromatography (HPLC) and the halquinol concentration was found to be at desired level.

Table 1:
The experimental design and dietary inclusion level of halquinol in broiler diet

Group	Inclusion level of halquinol (ppm)
Group I (Control C, n=100)	-
Group II (Treated T_1, n=100)	60 ppm
Group III (Treated T_2, n=100)	120 ppm
Group IV (Treated T_3, n=100)	240 ppm

From day 1 to day 20 of chicks, standard poultry starter feed mixed with halquinol at 60, 120, 240 ppm was fed to chicks of T_1, T_2 and T_3 treatment groups respectively and standard poultry starter feed free of halquinol was fed to chicks of control group. From day 21 to day 40, standard finisher poultry feed mixed with halquinol at 60, 120 and 240 ppm was fed to birds of T_1, T_2 and T_3 treatment groups respectively and standard finisher feed free of halquinol was fed to birds of control group. Birds had access to clean potable water *ad libitum* throughout the trial. From day 41 both control and treatment group birds were fed with standard poultry finisher ration free of halquinol for the rest of the period of study.

Collection of blood and edible tissues

In order to determine tissue distribution of halquinol upon incorporating it with poultry feed in broilers, six birds from each group were sacrificed on 5, 10, 20, 30 and 40 days during treatment. To determine tissue depletion of halquinol after withdrawal of treatment in broilers, six birds from each group were sacrificed at 2 h post-withdrawal of halquinol exposure through diet and on 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days after withdrawal of treatment. Edible tissue samples *viz.* liver, kidney, breast muscle of each bird were collected in separate self sealing polythene bags and plasma samples were collected in polypropylene micro centrifuge tubes and stored in deep freezer (-20 °C) until assayed.

Assay of halquinol

The concentration of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol of halquinol in plasma and tissue samples (*viz*: liver, kidney and breast muscles) was determined by using high performance liquid chromatography (HPLC; Shimadzu Prominence, Japan). Required studies were carried out to confirm the stability of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol under sample storage conditions. The standard procedures were adopted to confirm the recovery per cent, repeatability and assay precision. The extraction recovery of 5,7-dichloroquinilin-8-ol was 95.5, 89.5, 91.9 and 87.64 per cent for plasma, liver, kidney and breast muscle tissues respectively and that for 5-chloroquinolin-8-ol was 93.12, 90.56, 92.39 and 89.28 per cent for plasma, liver, kidney and breast muscle samples respectively. The intra and inter-day assay coefficients of variations were less than 10 % for both 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol.

i) ***HPLC-PDA conditions***

The HPLC system consists of single pump (LC-20AT), an auto sampler (SIL-20A), photo diode array detector (SPD-M20A) and LC solution® software for data analysis. A reverse phase C_{18} column (250 X 4.6 mm, particle size 5 µm, pH: 1.5-11, Purospher Star RP-18e, Merck, Germany) served as stationary phase. The mobile phase consisted of 0.1% v/v orthophosphoric acid and acetonitrile mixed at a ratio of 1:1 v/v. The flow rate of the mobile phase was one ml per minute and the injection volume was 20 µl. The detection wavelength was set at 247 nm with scanning range of 200-400 nm and the samples were analyzed for 30 minutes with column oven temperature set at 40 °C.

ii) ***Extraction of halquinol from plasma and tissues***

Extraction of 5,7-dichloroquinolin-8-ol and 5-chloroqunolin-8-ol from plasma samples was carried out by liquid-liquid extraction technique. Briefly, to 500 µl of plasma in a screw capped centrifuge tube was added a 0.2 ml of 0.02 M phosphate EDTA (disodium salt; 3%) buffer (pH adjusted to 6.5). Then 1.5 ml of acetonitrile was added, and vortexed for about a minute, then the mixture

was centrifuged (Eppendorf Centrifuge 5810R with swing bucket rotor, Germany) at 4000 rpm for 10 minutes. The clear supernatant thus obtained was transferred into a 2 ml micro centrifuge tubes and then filtered through a 0.45 µm PVDF syringe driven membrane filters (Millex HV, 33 mm). A 20 µl of filtrate was injected into HPLC system using auto sampler unit.

Frozen tissues (liver or kidney) samples were thawed and external fat and fascia was trimmed off. Five gram of tissue was cut into two to three small pieces and then homogenized using motor driven tissue homogenizer (Remi, India), equipped with a ground glass cylinder and teflon pestle. The homogenate was decanted quantitatively into screw capped polypropylene test tubes of 50 ml capacity. To it was added 0.5 ml of 0.02 M phosphate disodium EDTA buffer (pH 6.5) and mixed by shaking. The mixture thus obtained was subjected to extraction of 5,7-dichloroquinolin-8-ol and 5-chloroqunolin-8-ol by adding 20 ml of ethyl acetate and then vortexed for three minutes. After vortexing, the mixture was centrifuged (Eppendorf Centrifuge 5810R, Germany; fixed angle rotor) at 10,000 rpm for 5 minutes. The supernatant was transferred into 50 ml glass stoppered test tube. The extraction procedure was repeated with 10 ml ethyl acetate and the supernatant was transferred in to same test tube. The pooled organic fraction was evaporated to dryness in a gentle stream of nitrogen under water bath maintained at 50 °C. The residue left was reconstituted with one milliliter of methanol. This solution was then filtered though a 0.45 µm PVDF syringe driven membrane filter into an auto sampler vial. A 20 µl of this solution was injected into HPLC through auto sampler unit.

Extraction from breast muscles were carried out by taking 100g muscles cut into two to three pieces, later ground using household mixer grinder. Five gram of this ground muscle tissue was transferred to a 50 ml polypropylene screw capped centrifuge tubes. To this three ml of 0.02 M phosphate disodium EDTA buffer (pH adjusted to 6.5) was added and mixed by shaking, mixture was then homogenized with 20 ml of ethyl acetate using tissue homogenizer (Heidolph Silent Crucher M, Germany; dispersion tool is 18 F/M) at 10,000 rpm for 30 seconds which was then centrifuged (Eppendorf Centrifuge 5810R, Germany; fixed angle rotor) at 10,000 rpm for five minutes. The supernatant was then transferred into 50 ml glass stoppered test tube. The extraction procedure was repeated with 10 ml ethyl acetate and the supernatant was transferred in to same test tube. The pooled organic fraction was evaporated to dryness in a stream of nitrogen under water bath maintained at 50 °C. The residue left behind was immediately reconstituted with one milliliter of methanol. This solution was then filtered through 0.45 µm PVDF syringe driven membrane filters and 20 µl of this was injected into HPLC through auto sampler unit.

Phosphate disodium EDTA (3 %) buffer was added to each sample solutions to prevent the metal ions Cu^{2+}, Fe^{3+}, Zn^{2+} being extracted into the organic phase (acetonitrile fraction), thus a sharp chromatogram of 5,7-dichloroquinolin-8-ol and 5-chloroqunolin-8-ol was obtained. Similar technique was adopted by Hayakawa *et al.* (1982) for chromatography of clioquinol, a derivative of 8-hydroxyquinoline. Further, previous report (Bondiolotti *et al.,* 2006) indicates that addition of phosphate disodium ethylendiaminotetraacetate (Na_2EDTA) buffer prevents the formation of complexes that may reduce recovery of clioquinol in plasma and tissue samples of hamsters quantified by HPLC.

iii) ***Construction of standard curve***

The calibration curve was constructed in the range of 20 to 640 ng/ml for both the constituent molecules 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol which were spiked to drug free plasma sample. The standard curves were linear in the range of 20 to 640 ng/ml with r^2 value of 0.999 and limit of quantification was 20 ng/ml of plasma for both 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol.

For tissues matrices of liver, kidney and breast muscle, calibration curves were constructed in the range of 20 to 640 ng/ml for both 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol which were spiked to drug free liver, kidney and breast muscle samples. The standard curves were linear in the range of 20 to 640 ng/ml with r^2 value of 0.999 and limit of quantification was 20 ng/ml for a gram of tissue for all the three tissues: liver, kidney and breast muscle for both the constituent molecules 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol. The standard curves for 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol were prepared by plotting peak areas (x-axis) against concentration (y-axis) of respective molecules. The linear regression formula obtained from calibration curves were used to derive unknown concentration of 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol in plasma and tissue samples respectively.

Statistical analysis

All the experimental data obtained were expressed as mean±SE. The significant differences between the means of two groups were determined by one-way ANOVA with Tukey's post test or two-way ANOVA with Bonferroni post test whichever applicable using the GraphPad Prism Version 5 (USA).

RESULTS

The high performance liquid chromatographic (HPLC) assay system standardized for 5,7-dichloroquinolin-8-ol and 5-chloroquinolin-8-ol was used to quantify the constituent molecules of halquinol in plasma and tissue samples in the present study. However the plasma or tissue concentration of 5-chloroquinolin-8-ol was

less than the quantification limit of the assay system (20 ng/ml plasma or 'g' tissue). The plasma or tissue samples (*viz:* liver, kidney and breast muscle) of control group (C) collected at each sacrificing intervals in birds throughout the experimental period, upon HPLC analysis did not reveal presence of 5,7-dichloroquinolin-8-ol or 5-chloroquinolin-8-ol, thus eliminating chances of water and feed contamination of halquinol by unknown factors, if any.

Measurable quantity of 5,7-dichloroquinolin-8-ol was not found in plasma samples of experimental birds receiving halquinol either @ 60 or 120 ppm, however a concentration of 94.65±3.73 $ng.ml^{-1}$ plasma was observed on day '40' in samples belonging to T_3 (240 ppm). There was a significant (pd"0.05) increase in concentration of 5,7-dichloroquinolin-8-ol in liver and kidney between each successive sampling days of 5, 10, 20, 30 and 40 in T_1, T_2 and T_3 groups of experimental birds exposed to halquinol (Table 2). However, 5,7-dichloroquinolin-8-ol in breast muscle samples belonging to T_1 and T_2 group were below the quantification limit of assay system, while its concentration was significantly (pd"0.05) increased in experimental birds belonging to T_3 (240 ppm) group between each successive sampling days *viz.* 5, 10, 20, 30 and 40 of halquinol exposure.

Consequent to withdrawal of halquinol exposure in all the treatment groups (T_1, T_2 and T_3) there was significant decrease (pd"0.05) in concentration of 5,7-dichloroquinolin-8-ol in liver and kidney tissues on each successive sampling days and reached to non-quantifiable levels on day '4' (Table 3 and Figure 1, 2 & 3). The depletion of 5,7-dichloroquinolin-8-ol from breast muscles of experimental birds exposed to halquinol @ 240 ppm was relatively rapid and the residues were not quantifiable on day '3' following its withdrawal from diet (Figure 3).

DISCUSSION

The plasma levels of 5,7-dichloroquinolin-8-ol were below the limit of quantification of the developed assay system, except in four samples (out of six) belonging to 240 ppm group. Thus, the absorption and bioavailability of halquinol is poor in the present study. The bioavailability of halquinol was reported to be 5-10 per cent (Botsoglou and Fletouris, 2001). There exists a wide variation in lipid solubility among 8-hydroxyquinoline derivatives (Musiol *et al.*, 2010). The pKa value for 5,7-dichloroquinolin-8-ol is 1.81 and 7.62 and the log *'p'* value of 5,7-dichloroquinolin-8-ol is 3.31. Further, poor bioavailability of halquinol in the present study may be due to its glucuronide conjugation in the intestine itself as in the case of closely related compound clioquinol (Kotaki *et al.*, 1983). The 8-hydroxyquinolines are known to form chelation with several metal ions, especially with iron to a significant amount (Beltran *et al.*, 1985). The poor bioavailability observed in the present study may be due to combined effect of poor solubility, chelation and glucuronide metabolism with in the gastrointestinal tract.

In the present study, the significant increase (pd"0.05) in concentrations of 5,7-dichloroquinolin-8-ol in liver and kidney on each successive sampling days of 5, 10, 20, 30 and 40 in the three treatment groups were dose dependent (Table 2). On day 40 of halquinol exposure the tissue concentration of 5,7-dichloroquinolin-8-ol was relatively high in kidney (520.30 ± 10.09 $ng.g^{-1}$) in experimental birds which received halquinol @ 240 ppm. Bound residues may arise from incorporation of residues of the drug in to endogenous compounds, chemical retention of proven drug or its metabolites in to macromolecules or physical encapsulation in to tissue matrices (WHO 1989).

A metabolic study with ^{36}Cl labeled mixture of 5,7-dichloroquinoline-8-ol and 5-chloroquinoline-8-ol showed that distribution of ^{36}Cl in the urine and faeces to the extent of 39.3 and 60.4 per cent respectively, in rats following per-oral administration through stomach intubation, while their excretion were 80.5 and 10.5 per cent respectively in two month old French Friesian calf fed in capsule form (Bories and Tulliez, 1972). Thus, urinary excretion is predominant in the calf while faecal excretion is predominant in the rat. Urinary and faecal withdrawal of several quinoline compounds were very rapid with more than 90 per cent being eliminated within 48 h (Bories and Tulliez, 1972; Kotaki *et al.*, 1983).

When compared to liver and kidney tissue (60 and 120 ppm group), the mean residue concentration of 5,7-dichloroquinolin-8-ol in breast muscles was relatively less despite the experimental birds were fed halquinol at 240 ppm. This may be attributed to less vascular nature of skeletal muscles when compared to highly perfused organs (liver and kidney) in the present study.

There are no reports of either pharmacokinetic profile of halquinol or metabolic site (s) in poultry following extra -vascular administration. Depletion of tissue residues depends on terminal elimination phase of the drug in question. It has been reported that clioquinol, a quinoline compound mostly metabolised to clioquinol glucuronide and sulfate and these metabolites were excreted in urine and bile (Kotaki *et al.*, 1983). Rapid depletion of 5,7-dichloroquinolin-8-ol in all the treated birds (Table 3) might be due to combined effect of intestinal and/or hepatic metabolism and urinary or biliary excretion in the present study.

In the residue depletion study, the highest concentration of 5,7-dichloro-8-quinolin-ol was observed in kidney (34.50±1.29 $ng.g^{-1}$) in the 240 ppm group on day three following withdrawal of halquinol (Table 3). Halquinol is generally incorporated in the poultry diet at 30 or 60 ppm. The metabolic profile of halquinol in poultry is not understood, however, radio labeled studies in rat

Table 2:
Tissue concentration (ng.g^{-1}) of halquinol* at different time intervals during 40 days dietary exposure in broiler chickens

Day of halquinol exposure	Halquinol® concentration in diet						
	60 ppm (T_1)		120 ppm (T_2)		240 ppm (T_3)		
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Muscle
5	24.08±0.41^{A}	*30.11±1.49*^{a}	39.50±0.71^{F}	45.08±0.92^{f}	58.17±2.78^{K}	68.17±2.41^{k}	26.43±1.16$^{>}$
10	48.57±2.19^{B}	60.72±1.60^{b}	84.33±1.47^{G}	94.58±1.48^{g}	148.7±2.95^{L}	161.8±5.36^{l}	50.65±1.14$^{\#}$
20	103.22±2.34^{C}	129.0±2.62^{c}	180.0±3.65^{H}	190.5±4.06^{h}	319.2±5.83^{M}	332.7±8.55^{m}	98.67±2.33$^{\$}$
30	115.4±2.92^{D}	144.2±5.46^{d}	214.8±3.67^{I}	229.3±4.70^{i}	394.8±5.72^{N}	398.3±5.78^{n}	114.8±3.22$^{@}$
40	178.8±4.83^{E}	223.6±6.85^{e}	310.0±5.32^{J}	335.7±7.06^{j}	503.7±7.07^{O}	520.3±10.09^{o}	160.4±6.51$^{\wedge}$

All the values are expressed as mean ± SE; n= 6 birds sacrificed at a time; * = represents 5,7-dichloroquinolin-8-ol; Tissue concentration data (liver & kidney) between different dose groups and between different days during treatment period were analyzed by two way ANOVA followed by Bonferroni post tests, while muscle concentration data was analysed by one way ANOVA followed by Tukey's multiple comparison test; Values bearing different alphabets as superscripts both between rows (days) and columns (dose levels) for individual tissue vary significantly (*p*"0.05); Capital alphabets indicate comparison within liver tissue and small alphabets indicate comparison within kidney tissue and values bearing symbols as superscripts indicate comparison between rows with respect to muscle tissue.

Table 3:
Tissue concentration (ng.g^{-1}) of halquinol* at different time intervals following withdrawal of halquinol exposure through feed in broiler chickens

Day following withdrawal	Halquinol® concentration in diet						
	60 ppm (T_1)		120 ppm (T_2)		240 ppm (T_3)		
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Muscle
0.08$^{\#}$	179.6±4.71^{A}	224.4±7.38^{a}	310.8±4.72^{E}	336.7±6.38^{e}	504.2±6.82^{I}	520.7±10.26^{i}	160.8±6.36$^{\#}$
1	48.57±1.63^{B}	67.3±2.10^{b}	79.17±1.44^{F}	92.12±3.84^{f}	180.30±3.32^{J}	200.4±3.37^{j}	64.62±3.16$^{\wedge}$
2	35.54±2.21^{C}	44.44±1.19^{c}	59.17±1.24^{G}	63.83±1.16^{g}	83.17±2.70^{K}	91.87±2.87^{k}	27.8±1.22$^{@}$
3	24.24±0.92^{D}	30.90±1.04^{d}	28.33±0.44^{H}	29.83±0.94^{h}	31.50±1.18^{L}	34.50±1.29^{l}	ND
4	ND	ND	ND	ND	ND	ND	ND

All the values are expressed as mean ± SE; n= 6 birds sacrificed at a time ; * = represents 5,7-dichloroquinolin-8-ol ; No. of samples were collected at 2h post-withdrawal of dietary source of halquinol; Tissue concentration data between different days within a tissue of different dose groups, following withdrawal of halquinol exposure were analyzed by one way *anova* followed by Tukey's multiple comparison test; Values bearing different alphabets as superscripts between rows (days) for individual tissues vary significantly (pd"0.05); Capital alphabets indicate comparison within liver tissue, small alphabets indicate comparison within kidney tissue while values bearing different symbols as superscripts indicate comparison within muscle tissue; ND = not detectable

indicated that halquinol is excreted as glucoronide conjugate in the bile (Borries and Tulliez, 1972**)** similar to that of one of the closely related compound clioquinol which essentially undergo glucuronidation in the intestine (Kotaki *et al.*, 1983). In addition to poor absorption and metabolism in gastrointestinal tract, quinolines undergo aromatic hydroxylation and N-oxygenation based on regioselectivity among them (Dowers *et al.*, 2004).

In the present study, tissue residues of 5,7-dichloroquinolin-8-ol was determined, however the concentration of 5-chloroquinolin-8-ol was below the quantification limit of assay system. Hence, the withdrawal periods for halquinol in broiler chickens were made based on the tissue depletion profile of 5,7-dichloroquinolin-8-ol obtained in the present study.

By whatever procedures are applied, separate withdrawal periods (WPs) must be estimated from each edible tissue and the longest of these individual tissues WPs then becomes overall withdrawal period for the marketed product (Woodward, 2009).

Generally, MRL and tolerance values are employed to derive WPs for marketed veterinary medicines. Hence it can be derived by examining the time-dependent tissue depletion preferably in key tissues (*viz*: liver, muscle, kidney and fat) against the MRL or tolerance values. Upon extensive literature survey, it was found that there is lack of information on either residue kinetic data or other parameters of regulatory toxicological importance (*viz:* tolerance, ADI, NOAL, NOAEL) with respect to halquinol or its constituent molecules. However, in the absence of MRL, particularly keeping in view of developing countries where veterinary drug assessment and MRL values are difficult to generate, FAO/WHO joint expert committee on food additives (JECFA) has permitted the use of veterinary medicine products and trade at least based on scientific approach or those that develop and use their own national standards. However, there is no standard for halquinol residues in foods of animal origin set by Agricultural and Processed Food Products Export Development Authority or by Government of India under Prevention of Food Adulteration Act (GOI, 2004), thus, complicating the fixation of WPs.

In the present study, the residue profile of 5,7-dichloroquinolin-8-ol (major component of halquinol) was established and the withdrawal period was established based on its concentration equal or near to the analytical

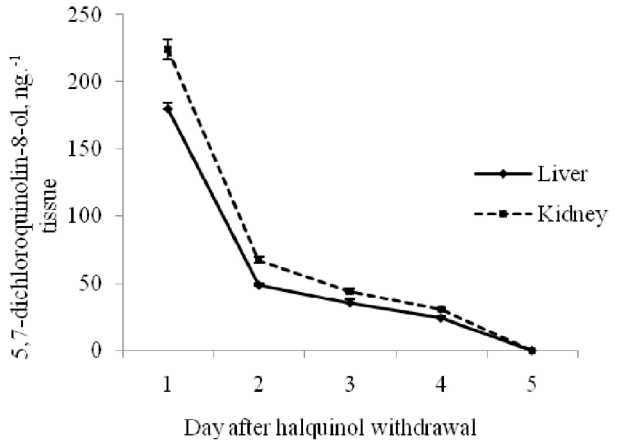

Fig.1

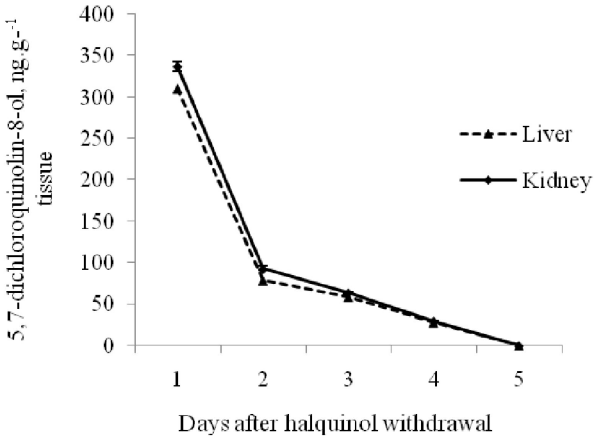

Fig.2

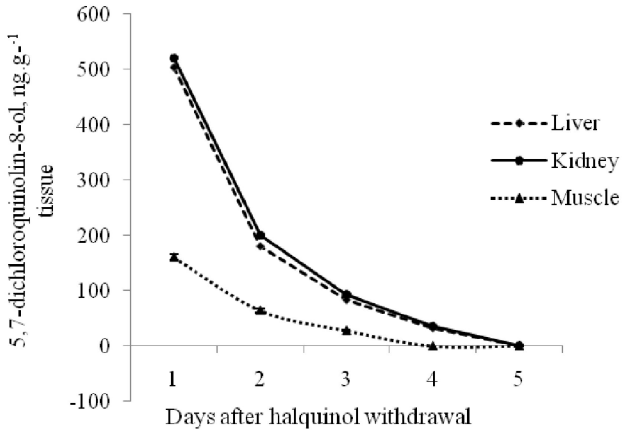

Fig.3

sensitivity (20.0 ng.g^{-1}). The residue concentration of 5,7-dichloroquinolin-8-ol found in liver, muscle and kidney were much below the levels of feed additives like apramycin, oxolinic acid and chlortetracycline used in poultry (Botsoglou and Fletouris, 2001).

In the case of substances for which MRL has not been established the issue of regulator may be proven based on presence or absence of residue in a food rather than the quantity present (Ellis, 2004). In such cases critical issues for validation may be the selectivity and sensitivity of the screening test or the lowest concentration at which a confirmatory test can detect and confirm the presence of residues. Keeping in view of the minimum required performance limit (MRPL) of the analytical device (Ellis, 2008) to detect residues of enrofloxacin and other agents (Botsoglou and Fletouris, 2001), the sensitivity, precision and repeatability of the present assay system to detect 5,7-dichloroquinolin-8-ol is acceptable.

Normally, halquinol is used at 30 and 60 ppm in poultry. The FDA has established that the edible tissue from which residues deplete most slowly could be considered as the target tissue (Ellis, 2004). It has been reported that WPs required to reach MRL could be five days for oxytetracycline and sulphadimidine and six days for ampicillin in chickens (Alhendi *et al.*, 2000).

Considering the poor bioavailability (~5-10 %) of halquinol (Botsoglou and Fletouris, 2001) and the depletion profile residues of 5,7-dichloroquinolin-8-ol in the present study, it can be hypothesized that even upon the higher dose levels (120 and 240 ppm) of halquinol, it did not result in extension of tissue depletion time when compared to recommended dose (60 ppm). In spite of its extensive use in India as a feed additive in broiler chickens there were no incidence of reported adverse effects. In the absence of systemic pharmacovigilance studies in India and continued use of halquinol in broiler chickens, the present study suggests that three days pre-slaughter withdrawal time for halquinol is the best option when used as feed additive at the @ 60ppm.

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Research Article

CLINICO-BIOCHEMICAL EVALUATION OF SUB-ACUTE TOXICITY OF INDOXACARB IN BUFFALO CALVES

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ABSTRACT

The experiment was undertaken to study the clinico - biochemical changes due to indoxacarb induced toxicity in buffalo calves for a period of 24 days. The study was conducted on seven male buffalo calves (9-12months) of age, weighing between 56-85 kg which were randomly divided into 2 groups (Group I and Group II) of 3 and 4 animal, respectively. The group 1 was kept as control without insecticide and group II was given indoxacarb to each animal@ 10mg/kg b.wt, po. The treated animals were observed regularly for clinical changes and blood was collected in heparin containing test tubes at day 0 before administration of indoxacarb, therafter on 3,7,10,14,17,21,24th day during treatment period and on day 7th during post treatment by jugular vein puncture to study the biochemical parameters such as aspartate animotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), acid phosphatase (ACP), plasma, erythrocyte and whole blood cholinesterase , blood glucose, blood urea nitrogen (BUN), creatinine and cholesterol levels. Indoxacarb produced mild clinical signs including reduction in body weight and feed intake, lameness, and nasal discharge. A significant ($P<0.05$) increase in the mean values of AST, ALT, ALP, AChE, BUN and creatinine in indoxacarb treated group as compared to control group indicated mild toxicity in buffalo calves.

Key words: Buffalo calves, biochemical, clinical, indoxacarb, transferases, cholinesterase

INTRODUCTION

Indoxacarb is a representative of the new pesticide class Oxadiazine. Indoxacarb [(S) - methyl 7 chloro -2, 5 dihydro -2- {(methoxy carbonyl) {4 (trifluoromethoxy) phenyl} amino]–carbonyl] indenol {1,2 - e} {1,3,4} oxadiazine - 4a -pH)-carboxylate] is used widely in agriculture and horticulture as an insecticide and exhibits strong activity against lepidopteron pests of vegetables, tree fruits, cotton, corn, peanut, soybean, alfalfa and other crops (Harder *et al.*, 1997). Activity of this compound has also been shown against some homopteran and coleopteran species (Wing *et al.*, 2000). However, the biological activity of indoxacarb is not just limited to insects, but its extensive use poses inadvertent serious health hazards in livestock, aquatic organism and man (National Registration Authority for Agriculture and Veterinary Chemical, 2000). It is a pro-insecticide, must undergo bioactivation to the N decarbomethoxyllated metabolite (Wing *et al.*,1998) which is much more toxic than parent compound. The activated metabolite of indoxacarb has a unique mode of action involving the blocking of sodium channels of nerve cells, resulting in paralysis and death of the target pest species (, Lapied *et al.,* 2001). The neuronal nicotinic AChR could be one of the primary target sites of the insecticide in mammals (Zhao *et al.,* 2003). Little information is available showing effect of indoxacarb in buffalo calves. Therefore, the present study was conducted to evaluate the toxic effect of indoxacarb on various biochemical parameters in buffalo calves.

MATERIAL AND METHODS

The study was conducted on seven male buffalo calves (9-12months) of age, weighing between 56-85kg which were randomly divided into 2 groups (Group I and Group II) of 3 and 4 animal respectively. The animals were dewormed and acclimatized to uniform environmental conditions and were provided seasonal green fodder and water *ad libitum*. Blood samples were collected for determining the normal values of various biochemical parameters. Groups I served as control to which no insecticide was given. Animals of Group II were administered indoxacarb in repeated oral dose of 10mg/kg /day for 24 consecutive days. Each animal was examined daily for general conditions, body weight and feeding habits. After the treatment period was over, animals were observed for recovery. The requisite amount of insecticide was suspended in 50 ml of water and drenched to the animals daily between 9.00-10.00 a.m. The animals were weighed every alternate day and doses of indoxacarb were corrected according to change in body weight.

Blood was collected in heparin containing test tubes on days 0,3,7,10,14,17,21,24 during treatment period and on day 7th post treatment by jugular vein puncture to study the biochemical parameters. For estimation of glucose, blood was collected without anticoagulant (Frankel *et al.*, 1970). Plasma enzymes, aspartate animotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and acid phosphatase (ACP) were estimated using the standard methods of Wotten

(1964). Plasma, erythrocyte and whole blood cholinesterase activity was measured according to the method of Voss and Sachsse (1970) as modified by Moroi *et al.*, (1976).Blood urea nitrogen (BUN), plasma creatinine and plasma cholesterol levels were determined by following the method of Wotten (1964). Statistical analysis of the data was performed by applying Student's't' test (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Daily oral administration of indoxacarb at dose rate of 10mg/kg/day produced mild toxicosis in buffalo calves. Indoxacarb produced mild signs of decrease in body weight, lameness, decrease feed intake and nasal discharge. However, Wing *et al.,* (2000) reported that neurotoxic symptoms lead to a rapid and irreversible cessation of feeding. Similar findings were observed by Goyal and Sandhu (2010) after subchronic oral exposure of Indoxacarb on buffalo claves. Indoxacarb at a dose rate of 10mg/kg/day for 24 consecutive days produced significant increase in plasma aspartate aminotransferase levels and alanine aminotransferase levels from 0 day to 24th day of treatment, respectively (Table 1). However, the aminotransferases concentration started decreasing after 7th day of withdrawal of indoxacarb treatment. Similarily, Kumar *et al.,* (2013) and Goyal and Sandhu (2010) observed the increase in aspartate aminotransferse and alanine aminotransferse levels due to subacute and subchronic oral exposure of indoxacarb in broilers and buffalo calves, respectively. Activity of AST is high in acute and chronic liver injury (Tennant, 1997) and muscular damage (Brar *et al.,* 2000). ALT is employed as a marker of hepatocellular damage and in general is considered a more sensitive indicator of liver cell injury than AST (Oser, 1976). Although it is difficult to point the damage to any particular organ by indoxacarb, but increase levels of aminotransferases in ruminants may be attributed to liver damage (Cornelius, 1989), as it is the primary organ of biotransformation of indoxacarb.

Repeated oral administration of indoxacarb @ 10mg/kg/day also produced an elevation in plasma alkaline phosphatase activity from a 0 day to 24th day of experiment (Table1). The present finding is in agreement with the study of Kumar *et al.*, (2013) in broilers. It has been reported that any damage to liver, bone, small intestine and kidney may liberate alkaline phosphatase in blood stream (Kaplan and Righeth, 1970). On the other hand, acid phosphatase is a lysosomal enzyme that gets stimulated in cases of imminent or prevalent tissue damage (Wilson *et al.,* 1970) or release of phosphatase usually occurs due to their increased synthesis brought about by variety of liver conditions (Seetharam *et al.,* 1986). There was elevation in plasma cholinesterase levels from 0 day to 24th which returned to normal after 7 days of withdrawl of insecticide

Table 1:
Mean values of biochemical parameters (Mean± SE) at different intervals of sub acute indoxacarb study in buffalo calves.

Time (Days)	0	3	7	10	14	17	21	24	Post treatment
Alkaline Phosphatase (nmol phenol liberated/min/ml)									
Group I [a]	68.34 ± 3.88	72.36 ±4.63	74.14 ±4.10	67.23 ±3.68	66.59 ±5.41	70.43 ±4.41	66.41 ±2.51	68.87 ±4.02	74.78 ±6.70
Group II	65.50 ± 2.25	67.14 ± 6.82	70.34 ± 2.66	72.84 ± 2.54	75.45 ± 5.24	78.78 ± 4.87	76.47 ±1.32*	79.84 ±3.33**	72.75 ±5.84
Acid Phosphatase(nmol phenol liberated /min/ml)									
Group I [a]	1.87 ± 0.09	1.93 ± 0.09	1.84 ± 0.13	1.92 ± 0.29	1.87 ±0.08	1.20 ±0.06	1.98 ±0.14	1.75 ±0.18	1.68 ±0.29
Group II	1.78±0.14	1.84 ± 0.08	1.87 ±0.20	1.94 ± 0.16	1.96 ±0.21	1.98 ±0.10	2.00 ±0.07	1.97 ±0.21	1.88 ±0.31
Aspartate Aminotransferase (nmol pyruvate formed/min/ml)									
Group I [a]	62.13 ± 3.75	62.50 ±3.37	60.44 ±2.05	62.32 ±2.69	60.80 ±4.52	67.29 ±2.99	62.86 ±4.37	65.54 ±5.27	63.83 ±6.24
Group II	54.48±2.65	56.48 ±3.77	62.36 ±1.81	61.68 ±2.21	65.37 ±3.18	67.89 ±4.49	70.21 ±5.32	71.63 ±3.34**	65.34 ±4.56
Alanine Aminotransferase(nmol pyruvate formed/min/ml)									
Group I [a]	48.15 ± 2.54	46.56 ±3.77	44.75 ±2.64	42.23 ±5.62	39.37 ±4.66	40.82 ±5.22	44.75 ±4.19	42.14 ±3.74	39.64±3.56
Group II	45.67 ± 4.51	48.24 ±3.84	49.32 ±2.66	50.78 ±4.32	54.87 ±3.48	52.64 ±4.83	56.38 ±3.72*	57.51 ±4.35**	49.72 ±5.32
Plasma Cholinesterase(nmol acetylthiocholine hydrolyzed/min/ml)									
Group I [a]	167.32 ± 6014	163.30± 7.54	172.30±2.24	167.2±3.18	169.2±2.56	171.9±2.31	168.2±4.14	171.2±2.53	165.24 ±4.52
Group II	166.54 ± 2.99	168.73±4.87	172.56±2.56	171.6±3.19	174.7±2.97	178.5±2.76	180.6±1.72*	184.4±2.40**	172.64 ±1.92
Erythrocyte Cholinetserase (nmol acetylthiocholine hydrolyzed/min/ml)									
Group I [a]	2255.2±120.3	2385.6±123.1	2448.1±156.3	2282.±84.76	2621.±101.4	2340.±126.4	2379.±130.2	2482.±128.3	2621.5±114.2
Group II	2445.5±183.3	2462.4±146.6	2621.5±95.48	2426.±120.6	2507.±98.42	2426.±110.4	2355.±182.2	2464.±140.2	2602.3±128.3
Whole Blood Cholinetserase(nmol acetylthiocholine hydrolyzed/min/ml)									
Group I [a]	674.32 ± 9.79	668.45±10.5	649.76± 9.32	651.8±8.25	667.2±7.39	632.1±9.28	648.3±5.63	637.9±6.33	658.34 ±9.41
Group II	658.39 ± 7.22	656.45±8.48	647.93± 7.67	642.1±6.30	659.9±9.54	657.6±7.18	652.2±9.94	648.8±6.65	637.94 ±7.45

The values are mean ± SE of 4 animals unless stated, a= values are mean ± SE of 3 animals. * P< 0.05 ; ** P< 0.01

Table 2:
Mean values of biochemical parameters (Mean± SE) at different intervals of sub acute Indoxacarb study in buffalo calves

Time(days)	0	3	7	10	14	17	21	24	Post Treatment (7days)
				Blood Urea nitrogen (mg/dl)					
Group I [a]	2.93 ± 0.08	2.88±0.04	3.12±0.19	2.84±0.07	2.87±0.13	2.70±0.12	2.92±0.08	2.81 ±0.04	2.76 ±0.10
Group II	2.65 ± 0.05	2.71±0.12	2.81±0.16	2.98±0.05	3.03±0.07	3.12±0.05	3.24±0.06*	3.32±0.05*	4.80 ±0.11
					Blood Glucose(mg/dl)				
Group I [a]	62.76 ± 2.46	60.56±1.83	58.36±2.76	59.43±3.17	57.34±2.29	57.56±1.34	58.34±1.94	60.42±1.97	68.71 ±3.29
Group II	58.72 ± 2.19	60.35±1.64	62.97±2.44	58.04±2.52	56.42±3.48	55.10±4.61	52.86±3.31	54.24 ±3.08	55.13 ±2.94
					Plasma Creatinine(mg/dl)				
Group I [a]	1.28 ± 0.06	1.30±0.04	1.32±0.05	1.36±0.02	1.30±0.07	1.26±0.03	1.24±0.04	1.28±0.03	1.34 ±0.06
Group II	1.32 ± 0.02	1.28±0.04	1.34±0.05	1.36±0.06	1.40±0.10	1.31±0.04	1.34±0.03	1.48±0.02**	1.40 ±0.09
					Plasma Cholesterol(mg/dl)				
Group I [a]	104.3± 6.54	109.67±5.49	112.95±7.98	118.92±8.20	110.26±8.79	104.23±6.64	112.74±7.78	116.32±9.11	108.70 ±7.98
Group II	104.4± 8.84	102.09±7.16	109.67±9.38	113.61±6.48	116.44±8.33	118.20±9.36	122.61±10.2	119.67±8.97	110.54 ±8.63

The values are mean ± SE of 4 animals, unless otherwise stated, a= values are mean ± SE of 3 animals. * P< 0.05 : ** P< 0.01

but there was no change in erythrocyte cholinesterase level and whole blood cholinesterase levels (Table 1) suggesting slight inhibitory effect of indoxacarb on neurological system of insects (Zlotkin, 2001).

Subacute oral exposure of indoxacarb did not produce any significant alterations in levels of blood glucose and cholesterol levels. There was significant elevation in levels of blood urea nitrogen and creatinine from 0 days to 24th day of the study (Table 2). However, the values became normal after the withdrawl of insecticide on 7th day. The findings are in agreement with Shit *et al.,* (2008) who conducted an experiment by administrating indoxacarb @ 24 mg/kg b.wt. in wistar rats for a period of 28 days and found an increase in plasma creatinine level in rats. The results indicated that indoxacarb has negative affect the renal functions. It can be concluded from the present study that indoxacarb is mildly toxic to buffalo calves when administered in repeated oral doses of 10mg/kg/day for 24 consecutive days.

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Research Article

DYNAMICS OF Ca^{2+} SENSITIVITY OF MYOMETRIA OF NON-PREGNANT AND PREGNANT BUFFALOES

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ABSTRACT

This study was proposed to delineate the physiological effects of calcium on gravid and non gravid myometrium of buffaloes. $CaCl_2$ was used to study the differential tonicity of buffalo myometrium isolated from non pregnant, early and late pregnancy stage buffaloes. Results revealed that, removal of extracellular calcium completely inhibited the normal rhythmic spontaneity of buffalo myometrium. Ca^{2+} produced dose-dependant contractile effect on buffalo myometrium in Ca^{2+} free RLS in non-pregnant, early and late stages of pregnancy. The pD_2 and the maximal efficacy (E_{max}) values produced by $CaCl_2$ were 1.92 ± 0.33, 2.38 ± 0.44 g, (n = 6); 2.20 ± 0.34, 1.45 ± 0.34 (n=6); 2.34 ± 0.17, 4.10 ± 0.61(n=6) in non-pregnant, early-pregnant and late-pregnant buffalo myometrium, respectively. From the study, it was concluded that myometrium of early pregnant uterus is least sensitive to extracellular calcium, presumably render the tissue less excitable, whereas, in late pregnancy, myometrium exhibited increased sensitivity to extracellular Ca^{2+}.

Key words: Calcium, myometrium, pregnant Buffalo and Non pregnant Buffalo.

INTRODUCTION

Uterus exhibits spontaneous myogenic contractile activity as a direct result of oscillations in intracellular calcium (Ca^{2+}_i) levels (Taggart and Wray, 1998). Myometrial resting membrane potential (RMP) is generally -35 to -60 mV, being low during most of the gestation period and increases near term. Extracellular calcium ions concentration is approximately 1.5 mmol/L and the estimated myometrial intracellular concentration is 0.13ìmol/L (Sanborn, 2000). Myometrium contains a significant intracellular pool of bound calcium ions stored in sarcoplasmic reticulum (SR), and the myometrial cells have a high concentration of SR. Calcium can be mobilized from SR as well as from the extracellular fluid. Formation of gap junctions occurs near term which simplifies coordination between smooth muscle cells (Guyton and Hall, 2000). The SR actively absorbs free calcium in the myometrial cells through Ca-ATPase (SERCA) (Tribe, 2001). As long as uterus is under progesterone dominance, uptake of calcium ions to the SR inhibits contractions (Bergstrom, 2009).

To evolve an effective therapeutic strategy for treatment or prevention of pre-term labour or other patho-physiological states of myometrium in buffaloes, it is essential to understand the sensitivity of myometrium to Ca^{2+} during different stages of pregnancy and its dynamics. It will help in controlling uterine contractions during the entire gestation period till parturition and also successful and also termination of pregnancy, if required. With this background information, the present study was undertaken on buffalo myometrium.

MATERIALS AND METHODS

Tissue source

Uteri along with ovaries were collected from both the cyclic and pregnant non-descript buffaloes immediately after slaughter from the local abattoir and transported to laboratory in chilled (4.0 ± 0.5æ%C) Ringer-Locke solution (RLS) of the following composition (mM/L): NaCl, 154; KCl, 5.6; $CaCl_2$, $2H_2O$, 2.2; $NaHCO_3$, 6.0; D-Glucose, 5.5 and having pH of 7.4. In Ca^{2+} free RLS, $CaCl_2$, $2H_2O$ was replaced by EGTA (0.38g/L). Di-estrous stage uteri were selected based on the well developed projected (crowned) corpus luteum on ovaries, genitalia with closed cervix and thick mucus. Further, uteri were cut open to ensure the pregnancy status and if pregnant, fetus was collected to determine the stage of pregnancy by measuring the curved-crown versus rump (CRV) length of fetus by applying the formula as suggested by Soliman *et al.* (1970).

Recording of isometric tension

Myometrial strips were prepared as described earlier (Choudhury *et al.,* 2010) and mounted in thermostatically controlled (37.0 ± 0.5æ%C) organ bath (Ugo Basile, Italy) of 10 ml capacity containing RLS continuously aerated with carbogen (95% O_2 + 5% CO_2) under a resting tension of 2 g. During the equilibration period of 2 hrs, bath fluid was changed after every 10 min. Isometric tension in tissue was recorded with the help of force transducer using Lab Chart Pro V6.1.3 software (Powerlab, AD Instruments; Australia).

Myometrial strips were made calcium-free by two successive washing in Ca^{2+}-free RLS solution with EGTA for 5 min each and there after left for 10 min in the same

Ca^{2+} free RLS. Tissues were again given two successive washings with Ca^{2+} free RLS solution without EGTA. After equilibration period of almost one hour, cumulative concentrations of calcium chloride were added from 10^{-7}M to 10^{-2}M to produce the maximal contraction. The concentration–response curves were constructed by measuring the height by using Labchart pro v 6.1.3 software (Aaronson *et al.,* 2006). EC_{50} and E_{max} value were determined by non linear regression analysis using Graph Pad Prism 4.0 (Graph Pad, La jolla, USA) and pD_2 value (potency) was calculated as – log of EC_{50}.

Data analysis

Results are expressed as mean ± SEM. Multiple mean values were analyzed using two-way ANOVA followed by Bonferroni post hoc test to compare between different treatments while one-way ANOVA followed by Tukey'spost-hoc test to compare between three groups.

RESULTS

In Ca^{2+}-free RLS, $CaCl_2$ produced concentration-dependent contractile effect on non-pregnant as well as pregnant buffaloes uteri (Fig. 1). Minimum threshold concentration of $CaCl_2$ required to initiate contraction was 10 µM and the maximal effect was observed at 10mM concentration on the uteri of both the non-pregnant and pregnant buffaloes irrespective of the stage of pregnancy as depicted in Fig. 2.

The pD2 value of $CaCl_2$ and maximal contraction produced by it (E_{max}) were 1.92 ±0.33 and 2.38±0.44 g; 2.20±0.34 and 1.45±0.34; and 2.34±0.17 and 4.10 ±0.61 in non-pregnant, early-pregnancy and late-pregnancy stage uteri, respectively. Compared to the effect of $CaCl_2$ on uteri of non-pregnant buffaloes, contractile effect on uteri of early pregnancy stage was markedly less as E_{max} value was almost 40 per cent lower compared to that on non-pregnant uteri and the DRC of $CaCl_2$ on early pregnancy uteri was on extreme right as shown in Fig. 2. But the DRC of $CaCl_2$ on uteri of late pregnancy stage buffaloes was on extreme left-side (Fig. 2) and the shift was significant ($P < 0.05$). Although pD2 values of $CaCl_2$ did not differ significantly during different stages but E_{max} value was significantly ($P<0.05$) higher on uteri of late pregnancy stage as compared to that on non-pregnant myometrium (Table 1).

DISCUSSION

Present study was carried out on buffalo myometrium collected from non-pregnant and pregnant buffaloes to see the effect of calcium on myometrial contractility. Salient observation included significantly ($P<0.05$) higher sensitivity of myometrium of late pregnancy stage and least of the early pregnancy stage buffaloes compared to that on non-pregnant uteri. Spontaneous rhythmicity of myometrial contraction was completely abolished in Ca^{+2} free RLS (-Ca^{+2}).

Muscular contractibility of myometrium in different stages of pregnancy is regulated by number of paracrine, autocrine and endocrine factors. These factors regulate uterine activities by the involvement of a number of cell to cell crosstalk pathways, which are complex and also show wide species variations (Sanborn, 2007). The regulation of myometrial contractibility in buffaloes is stage-dependant and molecular signalling pathways involved are obscure till date.

Like other ruminants, buffalo myometrium also exhibits differential contractibility pattern during different stages of pregnancy. During early pregnancy, myometrium remains under progesterone dominance which causes down-regulation of oxytocin receptors (OTR) and hence uterus remains quiescent (Challis, 2000). This quiescence of myometrium during early pregnancy is necessary for successful implantation of embryo and prevention of early embryonic abortion. Resting membrane potential was shown to increase from around –70 mV at 29 weeks to –55 mV at term and during labor (Parkington *et al.,* 1999) and these changes were associated with increased frequency of contractions *in vitro*.

Table 1:

Comparative pD_2 and E_{max} values of $CaCl_2$ in non-pregnant (n=6), early pregnant (1-3 months; n=6) and late pregnant (6-8 months; n=6) buffalo myometrium in Ca^{2+} free RLS.

Treatment	pD_2	E_{max} (gm)
Nonpregnant (n=6)	1.92 ± 0.33	2.38 ± 0.44
Early pregnancy(n=6)	2.20 ± 0.34	1.45 ± 0.34
Late pregnancy(n=6)	2.34±0.17	4.10 ±0.61*

* P<0.05 V/S non-pregnant.

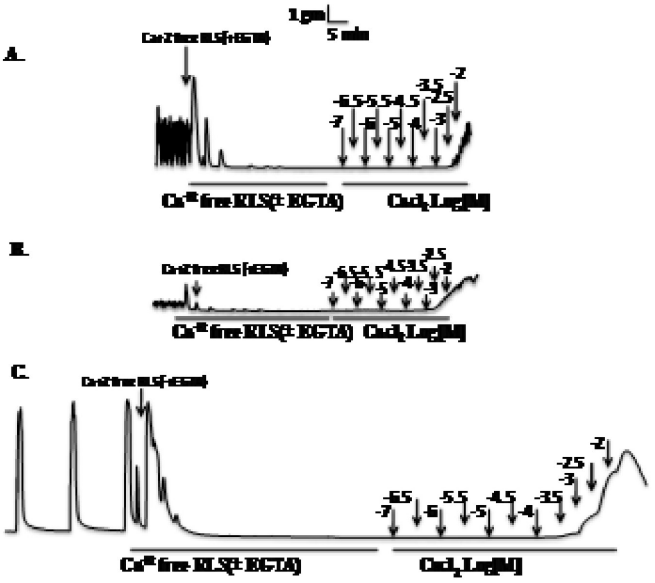

Fig.1:

Representative physiograph recording showing concentration-dependent contractile effect of $CaCl_2$ (10^{-7}–10^{-2} M) on myometrial strips of non pregnant (A), early pregnancy (0-3 month) (B) and late pregnancy (6-8 month) (C) stage buffaloes in the presence of Ca^{2+} free RLS.

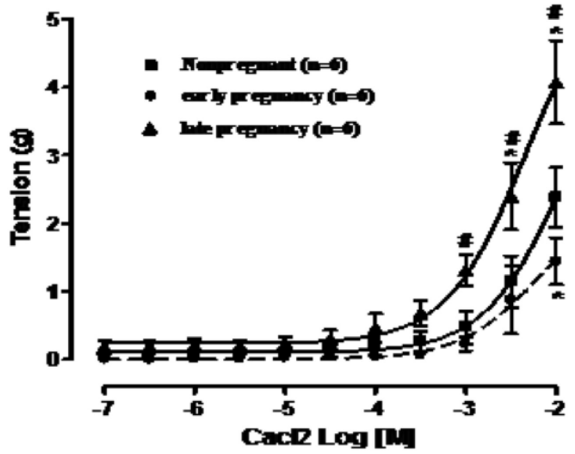

Fig. 2:
Cumulative concentration response curves of calcium chloride on myometrial strips from non-pregnant (n=6), early pregnancy (1-3 months; n=6) and late pregnancy (6-8 months; n=6) stage buffaloes. Vertical bars represents SEM. Data were analyzed by two-way ANOVA followed by Bonferoni post-hoc tests. * P<0.05 V/S non-pregnant, # P<0.05 V/S early-pregnant.

Progesterone dominance continues till the end of mid pregnancy. During late trimester of pregnancy, progesterone dominance gradually decreases and the locally secreted prostaglandins, endothelial factors and oestrogens gradually increase. With increase in oestrogen and simultaneous decrease in progesterone, myometrium start expressing OTRs and Ca^{2+} sensitivity is regulated dynamically through hormones (Fuchs *et al.,* 1992). In the present study, we observed highest sensitivity of myometrium of late pregnancy stage buffaloes to extracellular Ca^{2+} and this is in accordance with the literature in other animals. Increase in spontaneity of myometrium facilitates induction of labour and ease in parturition.

In pregnant human myometrium, slow wave depolarization alone is not sufficient to trigger contractions, but it will occur once the slow depolarization reaches the threshold for regenerative action potential generation (Kawarabayashi *et al.*1986). Excitability of tissue, in part, is governed by the resting membrane potential which is determined by opposing inward (Na^+, Ca^{2+}) and outward (K^+, Cl^-) ionic fluxes. The resting membrane potential gradually becomes more depolarized as term approaches. Contractions are elicited in pregnant myometrium by action potentials superimposed onto slow waves. In the present study, when the extracellular calcium was completely removed from RLS (- Ca^{2+}), entry of extracellular calcium was completely blocked. The DRC of calcium chloride was significantly (P<0.05) shifted towards left, with slight increase in potency, albeit, the increase in E_{max} value was found to be statistically significant (P<0.05) compared to control (non-pregnant). This observation indicates the entry of extracellular Ca^{+2} through VDCC (Coleman *et al.,* 2000). The increase in sensitivity to calcium may be attributable to an increase in the number and/or affinity to VDCC which may be changed during gestation.

Oxytocin receptors in increase myometrium during late gestation stage and it contributes to uterine contractions at delivery (Alexandrova and Soloff, 1980) or may be the influx of extracellular calcium activates ryanodine-sensitive 'calcium-induced calcium release' store in late pregnancy (Tribe, 2001). Increased sensitivity of myometrium to Ca^{2+} during late pregnancy may be associated with raised levels of cytokines (IL-6, IL-8 and IL-1β) in amniotic fluid and myometrial tissue of term and preterm labouring women (Brocklehurst, 1999).

During diestrous stage of estrous cycle, ovary shows presence of corpus hemorrhagicum and transient corpus luteum. The formed CL secretes progesterone which reaches peak if animal becomes pregnant. In the absence of pregnancy, the progesterone reaches to basal circulating levels and makes the uterus less sensitive to estrogen. This causes gradual decrease in the regulation and expression of OTR. During our study extracellular Ca^{2+} exhibited medium sensitivity of buffalo myometrium and may be due to both estrogen and progesterone effect.

From the study, it was evident that extracellular calcium is required for regulation of myometrial contractility and spontaneity, however, further studies are required to unravel the molecular mechanisms and signaling pathways of myometrial contractility in buffaloes which are still obscure.

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Research Article

AMELIORATION BY *ECLIPTA ALBA* IN ARSENIC EXPOSED COCKERELS

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ABSTRACT

The present study was designed to assess the effects of *Eclipta alba* when administered as dried powder with arsenic trioxide @100ppm in feed with the aim of achieving restoration of altered haematological, biochemical and serum enzymatic parameters. Thirty five White Leghorn 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 was fed with arsenic @ 100ppm + silymarin. IV and V groups were offered feed mixed 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. The haematological, biochemical and serum enzymatic parameters were studied following 90 days feeding trial. Arsenic exposure led to significant depletion (Pd"0.05) of Hb, PCV, TEC and TLC in cockerels fed on 100ppm arsenic. There was a significant increase (Pd"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 Pd"0.05) was observed in total protein, albumin and globulin levels indicative of hepatic and renal damage. Coadministration of *Eclipta alba* was quite effective almost parallel to silymarin in reducing arsenic induced haematological, biochemical and serum enzymatic alterations following 90 day exposure in WLH cockerels.

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

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 pollution in the environment has gained importance owing to its widespread toxic effects on aquatic and terrestrial animals. 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. Various plant products contain antioxidants, vitamins, flavonoids and polyphenolic compounds that are known to act as the scavengers of free radicals and inhibitors of lipid peroxidation (Flora *et al.,* 2008). Despite wide therapeutic potential of *Eclipta alba,* little information is available on the haematobiochemical alterations 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 into small pieces and was 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, four to six weeks old male white leghorn chicks, procured from Instructional Poultry Farm of the University, were randomly divided into five groups of seven birds each after 15 day acclimatization. The birds in poultry shed were kept in battery cage system under standard managemental conditions. After two weeks of adaptation period, the II group was offered feed mixed with arsenic (Reagent grade; Loba chemie) @100ppm and III group was fed with arsenic@ 100ppm + silymarin (Silybon-140, Microlabs). IV and V groups were offered feed mixed 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. After every 30 days blood was taken from each bird for study of different parameters. The research project was approved by IAEC for conducting this investigation.

Haematological examination

Blood samples were collected in 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 total erythrocyte count (TEC), total leucocyte count (TLC/), packed cell volume (PCV) and haemoglobin were estimated.

Biochemical profiles

The serum collected from blood samples was used for the estimation of biochemical parameters viz. total proteins, albumin, globulin, total cholesterol, bilirubin, glucose, triglycerides, creatinine and uric acid and 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 (Pd"0.05) reduction in Hb, TEC and PCV of cockerels in all groups as compared to control at 60 and 90 days interval. Hb concentration declined in all treatment groups except silymarin group in duration dependent manner (Table 1). 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. Prophylactic treatment groups showed restoration in values of haematological biomarkers.The anaemic condition of the arsenic exposed cockerels was possibly due to the depression of bone marrow activity and impaired folic acid absorption **(**Klassen, 2007). Padmaja *et al.* (2009) and Halder *et al* .(2009) reported reduced levels of Hb, PCV and following arsenic intoxication in chickens.

The decrease in PCV might be due to haemodilution to maintain normal cardiac output and pooling of blood cells in the blood reservoirs including spleen. The decrease in TEC levels could be due to the bone marrow suppression. A significant decrease in TLC observed in this study, could be attributed due to the damage of leucocytes by arsenic. Ramsajan *et al.* (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 (Radostits, 2000). Findings of the study indicate that DPEA 2000ppm ameliorated the haematological parameters like silymarin. Similar results regarding protective effect of *Eclipta alba* on haematological parameters were also reported by Pandey *et al.* (2012).

The total serum protein, albumin and globulin were significantly (P d" 0.05) reduced in all groups as

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 (Unit)	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	11.50 ±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	32.00 ±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	30.00 ±1.07^{y}	32.43 ±0.61^{Ax}	29.30 ±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}	3.50 ±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^{By}	23.377 ±0.90^{z}	32.04 ±0.36	31.03 ±0.11	30.48 ±0.26^{Bx}	32.50 ±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^{Dz}	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^{By}	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^{By}	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^{Bx}	289.67±12.66^{Ax}	271.32±10.85^{Cy}	334.90±11.78^{Bx}	380.53±11.10^{Az}	283.73±16.35^{Ay}	263.93±9.50^{C}	238.35±7.83^{D}	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)	150.20±1.74^{B}	152.32±1.69^{D}	156.33±1.28^{E}	176.49±1.63^{z}	208.02±2.52^{y}	227.00±7.56^{x}	150.94±0.90^{E}	156.55±0.78^{C}	171.00±1.34^{Dx}	173.55±3.14^{Ax}	190.10±2.48^{By}	204.02±2.47^{x}	151.41±1.24^{Bx}	162.84±2.62^{Cy}	188.12±1.75^{z}
Triglycerides (mg/dl)	85.22±2.58^{B}	85.86±1.62^{C}	83.63±3.08^{D}	91.91±1.79^{z}	120.80±1.20^{y}	143.10±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^{Ay}	92.14±1.67^{By}	93.36±3.43^{C}
Creatinine (mg/dl)	0.38±0.012^{A}	0.38±0.013^{B}	0.38±0.016^{D}	0.40±0.019^{z}	0.71±0.062^{y}	1.57±0.094^{x}	0.36±0.017^{Dy}	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^{Cz}
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}	35.20±0.19	7.80±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}	12.20±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}	1.50±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	123.10±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)	25.60±0.34^{C}	25.11±0.67^{D}	25.30±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}	27.00±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}	216.10±2.26^{Az}	245.86±1.37^{Ay}	256.88±1.89^{Ax}	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}	184.10±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 d" 0.05) among groups and x,y,z (P d" 0.05) superscripts in a row differ significantly among time intervals

compared to control at 90 day feeding interval (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 d" 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). Padmaja *et al.* (2009) reported similar findings in arsenic induced toxicity in broilers.

A significant (P d" 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 d" 0.05) reduction in serum glucose levels as compared to arsenic treated group II at 60 and 90 days interval. A significant (P d" 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 d" 0.05) decline in cholesterol values when compared with group II and IV throughout the trial. A significant (P d" 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 Ramsajan *et al.* (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. *Eclipta alba* is rich in saponins which are known to possess antidiabetic activity (Yoshikawa *et al.,* 2001). 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 d" 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 lines with the findings of Ramsajan *et al.* (2009) who reported similar findings in arsenic intoxicated cockerels. The flavonoids present in the *Eclipta alba* might be responsible in reducing the level of serum cholesterol (Anila and Vijayalakshmi, 2002**).**

There was a significant (P d" 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 d" 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 d" 0.05) increase in the serum uric acid and total bilirubin level was observed in all the groups at 30, 60 and 90 days feeding interval as compared to control.

Nephrotoxicities are associated with large elevations in 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 d" 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 poultry birds (Chen *et al.* (2009). The nephron protective effect of DPEA could be attributed due to the presence of phytocompounds as flavonoids and alkaloids. Our results are in accordance with the reports of Samundram *et al.* (2008) where elevated levels of uric acid and creatinine were restored towards normal level by ethanolic extract of *Eclipta alba*.

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 (Pandey *et al.,* 2005, 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. Similar findings have been reported (Samudram *et al.,* 2008) by giving ethanolic extract of *Eclipta alba* in carbontetrachloride intoxicated rats.

Serum AST activity was significantly (P d" 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 d" 005) decline in AST values at all intervals. Serum ALT and ALP levels were significantly (P d" 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 (Vandenberghe, 1995). 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 (Benjamin, 2010). 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

EFFECT OF *CARISSA CARANDAS* ON CLINICO-HAEMATOLOGICAL PROFILE ON MONOCROTOPHOS TOXICITY IN RATS

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ABSTRACT

The present investigation was conducted to access the ameliorative potential of hydroethanolic extract of *Carissa carandas* (HECC) Linn. on monocrotophos (MCP) induced toxicity in Wistar rats for a period of 28 days. In this study, effect of *Carissa carandas* leaf extract on body weight, organ weight and haematological alterations produced by monocrotophos, was determined in rats. Group T_1 was maintained as negative control which received vehicle ie: distilled water only. Group T_2, received monocrotophos an organophosphate compound @ 1.5 mg/kg b.wt. Group T_3 received MCP and Vit E @ 150 mg/kg b. wt. dissolved in distilled water. Group T_4, T_5 and T_6 received HECC @ 100, 200, 300 mg/kg b.wt. dissolved in distilled water along with MCP. All the treatment groups produced non significant changes in mean body weight, mean organ weight and relative organ weight except for mean organ weight of liver and lungs and relative organ weights of liver. Significant results were observed in haematological parameters by increased haemoglobin concentration, WBC and PCV level in treated groups.

Keywords: Hydroethanolic, *Carissa carandas*, monocrotophos, haemoglobin concentration, WBC, PCV

INTRODUCTION

Monocrotophos (MCP), is a broad spectrum contact and systemic organophosphate insecticide and acaricide widely used in India and is a potent neurotoxicant (Yaduvanshi *et al.*, 2010). It produces toxicity by inhibiting acetylcholinesterse (AChE) irreversibly which results in accumulation of ACh and persistent stimulation of cholinergic receptors and thus leading to increased secretions (Echobichon, 2001). Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants. *Carissa carandas* Linn. commonly known as “Karaunda” in Hindi, “Karvand” in Marathi and “Bengal Currants” in English, is a dichotomously branched evergreen shrub, belonging to the family Apocynaceae (Nadkarni, 1976). Various chemical constituents of plants are believed to possess anti-inflammatory, analgesic and antioxidant properties but there is paucity of literature related to their effect on body weight, organ weights and haematological profile in test animals. Hence, the present study was undertaken to study the effect of hydroethanolic extract of *Carissa carandas* (HECC) on clinical and haematological profile on monocrotophos induced toxicity in rats.

MATERIALS AND METHODS

HECC preparation

Hydroethanolic extract of dried leaves of *Carissa carandas* (HECC) was prepared and was used as herbal medicine as per the method described by Barua *et al.* (2009).

Experimental design

A total of thirty six female Wistar rats were divided randomly and equally into six groups T_1, T_2, T_3, T_4, T_5 and T_6. The rats were acclimatized for 15 days to the environment, before the start of the experiment. Group ‘T_1’ served as negative control and was treated with distilled water @ 0.5 ml/animal. Group ‘T_2’ served, as positive control, which received MCP @1.5 mg/kg b.wt. Group ‘T_3’ received monocrotophos @1.5 mg/kg b.wt. and Vit E @ 150 mg/kg b.wt. as a referral standard and Groups T_4, T_5 and T_6 were treated with HECC @ 100, 200, 300 mg/kg b wt, respectively, along with MCP @1.5 mg/kg b.wt.

Clinico-haematological examination

The body weight of each rat was recorded one day before initiation of treatment (Day 0) and thereafter at weekly intervals throughout the period of 4-week study. The organ weight of liver, kidney, brain and ovary of each rat was recorded after humanely sacrificing on 28th day of the experiment and the relative organ weight was calculated. After 28th day of experiment, the blood samples were collected in 1% EDTA and subjected to hematological studies such as hemoglobin (Hb), using Sahli’s method (acid hematin), total erythrocyte count(TEC), total leucocyte count (TLC) and packed cell volume (PCV) by micro tube method as mentioned by Benjamin (2001). All the values were presented as Means ± SEM and statistically analysed by completely randomized design at 5% level of significance.

RESULTS AND DISCUSSIONS

There was no significant ($P<0.05$) change in the

Table 1:
Effect of hydroethanolic extract of *Carissa carandas* on of Wistar rats exposed to monocrotophos (n=6).

Sr. No.	Organ	Groups						Significance
		T_1	T_2	T_3	T_4	T_5	T_6	
				Mean organ weight				
1	Liver	7.34±0.06^{bc}	7.179±0.58^{c}	7.65±0.05^{bc}	7.51±0.16^{bc}	8.243±0.319^{ab}	9.063±0.48^{a}	P < 0.01,0.05
2	Kidney	1.605±0.11	1.94±0.10	2.11±0.09	1.883±0.090	1.843±0.140	1.775±0.132	NS
3	Brain	1.838±0.056	1.698±0.089	1.866±0.172	1.593±0.07	1.66±0.108	1.628±0.096	NS
4	Heart	0.805±0.059	0.788±0.022	0.828±0.046	0.808±0.034	0.761±0.073	0.781±0.069	NS
5	Lungs	1.388±0.182^{c}	2.203±0.128^{a}	1.806±0.124^{ab}	1.764±0.05^{bc}	1.741±0.146^{bc}	1.582±0.164^{bc}	P < 0.01,0.05
6	Spleen	0.461±0.032	0.623±0.050	0.615±0.10	0.556±0.043	0.513±0.052	0.485±0.102	NS
				Relative organ weight				
1	Liver	3.00±0.07^{b}	2.92±0.22^{b}	3.08±0.06^{b}	3.02±0.11^{b}	3.325±0.14^{ab}	3.74±0.24^{a}	P < 0.05
2	Kidney	6.55±0.39	8.026±0.38	7.05±1.24	7.698±0.34	6.63±0.69	7.835±0.31	NS
3	Brain	7.549±0.34	7.025±0.39	5.863±1.20	6.505±0.24	6.813±0.56	5.448±1.15	NS
4	Heart	3.289±0.206	3.265±3.265	3.375±0.165	3.301±0.131	3.128±0.32	3.243±0.300	NS
5	Lungs	5.706±0.781	7.38±1.29	7.383±0.530	7.22±0.30	5.54±0.93	6.556±0.69	NS
6	Spleen	1.908±0.107	2.588±0.25	2.427±0.351	2.261±0.218	2.118±0.22	2.843±0.649	NS

Table 2:
Effect of hydroethanolic extract of *Carissa carandas* leaves on haematological parameters on monocrotophos induced toxicity in Wistar Rats

Sr. No.	Groups	Treatment	Hb (g/dl)	TEC (×10^6 cells/ dl)	PCV (%)	TLC (×10^3 cells/ dl)
1	T_1	Distilled Water @ 0.5 ml	15.76±0.158^{a}	8.435±0.433	47.66±3.190^{a}	13.583±0.543^{a}
2	T_2	Monocrotphos @1.5 mg/kg b.w	9.233±0.355^{d}	6.995±0.671	35.66±2.139^{b}	5.75±0.362^{c}
3	T_3	Monocrotophos @ 1.5 mg/kg b.w + Vitamin E @ 150 mg/kg b.w	14.36±0.265^{b}	8.376±0.405	41.66±2.10^{ab}	8.25±0.550^{b}
4	T_4	Monocrotophos @ 1.5 mg/kg b.w + *Carissa carandas* @ 100 mg/kg b.w	10.03±0.34^{cd}	7.563±0.7014	39.166±2.79^{b}	6.416±0.547^{c}
5	T_5	Monocrotophos @ 1.5 mg/kg b.w + *Carissa carandas* @ 200 mg/kg b.w	10.46±0.285^{c}	7.708±0.442	39.333±0.88^{b}	6.966±0.525^{bc}
6	T_6	Monocrotophos @ 1.5 mg/kg b.w + *Carissa carandas* @ 300 mg/kg b.w	10.71±0.322^{c}	7.858±0.460	39.333±1.76^{b}	7.083±0.802^{bc}

Values are mean ± S.E. for 6 rats in each group. Values not sharing a common superscript in a column differ significantly (P<0.05)

body weight gain in MCP and HECC treated groups throughout the study. The relative organ weight also did not reveal change except for mean organ weight of liver and lungs and relative organ weights of liver (Table1). The mean organ weight of liver, kidney, brain, heart, lungs and spleen were studied. The liver weight was found to increase whereas lung wt. was found to decrease significantly. Rests of the organs were unaffected. Janardhan and Sisodia (1990) reported significant reduction in weight gain and an increase in relative weights of liver, kidney, bladder and spleen in both male and female rats at all the levels of MCP @ 0.3 mg/ kg b wt .

Similarly, the relative organ weight of liver was found to be increased by treatment of HECC showing amelioration of toxicity produced by MCP. Reduction in mean and relative organ weights of liver might have occurred due to mono MCP crotophos induced interference in the metabolism and degenerative changes in vital organs (Garg *et al*., 2004; Kaur and Dhanju, 2005) .

Haematological parameters estimated in control and treatment groups and are presented in Table 2. MCP decreased TEC, Hb, PCV and TLC. Siddiqui *et al.* (1991) reported significant increase in TLC and significant decrease in PCV and TEC by MCP . The decrease in RBC count, Hb and PCV observed in the present investigation in monocrotophos treated group could be due to retarded haemopoisis, destruction and shrinkage of RBC. Haemolysis and RBC shrinking (Ahmad *et al*., 1995) and suppression in differentiation and proliferation of stem cells and bone marrow (Skripsky and Loosli. 1995) might have been responsible for reduction in haematological values in MCP treated rats.

Treatment with hydroethanolic extracts of *Carissa carandas* restored the Hb, PCV and TLC. So, the effect of the extract could be due to the boosting effects of the extract on the synthesis of haemoglobin and formation of red blood corpuscles due to their richness in iron and vitamin C (Loganayaki and Manian, 2010).Thus, the hydroethanolic extract of *C. carandas* @ 100, 200 and 300 mg/kg b.wt was found to be effective in restoring haematological values induced by monocrotophos toxicity.

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Short communication

BIOCHEMICAL CHARACTERIZATION OF RARE SEROVARS OF SALMONELLA ISOLATED FROM GANGETIC WATER

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ABSTRACT

The present study was conducted at the Animal Biotechnology Center, Pantnagar to check the level of *Salmonella* contamination in the river Ganga. Total 500 samples were collected from 10 stations (50 samples from each station) viz; Gangotri, Uttarkashi, Rishikesh, Haridwar, Hastinapur, Garhmukteshwer, Narora, Kanpur Allahabad and Varanasi. These samples were subjected to *Salmonella* specific 'his' gene PCR. Samples were then Biochemically screened and serotyped at National *Salmonella* Research Centre IVRI, Izatnagar U.P. These isolates were serotyped as *S*. Abuja, *S.* Lagos. *S*. Pontypridd, *S*. Chinkual, *S.* Zwickau, *S.* Goldenberg and *S*. Oritamerin.

Key words: *Salmonella*, rare serovars, primers, Tetrathionate (T.T.), Ethidium Bromide,

The water of natural sources is getting polluted day by day and now it has become a threat to whole world. In India hygienic conditions are not good and water born infection are very common. Ganga is the most important river of India and can be considered as backbone of Indian economy. This river is becoming contaminated day by day due to disposal of untreated savage in river (Hamner *et al*, 2007). Ganga river water is being utilized for drinking, bathing, and other religious occasions. Due to utilization of contaminated water there are several reports of water born diseases among the population residing on banks of Ganga. (Pandey et al, 2005). The objective of present study was to assess the presence of *Salmonella* in gang tic water from Gangotri to Varanasi.

Ganga Jal samples were collected from ten stations situated on the bank of river Ganga namely Gangotri, Uttarkashi, Rishikesh, Haridwar, Garhmukteshwar, Hastinapur, Narora, Kanpur, Allahabad and Varanasi .All of these stations were having either of religious of industrial importance . Fifty Ganga Jal samples (200 ml each) from each station were collected in autoclaved bottles. Samples were brought to laboratory at 4 °C and filtered from 0.45 ìm filters. Then this filter paper were added to the 2 ml of Luria Bertani broth (L.B. broth) and incubated at 37°C for over night (18 hrs). After that cells were harvested from 1 ml of this incubated culture and added to 100 ìl of autoclaved water and put in boiling water bath for 10 minutes Followed by the chilling on ice for same time. This cold and hot treatment caused lyses of the cells. The cell debris was removed and supernatant was collected.

For *Salmonella* specific PCR primers reported by (Cohen *et al.*, 1993) were used. PCR reaction mixture having 5ìl of genomic DNA, 20 pmol. Of each primer, 200Mm of dNTPs and 1.5Mm $MgCl_2$ all the chemicals were purchased from MBI (Fermentas). PCR was conducted in thermo cycler (T-personal M J Research) with standard conditions.

PCR programmed was as followed:

94°c	5min	1 cycle
94°c	1min	30cycle
60°c	1min	30cycle
72°c	1min	30cycle
72°c	5min	1 cycle

5ìl of amplicon mixed with 1ìl of 6x loading dye and loaded on 1% agarose gel having Ethidium Bromide. Then positive samples were selected for isolation of *Salmonella.* 1ml of culture in L.B. brothe was transferred in 9ml of Tetrathionate (T.T.) broth (Hi-media India) and incubated at 41°C for 18 hrs. Then culture was streaked on BGA plate having –Novobiocine (50ìg/ml) and incubated at 37°C for 18hrs.Then pink colonies were inoculated in 2ml of L.B. broth and retested for *Salmonella* by specific PCR.

Positive colonies were selected for biochemical characterization. The samples which showing positive were tested by Hi-*Salmonella* TM identification kit. Twelve biochemical tests named Methyl red, Vagas Proskev's test, Urease test, H_2s production test, Citrate utilization test, ONPG utilization test, and Lactose, Arabinose, Maltose, Sorbitol, Dulcitol fermentation test. Then isolates found positive in PCR and biochemical tests were sent to National *Salmonella* Research lab, Indian Veterinary Research Institute (Izatnagar) for serotyping.About 500 samples of Ganga Jal from 56 Ghats of 10 stations were tested for presence of *Salmonella.* Forty four samples were found positive by *Salmonella* specific PCR (Fig-1). The *Salmonella* was isolated from these samples and then serotyped. Twenty isolates were serotyped as *Salmonella*

Table1.
Isolates of *Salmonella* from Ganga water used in the study:

S.N	Isolate No.	Serovar	Place of isolation	S.N	Isolate No.	Serovar	Place of isolation
1	Ganga-2	*S*. Abuja	Hastinapur	11	Ganga-22	*S*. Abuja	Hastinapur
2	Ganga-4	*S*. Abuja	Hastinapur	12	Ganga-24	*S*. Chinkual	Varanasi
3	Ganga-6	*S*. Abuja	Hastinapur	13	Ganga-25	*S*. Zwickau	Narora
4	Ganga-7	*S*. Pontypridd	Kanpur	14	Ganga-26	*S*. Goldenberg	Narora
5	Ganga-9	*S*. Abuja	Hastinapur	15	Ganga-27	*S*. Lagos	Haridwar
6	Ganga-12	*S*. Lagos	Haridwar	16	Ganga-30	*S*. Pontypridd	Narora
7	Ganga-13	*S*. Lagos	Haridwar	17	Ganga-31	*S*. Abuja	Hastinapur
8	Ganga-15	*S*. Lagos	Haridwar	18	Ganga-32	*S*. Abuja	Hastinapur
9	Ganga-17	*S*. Chinkual	Varanasi	19	Ganga-39	*S*. Oritamerin	Narora
10	Ganga-19	*S*. Abuja	Hastinapur	20	Ganga-43	*S*. Orltamerin	Narora

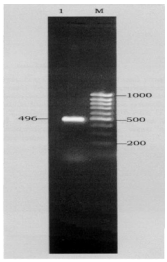

Fig. 1: Amplicon of *hisA* gene of salmonella specific PCR

(Rare) as shown in Table-1.

Salmonella isolates (Rare) were isolated from Haridwar, Hastinapur, Narora, Kanpur and Varanasi. But most of the isolates were from Hastinapur and Haridwar. The most probably reason could be the historical and religious background of both the stations, It could be possible because of visit of the large number of foreign tourists to both places and contamination of such rare *Salmonellas* may be due to them.

In past also there are some (Hamner *et al* 2006, 2007, Pandey *et al.* 2005, CDC 2001, 2002, 2004,) reports of isolation of *Salmonella* in Ganga Jal food material and domestic animals. The main cause of occurrence of *Salmonella* isolates (Rare) may be as the frequent visiting of foreign visitors to such religious points. Because of these *Salmonella* strains are generally found in other countries not in India.

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Short Communication

EFFECT OF *BACOPA MONNIERIHYDROETHANOLIC* EXTRACT ON ISOLATED RAT ILEUM

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ABSTRACT

The study was conducted to evaluate the effect of hydroethanolic extract of *Bacopa monnieri* (HEBM) on isolated rat ileum. Plant extract at a concentration of 1000µg/ml induced relaxation of isolated rat ileum. The spasmolytic action was further affirmed when the plant extract (1000µg/ml) attenuated the contractile responses shown by acetylcholine(10^{-4}M), calcium chloride(10^{-4}M) and potassium chloride ($4x10^{-4}$M) on rat ileum and enhanced the relaxing activity of epinephrine(10^{-6}M).It is thus suggested from the present study that HEBM induced spasmolytic effect might have beeen due to HEBM induced calcium channel blockade or inhibition of mucarinic receptors.

Key words: *Bacopamonnieri*, rat ileum, calcium channel, adrenergic and muscarinic receptors

Bacopa monnieri commonly known as Brahmi has been used by Ayurvedic medicinal practitioners in India for almost 3000 years due to its medicinal properties (Satyavati *et al.*, 1976). The plant possesses antioxidative, hepatoprotective, sedative, antiinflammatory, immunomodulatory, neuroprotective and antimicrobial properties (Nadkarni, 1988). The present study was conducted to evaluate the effect of the extract on isolated smooth muscles of rats.

For the present study, five healthy adult male rats weighing 100-150 gms were procured from experimental animal house, Pantnagar and acclimatized for 2 weeks in departmental animal shed. The rats were kept off-fed overnight before commencement of the study. The rats were anaesthetized with ether and then ileum was dissected out, free of fat and connective tissue and then was immediately transferred to cold (4°C) aerated De-Jalon's solution(sodium chloride9 gm/l, potassium chloride0.42 gm/l, calcium chloride0.06 gm/l, glucose0.50 gm/l and sodium bicarbonate0.5gm/l) (Ghosh, 1984). The tissue was cut into length of 10-15 mm and then immediately mounted. The temperature of the organ tube was thermostatically controlled and the tissue was continuously aerated by an aerator attached to the oxygen tube. After mounting, the tissue was kept for an equilibration period of 90 minutes. During this period, the bathing fluid was changed every 15 minutes. After equilibration period, the drugs were added as per the experimental protocol. The response was recorded on student's physiograph (BSL Pro 3.7) and analysed by comparing the deviation from baseline.

The solutions of acetylcholine (10^{-4}M), epinephrine(10^{-6}M), atropine (10^{-4}M), prazosin (10^{-4}M), propranolol($7x10^{-7}$M), calcium chloride (10^{-4}M), potassium chloride($4x10^{-4}$M) and HEBM (1000µg/ml)were prepared fresh in triple glass distilled water in desired concentration for use in the experiment.

The study aimed at observing the effect of agonist drugs like acetylcholine, epinephrine, calcium chloride and potassium chloride in presence of their respective antagonists and HEBM. Isolated rat ileum exhibited normal peristaltic activity in DeJalon solution. After equilibration period of 90 minutes, the agonist was added to organ tube, allowed to act for one minute and then washed. After 5 minutes, the antagonist was added and allowed to act for one minute. This was followed by addition of agonistso as to observe the effect shown by agonist in presence of antagonist. The similar protocol was followed for HEBM (1000µg/ml) where antagonist was replaced by plantextract.

The present study demonstrated the antispasmodic activity of hydroethanolic extract of *Bacopa monnieri*(HEBM) on isolated rat ileum. The contractile response shown by acetylcholine (10^{-4}M) was completely blocked by atropine sulphate (10^{-4}M). Administration of HEBM (1000µg/ml) attenuated the contractile response produced by acetylcholine indicating spasmolytic property of the extract as shown in Figure1. Administration of epinephrine (10^{-6}M) caused relaxation of the isolated ileum. However, the relaxing effect of epinephrine (10^{-6}M) was abolished in presence of propranolol ($7x10^{-7}$M). When epinephrine was given in presence of HEBM (1000µg/ml) the relaxing action of epinephrine was more pronounced as depicted by lowering of physiogram below base line (Figure 4.2a). As Figure 4.22b shows, administration of epinephrine (10^{-6}M) caused relaxation of the isolated ileum. The relaxing effect of epinephrine (10^{-6}M) was abolished in presence of prazosin in a similar manner as shown by propranolol ($7x10^{-7}$M). The relaxing action of epinephrine was more pronounced when epinephrine was given in presence of HEBM (1000µg/ml)

Treatment of the tissue with calcium chloride (10^{-4}M) caused a slight contraction of the tissue. Pretreatment of the tissue with HEBM (1000µg/ml) caused no contractile response of the tissue on addition of calcium chloride (10^{-4}M) as shown in Figure 3.

Figure 4 shows a contractile effect on rat ileum on addition of potassium chloride ($4x10^{-4}$M). Pretreatment

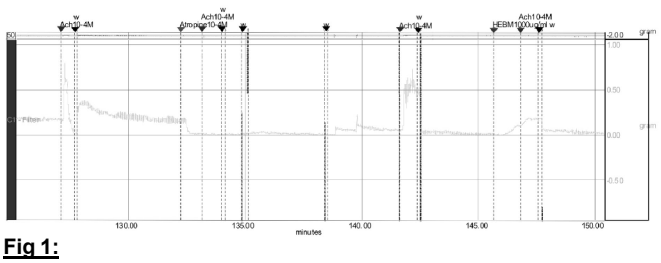

Fig 1:
Effect of HEBM on response of acetylcholine and atropineon rat ileum.

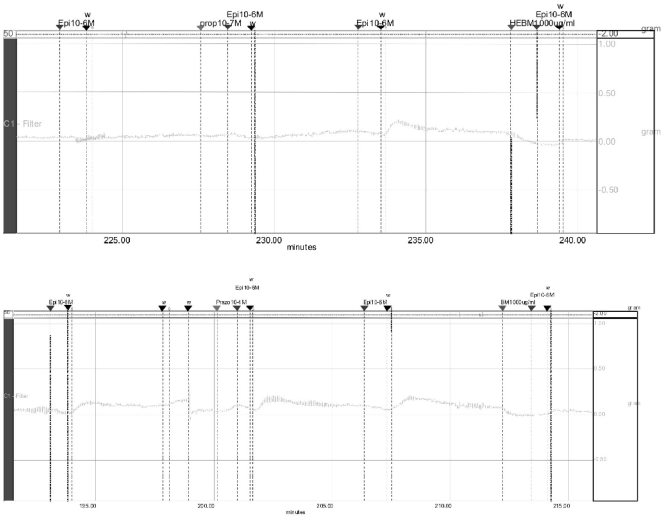

Fig 2a&b:
Effect of HEBM on response of epinephrine and prazosin and propranolol on rat ileum.

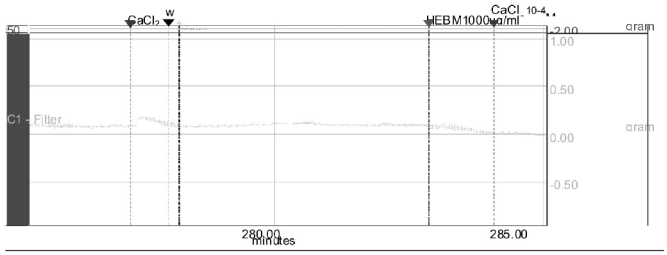

Fig 3:
Effect of HEBM on response of calcium chloriderat ileum.

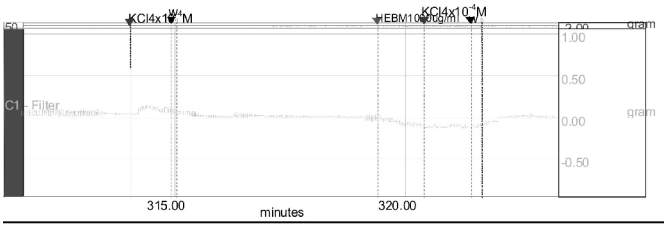

Fig 4:
Effect of HEBM on response of potassium chloriderat ileum.

▼: Acetylcholine, epinephrine, calcium chloride and potassium chloride
▼: Washing
▼: Atropine, prazosin and propranolol
▼: HEBM

of the tissue with HEBM (1000µg/ml) abolished the contractile effect on further addition of potassium chloride ($4x10^{-4}$M).

The action of acetylcholine on GIT is mediated through muscarinic receptors. Since, the extract attenuated the contractile response produced by acetylcholine on rat ileum; it is likely that HEBM possessed antimuscarinic properties like atropine. Antimuscarinic property might be due to presence of alkaloids in the extract as evident from phytochemical analysis conducted in this investigation. Other possible mechanism could be inhibition of calcium influx. Intestinal smooth muscles showed spontaneous rhythmic movements regulated by periodic depolarization and repolarization. At the height of depolarization, there is a rapid influx of Ca^{2+} via voltage-operated calcium channels. The inhibition of spontaneous activity of smooth muscle by HEBM could be either due to interference in the depolarization process or with the calcium influx through voltage-operated channels. The findings on smooth muscle activity observed in this study are in accordance with the reports of Aithal and Sirsi (1961) and Channa and Dar (2012).

The extract inhibited potassium chloride induced contractions of ileum. Similar reports regarding inhibition of potassium chloride induced contractions by *Bacopa* extract were given by Channa and Dar (2012).Epinephrine causes relaxation of GI smooth muscles mediated through activation of both á and β receptors. Muscle relaxant action of norepinephrine is less pronounced than epinephrine and is mediated by á-adrenoreceptors. Our findings revealed that in presence of HEBM, the relaxing effect of epinephrine and norepinephrine was more pronounced. This strengthened the fact that *Bacopa* showed synergistic action with adrenergic drugs as also reported in previous reports on the relaxant effect of *B.monnieri* on rat ileum **(Aithal and Sirsi, 1961).**It is concluded from the present study that HEBM induced spasmolytic effect might be due to calcium channel blockade or inhibition of mucarinic receptors.

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Short Communication

EVALUATION OF *ELETTARIA CARDAMOMUM* ON ISOPROTERENOL INDUCED CARDIAC INJURY IN RATS

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ABSTRACT

Present study was done to assess the effect of ethanolic extract of *Elettaria cardamomum* (ECE) in isoproterenol (ISP) induced cardiac injury in rats. Assessment of two concentrations (250 and 500 mg/kg of b wt) of ECE were done in ISP-treated rats. Evaluation of cardiac injury was done by estimation of different cardiac injury markers like LDH, CK-MB, lipid profile and myocardial infarcted area. Both the doses (250 and 500 mg/kg of body weight) of AEEG significantly ($p<0.05$) improved the heart weight of cardiac injured group but pre-treatment of AEEG did not change heart weight to body weight ratio. ECE showed significant ($p<0.05$) improvement in serum LDH, CK-MB and triglyceride level at both the concentrations. Heart weight and myocardial infarcted area were also significantly ($p<0.05$) improved in ECE pre-treated rats. No improvement was observed in serum cholesterol, LDL, HDL and heart weight to body weight index in these rats. The study suggests that ECE showed partial protective effect in ISP-induced cardiac injury in rats.

Key words: *Eletteria cardamomum*, isoproterenol, cardiac

Cardiac injury is one of the leading causes of mortality and morbidity in the Indian subcontinent (Gupta *et al.,* 2008). Conventional therapy has better efficacy but possess various side effects also. A lot of attention has been drawn towards use of herbal drugs as potential therapeutic agents in the prevention and management of various cardiovascular diseases (Sakat *et al.,* 2009). *E. cardamomum cardamom* has been used in India since many years. *E. cardamomum* is used as a cuisine in curry, cakes, bread, coffee and a flavouring agent in different foods (Malti *et al.*, 2012). It has been reported that *Elettaria cardamomum* showed beneficial effect in stress-induced myocardial injury, high blood pressure and oxidative stress induced blood lipids and fibrinogen levels (Gilani *et al.*, 2008; Verma *et al.*, 2009, 2010; Khan *et al.*, 2011). But, the effect of *E. cardamomum* is not studied in the isoproterenol (ISP)-induced cardiac injury. The aim of the present study was to determine cardioprotective efficacy of ethanolic extract of *E. cardamomum* in ISP-induced cardiac injury in rats.

Fruits of *E. cardamomum* were purchased from the local market and were identified botanically before use. Fruits of *E. cardamomum* were powdered and then extracted with 70% alcohol under reflux. Ethanolic extract of *E. cardamomum (ECE)* was concentrated under reduced pressure to a semi-solid mass and made free from solvent. Wistar male rats (150-250g) were used in this study. Recommended guidelines for care and use of animals were followed. Rats were divided into following four groups: control group; isoproterenol (ISP)-treated group. In this group, ISP was administered at the dose rate of 60 mg/kg of b wt, s.c., at an interval of 24 hours for 2 consecutive days. In third group, ECE was given at the dose of 250 mg/kg bw orally for 30 days while in fourth group ECE was given @ 500 mg/kg b wt orally for 30 days. In third and fourth groups, ISP was provided @ 60 mg/kg bw, s.c. on 29th and 30th day of the treatment. Rats were killed after 24 hours of last dose of ISP administration by bleeding from abdominal aorta under pentobarbitone (60 mg/kg bw i.p.) anesthesia. Blood was collected for biochemical estimation. Heart was collected for heart weight and myocardial infarcted area analysis.

LDH, CK-MB, triglyceride cholesterol, LDL and HDL were analyzed in serum by using commercial kits (Span Diagnostics, Coral Diagnostics, Surat, India). Measurements of heart weight and heart weight to body weight index were also done. Myocardial infarcted was estimated by TTC staining.

Results are expressed as Mean ± SEM with n equal to number of animals. Data were analyzed by Newman-Keuls multiple comparison test. Differences in values were considered statistically significant at $p<0.05$.

LDH and CK-MB levels were significantly ($p<0.05$) higher in ISP-treated rats in comparison to control group. Levels of LDH and CK-MB have shown significant ($p<0.05$) improvement in ECE pre-treated rats at both the doses (Table 1). Results of present study are supported by previous reports in which cardamom was reported to show protective effect against stress-induced myocardial injury (Verma *et al.,* 2010). Continuous consumption of cardamom may be beneficial in treatment ischemic heart disease condition which is induced by facing regular stress

Table 1:
Effect of ECE on heart weight, HW/BW ratio and serum cardiac marker enzymes

Treatment and Dose (mg/kg of body wt.)	Heart Weight	HW/BW Ratio	LDH (IU/L)	CK-MB (IU/L)
Control	0.698±0.05[a]	0.0034±0.0002[a]	72.94±9.57[a]	166.90±44.02[a]
Isoproterenol (60)	1.089±0.04[b]	0.0044±0.0001[b]	195.30±28.39[b]	460.00±76.88[b]
ECE (250)+ISP (60)	0.879±0.03[c]	0.0045±0.0001[b]	130.60±.3.67[a]	216.60±44.49[a]
ECE (500)+ISP (60)	0.887±0.05[c]	0.0045±0.0001[b]	117.30±8.59[a]	269.60±53.03[a]
	Lipid profile			
	Triglyceride (mg/dl)	Cholesterol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)
Control	31.67±6.38[a]	31.07±4.65[a]	13.74±1.92[a]	30.86±5.56[a]
Isoproterenol (60)	71.54±9.29[b]	55.29±7.41[b]	28.83±4.83[b]	18.52±4.25[b]
ECE (250)+ISP (60)	43.93±5.56[a]	50.47±5.16[b]	29.55±2.82[b]	28.52±4.31[ab]
ECE (500)+ISP (60)	46.20±3.10[a]	50.52±4.18[b]	29.38±4.38[b]	34.01±2.52[ab]

Values are represented as Mean ± SE. [b]P<0.05 compared to control group; [c]P<0.05 compared to ISP-treated and control group.

(Darwish and Sh, 2012).

Heart weight was increased significantly (p<0.05) in isoproterenol (ISP) treated rats in comparison to control rats. Pre-treatment of ECE at dose rate of 250 and 500 mg/kg of body weight significantly (p<0.05) reduced the heart weight in cardiac injured rats but it was not comparable to control animals. In ISP-treated animals, heart weight to body weight ratio was significantly (p<0.05) increased in comparison to control group. However, both the doses of EGE have not shown any significant improvement in cardiac injured animals (Table 1).

In ISP-treated rats, triglyceride level in serum was significantly (p<0.05) increased in comparison to normal control rats. Oral administration of ECE for 30 days at dose rate of 250 and 500 mg/kg of body weight significantly (p<0.05) decreased triglyceride level in cardiac injured rats, which was comparable to control animals. Levels of serum cholesterol, LDL and HDL were significantly higher in ISP-treated animals in comparison to control rats. Rats treated with ECE did not show any significant improvement in serum cholesterol, LDL and HDL levels in cardiac injury (Table1)**.** In a previous report, it has been shown that prolonged treatment of cardamom before exposure to whole body gamma irradiation has significantly lowered the alteration in the serum lipid profile levels when compared with the irradiated rats (Darwish and Sh, 2012). In present study serum triglyceride level was decreased in cardiac injured rats which are in agreement of previous report but serum cholesterol, LDL and HDL levels were not altered in present study. In some studies, it has been reported that cardamom has the blood pressure lowering activity (Gilani *et al.,* 2008; Verma *et al.,* 2009).

In ISP-treated rats, myocardial infarcted area was increased significantly (p<0.05) in comparison to normal control rats. ECE (250 and 500 mg/kg b wt) pre-treated rats showed significant (p<0.05) improvement in myocardial infarcted area in cardiac injury induced by ISP. These results are in good agreement with previous report in which cardamom showed protective effect against stress- induced myocardial injury (Verma *et al.,* 2010).

In conclusion, the results of present study suggest that the pre-treatment of ethanolic extract of *E.cardamomum* has partial protective effect in isoproterenol induced cardiac injury in rats.

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Short Communication

IN VITRO EVALUATION OF ANTIOXIDANT ACTIVITY OF *CURCUMA LONGA* AND *BERBERIS ARISTATA*

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ABSTRACT

Rhizomes of *Curcuma longa* and stems of *Berberis arista* were used to study antioxidant activity by *in vitro* assays viz. DPPH free radical scavenging assay and total antioxidant activity by ABTS method. BHT and Trolox were used as reference standards for DPPH and ABTS assays respectively and IC_{50} values were calculated. The results revealed that the recovery of methanolic extract of *C. longa* rhizomes and *B. aristata* stems was 16.2% and 9.79% respectively. The extracts were found to scavange free radicals; DPPH and ABTS in a dose dependent manner. Comparatively, the methanolic extract of *B. arista* stems was found to have significantly lower IC_{50} values than *C. longa* in both the assays, indicating its better antioxidant activity. The results obtained herein indicate that methanolic extracts of *Curcuma longa* and *Berberis aristata* are potential source of natural antioxidants and may provide potential therapeutic intervention against oxidative threats, both in healthy and diseased conditions.

Key words: Antioxidant activity, *Berberis aristata, Curcuma longa*, Free radicals, ABTS, DPPH

A vast amount of circumstantial evidence implicates free radicals as the mediators of wide range of diseases including diabetes, ageing, cancer etc. *Curcuma longa* (*Turmeric*) and *Berberis aristata* (*Tree Turmeric*) are two among the many herbs that have been widely used in traditional medicine for centuries. Both the herbs are well known for their anti-inflammatory activity. *Berberis aristata* commonly known as “Daru haldhi” is a herb native to northern Himalayan region. The stem of *Berberis aristata* is used in Indian traditional medicine to treat antibacterial, antiperiodic, antidiarrhoeal, ophthalmic, skin diseases and diabetes mellitus (Upwar *et al*., 2010). Thus, *Curcuma longa* and *Berberis aristata* have the potential for the development of modern medicine for the treatment of various diseases. The neead of the hour is to verify and validate these traditional uses by subjecting them to proper experimental studies. Thus, the present study was aimed to evaluate antioxidant activity of methanolic extracts of *Curcuma longa* and *Berberis aristata*.

Rhizomes of *Curcuma longa* were procured from Dept. of Vegetable Science and Floriculture, CSKHPKV, Palampur and stems of *Berberis arista* were procured from Distt. Mandi, H.P. and got identified at the Dept. of Biodiversity, IHBT, Palampur. The collected plant parts were shade dried, powdered and subjected to methanolic extraction. The extracts were filtered and filtrate were concentrated *in vacuo* using rotary evaporator at 45^0C. The concentrated extracts were subjected to freeze drying to obtain dry powdered extracts and percent recovery was calculated. The lyophilized samples were used for further studies.

The ability of extracts to scavenge DPPH radical was determined according to the method of Hsu *et al*. (2006) at different concentrations ranging from 0.0625-0.500 mg/ml. BHT was taken as a reference standard. The absorbance of the test and control solutions was determined at 517 nm. The per cent DPPH radical scavenging was determined by using the following formula and further IC_{50} value was calculated:

% DPPH radical scavenging activity = [1-(A_{517nm} test/A_{517nm} control)] x 100

IC_{50} value denotes the concentration of sample required to scavenge 50% DPPH free radicals.

The total antioxidant activity was determined according to the method of Re *et al*. (1999) at different concentrations of the sample ranging from 0.0625-0.500 mg/ml. Trolox was taken as a reference standard. The absorbance of the test and control solutions was determined at 734nm. The percentage of inhibition of $ABTS^+$ radicals at different concentrations was determined by using the following formula and further IC_{50} value was calculated:

% $ABTS^+$ inhibition = [1-(A_{734nm} test/A_{734nm} control)] x 100

IC_{50} value denotes the concentration of sample required to scavenge 50% $ABTS^+$ radicals.

The percent activity was plotted against the sample concentration and a linear regression test was done using Graph Pad INSTAT and IC_{50} value was interpolated from standard curve. Lower IC_{50} values indicate higher antioxidant activity. All the tests were done in triplicates and the result were expressed in mean ± SEM.

The recovery of methanolic extracts of *C. longa* and *B. aristata* was found to be 16.2% and 9.79% respectively. The results of antioxidant activity using DPPH free radical scavenger method and ABTS total radical scavenging activity is shown in Table 1. The results showed

Table 1.
IC_{50} values (Mean ± SEM)of methanolic extracts of *C. longa* and *B. arista* by DPPH and ABTS free radical scavenging method.

Extract	IC_{50} Value (mg/ml)	
	DPPH method	ABTS method
Curcuma longa	0.119 ± 0.019[a]	0.821 ± 0.016[a]
Berberis aristata	0.043 ± 0.017[b]	0.748 ± 0.006[b]
Reference Standard	0.040 ± 0.011[b]	0.53 ± 0.004[c]

Values are Means with different superscripts vary significantly ($p<0.05$) with each other within a column.

that both the extracts exhibited free radical scavenging activity in concentration dependent manner. The amount of sample needed to decrease the initial DPPH concentration by 50 % (IC_{50}) is a parameter widely used to measure the antioxidant activity. The extract of *Berberis aristata* was found to be more active with IC_{50} value of 0.043 mg/ml as compared to *Curcuma longa* with IC_{50} value of 0.119 mg/ml. BHT was taken as reference standard for this test with IC_{50} value of 0.040 mg/ml, which was found to be significantly equal to that of *B. aristata.* ABTS reacts with potassium persulphate to produce ABTS radical cation ($ABTS^{+}$), a blue green chromogen with absorption maxima at 734 nm. The extent of decolorization is significant indicator of antioxidant activity of the sample. The effects of antioxidants, reductants on ABTS radical cation is due to its hydrogen donating availability which is observed by a change in color radical $ABTS^{+}$ to colorless ABTS. Like DPPH assay, the IC_{50} value for ABTS radical scavenging activity of *Berberis aristata* (0.748 mg/ml) was found to be significantly lower than *Curcuma longa* (0.821 mg/ml), indicating its higher antioxidant potential.

The methanolic extracts are one of the most active of all extracts. The inhibitory effect on lipid peroxidation has been reported to be best shown by methanolic extracts (Kumar and Muller, 1999). Therefore, for the present study, the selected herbal plants were subjected to methanolic extraction. Our results corroborate with the findings of earlier workers who observed antioxidant and hepatoprotective activity of the alcoholic extracts of *C. longa* and *B. aristata* respectively (Lans *et al.,* 2007). Mishra *et al*. (2011) have also reported that various herbal formulations of *Berberis aristata* and *Curcuma longa* have anti-diabetic effects. Wang *et al.* (2012) suggested that Curcumin obtained from rhizome of *Curcuma longa* has various beneficial effects acting as an antioxidant, anti-inflammatory and anti-fibrotic. Pasrija *et al*. (2011) screened extract of *Berberis aristata* by HPTLC fingerprinting and showed that berberine was present in *Berberis aristata* sample that observed antimicrobial activity. Such reports implied that the phytochemicals Berberin and Curcumin present in the extract of *Berberis aristata* and *Curcuma longa* respectively, may be responsible for the antioxidant activity reported in the present investigation.

In this study we conclude that the methanolic extracts of *Curcuma longa* and *Berberis aristata* have antioxidant potential and *Berberis aristata* possess better antioxidant activity as compared to *Curcuma longa.* The findings of present study thus validate the claims of traditional healers for the therapeutic use of *Berberis aristata* and *Curcuma longa* in various diseases.

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