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Review Article

SCREENING METHODS FOR ANTICANCEROUS DRUGS

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ABSTRACT

Plant-derived compounds have been an important source of several clinically useful anti-cancer agents. Standardized, well-established *in vitro* and *in vivo* methods are available for the experimental evaluation of new anticancer agents. *In vitro* methods are usually easy to set up, yield results quickly, readily permit replication and good quantification, are inexpensive, and are simple to run and automate. *In vivo* methods are necessary to confirm the principal mechanism of drug action, and then to determine the general pharmacology, pharmacokinetics and toxicology of selected compounds. A step-wise procedure from *in vitro* to *in vivo* experiments using non-functional, functional non-clonogenic and, if applicable, clonogenic assays allows reduction of the number of promising agents for further clinical testing.

Key words: Anticancer drugs, screening methods, *in vitro* methods, *in vivo* methods.

Introduction

Cancer is one of the leading causes of death and globally the number of cases of cancer is increasing gradually. A tumor or cancer is any abnormal proliferation of cells, which may be either benign or malignant. A benign tumor remains confined to its original location neither invading surrounding normal tissues nor spreading to distant body sites. A malignant tumor, however, is capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems (metastasis) (Bishop and Weinberg, 1996). Only malignant tumors are properly referred to as cancers, and it is their ability to invade and metastasize that makes cancer so dangerous. Despite considerable efforts, cancer still remains an aggressive killer worldwide. Moreover, during the last decade, novel synthetic chemotherapeutic agents currently in use clinically have not succeeded in fulfilling expectations despite the considerable cost of their development (Solowey *et al.,* 2014).

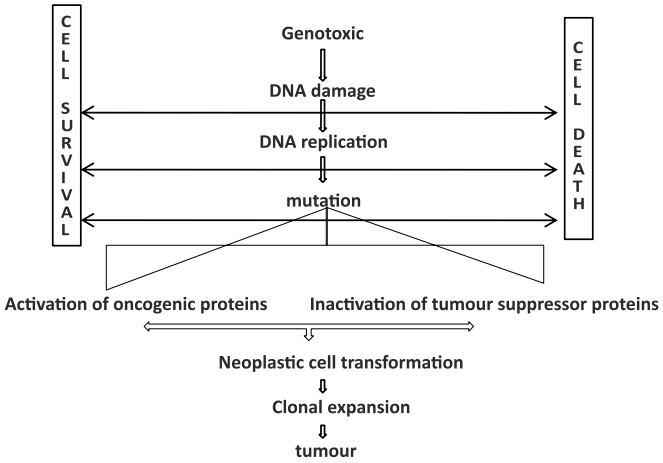
Tumour development

The stages in tumorigenesis have been designated as initiation, which corresponds to damage and then division of exposed cells such that their growth potential is changed irreversibly, and progression, denoting multiple rounds of cell replication mediating the gradual transition of an initiated cell towards autonomous, cancerous, growth. Ultimate spread of malignant cells resulting in multiple tumour sites has been termed as metastasis (Anand *et al.,* 2008).

Chemicals that react with DNA may cause damage such as adduct formation, oxidative alteration, and strand breakage. In normal conditions, these lesions are repaired or injured cells are eliminated. If neither event occurs, a lesion in the parental DNA strand may induce a heritable alteration, or mutation, in the daughter strand during replication. The mutation may remain silent if it does not alter the protein encoded by the mutant gene or if the mutation causes an amino acid substitution that does not affect the function of the protein. Alternatively, the genetic alteration may be incompatible with cell survival. The most unfortunate scenario for the organism occurs when the altered genes express mutant proteins that reprogram cells for multiplication and avoidance of apoptosis (i.e., immortalization). When such cells undergo mitosis, their descendants also have a similar propensity for proliferation. Moreover, because enhanced DNA replication and cell division increases the likelihood of mutations, these cells eventually acquire additional mutations that may further increase their growth advantage over their normal counterparts. The final outcome of this process is a nodule, followed by a tumor consisting of transformed rapidly proliferating cells (Klaassen, 2008).

Incidence of cancer in animals

Cancer related problems gained much importance in pet animals owing to the affection, love and increased



awareness among the people towards animal sufferings and pain. Cancer is an important disease in dogs and represents one of the major causes of canine death accounting for 27% of all deaths in purebred dogs in developed countries (Adams *et al.,* 2010). Dogs develop neoplasms mainly skin and mammary tumours twice as frequently as humans (Rungsipipat, 2009). In India, the incidence of tumours in animals is found to be highest in canines followed by equines and bovines (Shekhar *et al.,* 2001). The Boxer dog is, according to many authors, a high- risk breed for several tumours: sarcomas of bone and soft tissues, malignant lymphoma, glioma, testicular tumours, and thyroid carcinoma. Skeletal osteosarcomas are also found in large dogs (Misdorp and Hart, 1979). White unpigmented skin in cattle, cats, dogs, and goats is associated with an excessive risk for the development of squamous cell carcinoma of the skin (Dobson, 2013). Mukhopadhayay and Som (1992), in their study on 136 canine tumours from Calcutta found highest incidence of skin tumours (46.6%), followed by genital organs (23.5%), mammary gland (20.6%) and other organs (13.2%). Shekhar *et al.* (2001), in an epidemiological study on canine mammary tumours, reported a high incidence of malignant mammary tumours (59 out of 72).

New anticancer agents are either designed for tumor specific targets based on a biological rationale or are generated by large-scale drug screening programs. The question of whether a new drug improves cancer therapy in patients can ultimately only be answered in a clinical trial, since it generates data on safety and efficacy. But for conducting a clinical trial, there are many ethical, medical and economic constraints. So, most of the researches on new drugs have to be done in experimental systems. Over many decades, researchers in experimental tumor therapy have developed well-proven, reliable *in vitro* and *in vivo* methods to evaluate treatment response. The limiting factor in these affairs is time, consequently there is a gap between the quantity of new agents and the resources available for their proper clinical evaluation. So standardized experimental methods for the evaluation of new drugs is an important part of cancer research.

Functional *versus* non-functional assays

Functional assays evaluates of new anticancer agents by means of *in vitro* and *in vivo* methods to describe whether or not a new drug is effective against cancer cells. It basically measures survival of tumor cells with and without therapy, *e.g.* as a total number of cells, a number of colonies, tumor volume or tumor cure rate. Non-functional assays are often also referred to as mechanistic investigations, and are important to improve our understanding of the underlying mechanisms of action. For example, assessment of the new drug's effect on apoptotic pathways or intracellular signaling will give us more information about the possible mechanism of action of the drug. Both functional and non-functional assays are essential for the evaluation of anticancer agents.

Clonogenic assays

Clonogenicity is the ability of a tumor cell to produce an expanding family of descendents (Steel, 2002; Tannock and Hill, 1998). In most of the cancerous tissues, only a small percentage of cells are clonogenic cells. Non-clonogenic tumour cells die without any therapy after some cell divisions. So to cure a tumor, it is necessary to inactivate all clonogenic cells either by inducing cytotoxicity or a permanent state of dormancy, *i.e.* the loss of clonogenic capacity. The evaluation of whether a drug has a curative potential, *i.e.* effectively inactivates clonogenic tumor cells, requires the use of experimental endpoints that represent the response of clonogenic cells. Clonogenic endpoints are of particular importance when new anticancer agents are integrated into curative therapeutic settings, *e.g.* in combination with radiotherapy or chemotherapy.

***In vitro* methods**

There are many in vitro methods available for testing anti cancerous activity of a compound and the primary evaluation technique is cell culture. Compared to animal tumor models, *in vitro* methods are less expensive and less time-consuming, thereby allowing evaluation of large quantities of new anticancer agents. Molecular methods prove and quantify the potential of several drugs to affect the molecular target, *e.g.* to decrease the activity of a specific kinase, facilitate the selection of promising candidate drugs. Sophisticated *in vitro* experiments provide data on mechanisms of action, by how the compounds act on tumour entities when combined with the specific cell lines. Based on these data, further selection of promising drugs for *in vivo* testing can be obtained. In general, for functional assays cells are exposed to different drug concentrations and the response is monitored. Cell counting or dye-based assays such as MTT are quick and robust methods to estimate the total number of surviving cells. Assessment of the fraction of surviving clonogenic cells can be done either by the colony forming assay or by the dilution assay (Freshney, 2000).

Ionogenic assays are laborious and require experience. As a consequence, nonclonogenic and non-functional tests are preferred. However, data obtained from non-clonogenic and clonogenic assays are not necessarily consistent (Brown and Wouters, 1999; Baumann *et al,* 2003; Eshleman *et al.,* 2002). Thus, for evaluation of the effects of a new drug on clonogenic tumor cells, nonclonogenic assays cannot replace clonogenic assays. Data obtained from these in vitro tests will substantiate the in vivo tests. For example, the IC_{50} value describes the drug concentration necessary to reduce the number/fraction of cells to 50% compared with the controls. The IC_{50} value allows comparison with results obtained with other drugs and other cell lines. Moreover, from IC_{50} and

pharmacokinetic data, it can be estimated whether effective drug concentrations are achievable *in vivo*. Thus, quantitative *in vitro* evaluation of anticancer drugs is fundamental for further testing in animal models.

Unlike the situation *in vitro*, a tumor is a 3-dimensional complex consisting of interacting malignant and non-malignant cells. Vascularisation, perfusion and, thereby, drug access to the tumor cells are not evenly distributed and this fact consists an important source of heterogeneity in tumor response to drugs that does not exist *in vitro*. Therefore, prediction of drug effects in cancer patients based solely on *in vitro* data is not reliable and further evaluation in animal tumor systems is essential. Given that it is practically impossible to test large quantities of new anticancer agents *in vivo*, the most important function of *in vitro* experiments is to select promising candidates for further testing and to gain insights into cellular mechanisms of action. To reduce the number of potential candidates for *in vivo* testing, it seems reasonable to proceed step-wise from non-functional to functional *in vitro* tests and, if applicable, from nonclonogenic to clonogenic assays. Although this procedure might exclude drugs that have no activity at a certain level, but which would have an anticancer effect at the next level, there is no obvious alternative to this strategy.

Various *in vitro* methods for screening anti cancerous activity:

1.Tryphan blue dye exclusion assay

The trypan blue dye exclusion assay is the most commonly utilized test for cell viability. In this assay, the cells are washed with HBSS (Hank's Buffered Salt Solution) and centrifuged for 10 -15 min at 10,000 rpm. The procedure is repeated thrice. The cells are suspended in known quantity of HBSS and the cell count is adjusted to 2 x 10^6 cells /ml. The cell suspension is distributed into Eppendorf tubes (0.1 ml containing 2 lakhs cells). The cells are exposed to drug dilutions and incubated at 37 °C for 3 h. After 3 h, dye exclusion test, that is, equal quality of the drug treated cells are mixed with tryphan blue (0.4 %) and left for 1 min. It is then loaded in a haemocytometer and viable and non-viable count are recorded within 2 min. Viable cells do not take up colour, whereas dead cells take up colour. However, if kept longer, live cells also generate and take up colour. The percentage of growth inhibition is calculated using the following formula:

$$\text{Growth Inhibition } (\%) = 100 - \frac{Total\ Cells - Dead\ Cells}{Total\ Cells}\ x\ 100$$

2. *LDH (Lactic dehydrogenase) Assay*

Lactic dehydrogenase activity is spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate lactate transformation. Cells are lysed with 50 mM Tris-HCl buffer, pH 7.4 + 20 mM EDTA + 0.5 % Sodium Dodecyl Sulfate (SDS), further disrupted by sonication and centrifuged at 13,000 g for 15 min. The assay mixture (1ml final volume) for the enzymatic analysis consists of 33 µl of sample in 48 mM PBS, pH 7.5 + 1 mM pyruvate and 0.2 mM NADH. The percentage of LDH released is calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium.

3. In vitro Cytotoxicity Assays

Cell lines are obtained from any standard laboratory and they are grown and maintained in a humified incubator at 37ÚC under 5% CO_2 atmosphere in MEM medium (Minimal Essential Media) supplemented with TPVG & 10% Fetal calf serum and 100 units/ml penicillin. After 25hrs incubation, to allow cell attachment, the cells are treated with fresh medium containing the drug ranging from 10-100 µg/ml, dissolved in DMSO and incubated for 48hrs.

MTT Assay

It is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells. The assay is based on the capacity of the cellular mitochondrial dehydrogenase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue/purple formazan product which is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cells lines. At the end of 48hrs incubation, the medium in each plate containing the extract of plant was added with 200µl of MTT solution and incubated for another 4hrs.The supernatant was then removed & replaced with 500µl of DMSO to dissolve the resulting MTT formazan crystals followed by mixing & measuring the absorbance at 590nm.

XTT assay

In order to measure the proliferation response, the (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2Htetrazolium- 5-carboxyanilide inner salt (XTT) assay is used. The tetrazolium salt, XTT, is especially useful in quantifying viable cells. This assay quantifies spectrophotometric cell growth and viability without the use of radioactive isotopes and is based on the cleavage of yellow tetrazolium salt, XTT, to form an orange formazan dye by metabolically active cells. XTT cleavages into an orange formazan dye by the mitochondrial enzyme, dehydrogenase, occurs exclusively in living cells. Cells are grown in growth medium plus 10 % FBS in 96-well plates until 70-80 % confluence. They are then treated with the appropriate drug sample for 24 h. An XTT assay is performed at the end of incubation. Briefly, 50 mL of XTT labeling mixture solution is add to each well, and the cells are incubated at 37 °C for 4 h. The formazan dye formed is soluble in aqueous solutions and the optical density at 450 nm is compared with that of control wells

with a screening multiwell spectrophotometer enzyme linked immunosorbent assay (ELISA) reader. The reference wavelength is 650 nm.

Sulforhodamine B assay

Sulforhodamine B assay is a bright pink aminoxanthene dye that binds to basic amino acids in mild acidic conditions and dissociates under basic conditions. Cells are plated in 96-well flat bottom plates at 5000-10000 cell/well. The difference in cell numbers plated adjusts for differences in the growth rates of the various cell lines. Cells are allowed to adhere to the wells overnight, then the samples are added to triplicate wells in serial 3-fold dilutions. Water is added to the control wells at a 1:10 dilution in medium. These plates are incubated at 37 °C, 5 % CO2 for 3 days, then assayed for growth inhibition using a sulforhodamine B (SRB) assay. The cells are fixed by the addition of cold 50 % trichloroacetic acid to a final concentration of 10 %. After 1 h incubation at 4 °C, the cells are washed five times with deionized water. The cells are then stained with 0.4 % SRB dissolved in 1 % acetic acid for 15-30 min and subsequently washed five times with 1 % acetic acid to remove unbound stain. After the plates are air dried at room temperature, the bound dye is solubilized with 10 mm Tris base and the plates are analysed on a microplate reader at 595 nm.

4. Hollow Fiber Assay

The HF assay is composed of 2 cm tubes filled with tumor cell lines. These fibers are implanted into mice at two sites (intraperitoneal and subcutaneous). The fibers are removed after 4–6 days in the animal and processed in vitro for quantification of tumor cell growth. By determining net cell kill, one can examine whether drugs administered via different routes are bioavailable and can reach the tumor sites.

***In vivo* methods**

A variety of tumor systems for *in vivo* evaluation of new anticancer agents are available. Murine host systems are most suitable for experimental tumor therapy because of the availability of in-bred lines at relatively low costs, the ease of obtaining tumor models and established, widely accepted experimental endpoints (Taghian and Suite, 1999). Spontaneous or transplanted murine tumors can be studied in immunocompetent mice whereas investigation of human tumors requires an immunodeficient host, *e.g.* nude mice, to avoid tissue rejection. Genetically engineered mice may help to improve this situation (Tuveson and Jacks, 2002). For practical reasons, scientists mainly use ectopically-implanted, subcutaneously-growing tumor models. Thus, these models can provide proof of principle, but the magnitude of effect does not necessarily correspond to the clinical situation. Animal tumor systems have to meet several requirements to be suitable for experimental tumor therapy (Taghian and Suite, 1999; Sieman, 1987). It is very important that the tumor precisely reflects treatment response, and that the natural history of the host allows the study of the experimental endpoint, *e.g.* a sufficient life-span for follow-up to assess local tumor control. Stable biological characteristics of the tumor system such as expression of the molecular target, growth rate, differentiation and immune response are also required to assure the high quality of experiments. To avoid undetected changes of characteristics of tumor systems, it is indispensable that each researcher follows strict quality assurance protocols (Pathak *et al.,* 1998).

Tumor growth delay assay

This functional assay is robust, standardized, widely accepted and used in most experiments to study anticancer agents *in vivo* (Begg, 1987). A delay in tumor growth is an experimental endpoint of clinical relevance for new anticancer agents in animals. The tumor growth delay assay provides evidence for further drug evaluation in clinical trials. Moreover, results from pre-clinical investigations in animal tumor models may help to design clinical trials, while detailed *in vivo* experiments may help in understanding the results from clinical trials. The results from pre-clinical and clinical studies on inhibitors of EGFR and angiogenesis demonstrate that animal models can prove the principle of a new therapeutic approach. Knowledge and experience is required to correctly perform and interpret the growth delay assay. Usually tumors are allocated to two experimental groups. Animals of the first group are treated with the anticancer agent. Animals receiving the so-called vehicle, *e.g.* the compounds and solutions that were used to prepare the drug solution, are controls. There is no consensus about the minimal group size to perform the tumor growth delay assay. Of course, the group size to detect a difference in tumor growth times depends on the magnitude of effect and intertumoral heterogeneity. Unfortunately, both factors usually are unknown when the experiment is designed. To account for intertumoral heterogeneity, it is useful to randomize the animals over the experimental matrix and to treat both experimental groups in parallel. As many tumor characteristics such as growth rate, cell loss, hypoxia, angiogenesis and response to anticancer agents may change with increasing tumor volume, it is necessary that the tumors in both experimental groups be enrolled into the treatment protocol at a similar tumor volume. Apparently most anticancer agents are more effective in smaller than in larger tumors. This is an important caveat because in clinical trials often patients with advanced stages and tumor masses are treated. To determine tumor growth delay, the tumor volume is repeatedly measured, and for each individual tumor the time to reach a multiple of the starting volume, *e.g.* two, five or ten times the starting volume, is recorded. The calculated growth delay (tumor

growth time of treated tumors minus tumor growth time of control tumors) is a direct measure of the drug effect on tumor growth. To generalize the data for comparison with other tumor models and drugs, the so-called specific tumor growth delay (ratio of growth delay to growth time of control tumors) is calculated. It is important to note that the endpoint of the tumor growth delay assay is a *time* to reach a volume but not a *volume* at a given time point. For many drugs the tumor growth delay increases with increasing endpoint sizes, because, in experiments with multiple drug administrations, the tumor growth delay increases with time because of the accumulation of drug effect. There is no consensus about the optimal endpoint size to report data from growth delay assays. If tumor cell kill is the major mechanism of action of an anticancer agent, dead and doomed cells and their clearance will contribute more and more to the tumor volume. Especially in slow shrinking tumors, this may mask the rapid regrowth of surviving tumor cells. Therefore, it appears that the smaller the endpoint size the more closely this will reflect the actual anticancer effect of the drug (Begg, 1987; Beck-Bornholdt *et al.,* 1987). Multiple administrations of antiproliferative agents probably result in an increasing tumor growth delay with time. In fast growing tumors, the effect on tumor growth rate is detectable only after some drug administrations and, thereby, at later time points. Thus, for antiproliferative agents, larger endpoint sizes seem preferable. As the mechanisms of action of new drugs are usually unknown before the experiment, it is reasonable to analyze and report tumor growth delay with multiple endpoints.

Anticancer drugs may prolong tumor growth by several mechanisms. Agents may affect tumor cells directly or indirectly, *e.g. via* targeting stromal cells by inhibiting angiogenesis. Both directly and indirectly acting anticancer agents can reduce the tumor growth rate by inhibition of tumor cell production, increased tumor cell death, or improved clearance of dead and doomed cells. Determination of the mechanism underlying the anticancer effect of an anticancer agent by a simple tumor growth delay assay is impossible and requires more detailed *in vitro* and *in vivo* experiments. Whether a new drug affects proliferation or survival is of particular significance for designing more complex *in vivo* experiments and clinical trials.

Tumor control assay

In contrast to tumor growth delay, the results from the tumor control assay solely depend on the therapeutic effect on clonogenic cells. Permanent tumor control is the most relevant experimental endpoint for testing of potentially curative settings (Suit *et al.,* 1987). In practical terms, after therapy tumors are followed-up and regrowth of the recurrent tumor is recorded. This requires sufficient follow-up times to detect virtually all recurrences.

An alternative to this time consuming procedure is the tumor-excision assay. For this, tumors are excised after treatment, a single cell suspension is prepared and cells are seeded into flasks or multi-well plates. After incubation, the fraction of surviving clonogens can be determined and compared with control tumors without treatment. Although this assay has the limitation that the survival of clonogenic cells is not determined in their original environment, the tumor-excision assay is less expensive than the tumor control assay because no follow-up is necessary and the number of animals required is smaller.

Many of the new anticancer drugs reduce tumor growth but do not eradicate the tumor. Combination of new anticancer agents with potentially curative therapy modalities, such as radiotherapy, can improve the results compared with radiotherapy alone. For example, inhibitors of the epidermal growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF)-dependent angiogenesis are not curative as a monotherapy. However, the combination of these inhibitors with irradiation in animal models consistently resulted in longer tumor growth delay than either treatment alone (Baumann and Karuse, 2004; Zips and Baumann, 2003; Zips *et al.,* 2003). Administration of the VEGFR2 mAB DC101 to tumor bearing animals exposed concomitantly to fractionated irradiation improved the results of the tumor control assay (Kozin *et al.,* 2001). However, results from tumor growth delay and tumor control assays are not necessarily consistent. BIBX1382BS is an potent inhibitor of the receptor tyrosine kinase of EGFR, resulting in clear-cut effects on tumor cell proliferation *in vitro* and *in vivo* using the human squamous cell carcinoma FaDu, which shows membranous expression of the molecular target, *i.e.* the EGFR (Baumann *et al.,* 2003). In combination with fractionated irradiation, tumors treated with BIBX1382BS show a longer tumor growth delay than irradiated tumors or tumors treated only with BIBX1382BS. This clearly shows that the drug is also effective on the growth of irradiated tumors. However, BIBX1382BS did not improve the tumor control probability in the same tumor model (Baumann *et al.,* 2003). The underlying reason for the discordance of the growth delay assay and the tumor control assay is unclear. From this example, it is quite obvious that an extrapolation of results from non-clonogenic assays to predict response of clonogenic cells can be misleading and may cause incorrect conclusions with far-reaching consequences for clinical trials.

In our opinion, tumor control is the most relevant endpoint for preclinical testing of anticancer agents. Alternatively, large growth delay studies using different dose levels may yield results similar to those obtained from tumor control assays. Monoclonal antibodies against EGFR have been shown to improve tumor control after radiotherapy in patients with head and neck cancer (Bonner *et al.,* 2004). Interestingly, xenografted FaDu tumors also

showed a higher local tumor control rate after anti-EGFR antibody therapy with C225 and irradiation. Comparison of pre-clinical and clinical data of EGFR inhibition and radiotherapy corroborates the importance of detailed *in vivo* studies with suitable, well-characterized tumor models in a clinically relevant setting. Neglect of clonogenic endpoints might result in misleading strategies for further clinical testing. Although failure of new approaches in the clinic cannot be prevented by *in vivo* animal studies, consideration of data from carefully performed *in vivo* studies on efficiency, curative potential and optimal regimen are valuable for the design of clinical trials and the investigation of mechanisms of action.

CONCLUSION

Standardized, well-established *in vitro* and *in vivo* methods are available for experimental evaluation of new anticancer agents. A step-wise procedure from *in vitro* to *in vivo* seems reasonable to reduce the large quantity of potential drugs to a few promising agents for further clinical testing. The clinical application for which the drug is aimed, *e.g.* palliative, curative, tumor entity, or combination with other modalities, needs to be considered in the experimental evaluation. For evaluation of new anticancer agents, we advocate *in vitro* and *in vivo* experiments with at least two or three different tumor cell lines, applying functional nonclonogenic and, if applicable, clonogenic assays.

REFERENCES

Adams, V.J., Evans, K.M., Sampson, J. and Wood, J.L.N. (2010). Methods and mortality results of a health survey of purebred dogs in the UK. *J Small Ani Prac*. **51:** 512–524.

Anand,, P., Kunnumakara, A.B., Sundaram, C., Harikumar, B.K., Tharakan, S.T., Lai, O.S., Sung, B. and Aggarwal, B.B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharm Res.* **25**: 2097–2116.

Baumann, M. and Krause, M. (2004). Targeting the epidermal growth factor receptor in radiotherapy: radiobiological mechanisms, preclinical and clinical results. *Radiother Oncol.* **72:** 257-266.

Baumann, M., Krause, M., Zips, D., Eicheler, W., Dorfler, A., Ahrens, J., Petersen, C., Bruchner, K. and Hilberg, F. (2003). Selective inhibition of the epidermal growth factor receptor tyrosine kinase by BIBX1382BS and the improvement of growth delay, but not local control, after fractionated irradiation in human FaDu squamous cell carcinoma in the nude mouse. *Int J Radiat Biol.* **79:** 547-559.

Beck-Bornholdt, H.P., Wurschmidt, F. and Vogler, H. (1987). Net growth delay: a novel parameter derived from tumor growth curves. *Int J Radiat Oncol Biol Phys.* **13:** 773-777.

Begg, A.C. (1987). Principles and practice of the tumor growth delay assay. *In*: Kallmann RF (ed.), Rodent Tumor Models in Experimental Cancer Therapy, pp. 114-121. Pergamon Press, New York.

Bishop, M. and Weinberg, R.A. (1996). Scientific American Molecular Oncology. New York, Scientific American, Inc.

Bonner, J.A., Harari, P.M. and Giralt, J.L. (2004). Cetuximab (Erbitux TM) prolongs survival in patients with locally advanced squamous cell carcinoma of the head and neck: a phase III study of high dose radiation therapy with and without cetuximab. *Proc Am Soc Clin Oncol Abstract.* 5507.

Brown, J.M. and Wouters, B.G. (1999). Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res.* **59:** 1391-1399.

Dobson, J.M. (2013). Breed-Predispositions to Cancer in Pedigree Dogs. *ISRN Vet Sci.* **2013** (941275): 1-23.

Eshleman, J.S., Carlson, B.L., Mladek, A.C., Kastner, B.D., Shide, K.L. and Sarkaria, J.N. (2002). Inhibition of the mammalian target of rapamycin sensitizes U87 xenografts to fractionated radiation therapy. *Cancer Res.* **62:** 7291-7297.

Freshney, R.I. (2000). Culture of Animal Cells: A Manual of Basic Techniques, 4th Edn., Wiley-Liss, New York.

Klaassen, Curtis D. (2008). Casarett and Doull's toxicology : the basic science of poisons. 7th Edn, McGraw-Hill Medical, New York.

Kozin, S.V., Boucher, Y., Hicklin, D.J., Bohlen, P., Jain, R.K. and Suit, H.D. (2001). Vascular endothelial growth factor receptor-2-blocking antibody potentiates radiation-induced long-term control of human tumor xenografts. *Cancer Res.* **61:** 39-44.

Misdorp, W. and Hart, A.A.M. (1979). Some prognostic and epidemiologic factors in canine osteosarcoma. *Nati Cancer Inst*. **62**: 537-45.

Mukhopadhayay, S. and Som, T.L. (1992). *Ind J Vet Pathol. 16* (2): 122-123.

Pathak, S., Nemeth, M.A. and Multani, A.S. (1998). Human tumor xenografts in nude mice are not always of human origin: a warning signal. *Cancer.* **83:** 1891-1893.

Rungsipipat, A. (2009). Feline Neoplasm. *J. Thai. Vet. Prac*. **21**(3): 98-105.

Shekhar, C.S., Vijaysarathi, S.K., Gowda, R.N.S., Suguuna, R. and Rao, S. (2001): An epidemiology study on canine mammary tumors. *Ind Vet J*. **78**: 107–109.

Siemann, D.W. (1987). Satisfactory and unsatisfactory tumor models: factors influencing the selection of a tumor model for experimental evaluation. *In*:

Kallmann RF (ed.), Rodent Tumor Models in Experimental Cancer Therapy, pp. 12-15. Pergamon Press, New York.

Solowey, E., Lichtenstein. M., Sallon, S., Paavilainen, H., Solowey, E. and Haya Lorberboum-Galski (2014). Evaluating Medicinal Plants for Anticancer Activity. *Scientific World J*, **2014** (721402): 1-12.

Steel, G. (2002). Basic Clinical Radiobiology, 3rd. edition. London: Arnold.

Suit, H.D., Sedlacek, R. and Thames, H.D. (1987). Radiation doseresponse assays of tumor control. *In*: Kallmann RF (ed.), Rodent Tumor Models in Experimental Cancer Therapy, pp. 138-148. Pergamon Press, New York.

Taghian, A.G. and Suit, H.D. (1999). Animal systems for translational research in radiation oncology. *Acta Oncol.* **38:** 829-838.

Tannock IF and Hill RP (1998). The Basic Science of Oncology, 3rd. edition, New York: McGraw-Hill.

Tuveson, D.A. and Jacks (2002). Technologically advanced cancer modeling in mice. *Curr Opin Genet Dev.* **12:** 105-110.

Zips, D. and Baumann, M. (2003). Anti-VEGF strategies in combination with radiotherapy. *In*: Nieder C, Milas L and Ang KK (eds.), Modification of Radiation Response: Cytokines, Growth Factors and other Biological Targets, pp. 179-188. Springer, Berlin, Heidelberg.

Zips, D., Krause, M., Hessel, F., Westphal, J., Brüchner, K., Eicheler, W., Dörfler, A., Grenman, R., Haberey, M. and Baumann, M. (2003). Experimental study on different combination schedules of VEGF-receptor inhibitor PTK787/ZK222584 and fractionated irradiation. *Anticancer Res.* **23:** 3869-3876.

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Research Article

ANTI-INFLAMMATORY ACTIVITY OF A POLYHERBAL FORMULATION IN LABORATORY ANIMALS IN ACUTE AND SUB ACUTE MODELS

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ABSTRACT

The objective of the study was to explore the role of various ratios of *Piper longum* extract for optimum anti-inflammatory effect. Subsequently, a ratio of 1:1:3 showed maximum potency as an anti-inflammatory compound in our study. To evaluate this, various irritants like carrageenan, formaldehyde, cotton pellet and granuloma pouch models were used in rat models. Polyherbal formulation was administered in graded doses (50,100 and 200mg/kg, p.o.) and indomethacin was used as standard drug. Percent (%) inhibition of paw edema, blood and biochemical parameters, were studied and compared with standard drugs. Inhibition of paw edema was seen at 200mg/kg in all the models. There was reduced RBC count in control group, but elevated RBC counts were seen in treated groups. Similarly, haemoglobin (Hb) levels were elevated in all the models than their respective control groups. Likewise, decreased ESR and recovery from the induced anemia were evident in all the groups. An indicator of infectious and inflammatory diseases, the DLC count was increased in arthritic rats. Results of this study strongly support the anti-inflammatory potential of the formulation due to presence of various phytoconstituents like flavonoids, phenolics and steroids.

Key words: Carrageenan, granuloma pouch, polyherbal, flavonoids.

INTRODUCTION

Piper longum Linn. (Piperaceae) is widely distributed in Assam, Tamil Nadu and Andhra Pradesh in India, Malaysia, Indonesia, Singapore, Sri Lanka and South Asian regions. A bioassay-guided isolation of an ethanol extract of the fruit of *Piper longum L*. yielded piperlonguminine, piperine and pipernonaline, as the main anti-hyperlipidemic constituents from *Piper longum* (Jin *et al.,* 2009). Pharmacological and clinical studies have revealed that piperine has CNS depressant, anti-pyretic, analgesic, anti inflammatory, antioxidant, and hepatoprotective activities (Ratner *et al.,* 1991).

Clerodendrum indicum (L.) has shown antinociceptive and antimicrobial activities. The juice of the fresh leaves has been prescribed by the folk practitioner as blood purifier to treat gastrointestinal tract aliments (Pal *et al*., 2012; Raihan *et al*., 2012). The alcoholic extract and the essential oil of *Acorus calamus* Linn., a semi- aquatic, perennial, aromatic herb with creeping rhizomes, have been reported to possess antimicrobial, antiulcer, expectorant, carminative, anti-spasmodic, emetic, laxative and diuretic activities (Dastur, 1951; Elaya *et al.,* 2009).

The authors have reported the *in vitro* antioxidant activity of the extracts of the three plants (Barua *et al*., 2014a and 2014b). The plants *Clerodendrum indicum* and *Acorus calamus* have been traditionally used in various disease conditions. *Piper longum* is known to increase bioavailability of other drugs, hence, a combination of hydroethanolic extract of *C.indicum* and ethanolic extract of *A. calamus* and ethanolic extract of seeds of *Piper longum* were used in different proportions in order to establish the best formulation against arthritis.

MATERIALS AND METHODS

Experimental animals

Male Wistar albino rats (6-8 week of age, 200-250 g, b wt) were used. Breeding and maintenance of the animals were done according to the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) and animal welfare division, Government of India for the use of laboratory animals. This work has been carried out under the supervision of institutional animal ethical committee (No CPCSEA/770/ac/ IAEC/118). All the animals were housed in polypropylene cages using paddy husk bedding at 28 ±1°C temperature and 50 ± 5% humidity. Animals were fed on laboratory feed and water *ad libitum*.

Preparation of the plant extract

Whole plants of *C. indicum, A. calamus* and fruits *of P. longum* were collected and were authenticated by Dr. I.C. Barua, Taxonomist, AAU, Jorhat. Voucher specimens were deposited. They were shade dried and blended to fine powder. The powdered plants were extracted

in rotary evaporator (Buchi) using 95% and 50% ethanol as the case may be. The extract was concentrated by distillation under reduced pressure and evaporated to dryness and stored at 40^0c until use.

Acute toxicity test

Groups of six albino male Wistar rats were administered with the extracts in the doses of 0.25, 0.5, 1.0, 1.5, 2.0 g/kg body weight respectively as per OECD guidelines 425. Rats were continuously observed for mortality and behavioral responses for 48 h and once daily thereafter until the 14th day. Three doses *viz.* 50, 100 and 200mg/kg doses were selected for the study.

Evaluation of anti-inflammatory activity

Grouping of animals

Rats consisting of 5 animals in each group were divided into 5 groups , as Group I, control, administered with normal saline, Group III, administered with 200mg/kg of PHF, Group IV, administered with 100mg/kg of PHF, Group V, with 50mg/kg oral dose of the extract as PHF. Finally, Group II consists of the standard drug, indomethacin (10 mg/kg, i.p.). The drugs were administered as per protocol.

Carrageenan induced arthritis in rats

Extracts were administered 1 hour before induction of paw edema in rats with injection of 100µl of 1 % carrageenan in the left hind paw. Percent inhibition of paw edema was calculated along with various biochemical, blood parameters (Winter *et al*., 1962) were calculated along with haematological and biochemical parameters in all the groups.

Formaldehyde induced arthritis in rats

Arthritis was induced by injecting 0.1 mL of 2% formaldehyde on day 1 and 3. Extract and standard drug was fed and paw volume was measured once daily for the next 10 days. Percentage inhibition of paw edema and other biochemical and haematological parameters (Turner, 1965) were measured and calculated as above.

Cotton pellet Granuloma model in rats

Sub-acute inflammation was induced using sterilized cotton pellets (10±1mg) implanted subcutaneously on either side into the dorsal region in each rat under light anaesthesia. Extracts, standard drug and vehicle were administered for 7 days after implantation. On day 8, percent inhibition of exudates formation and reduction in weight of granuloma was calculated as dry weight, along with biochemical and blood parameters (D'Arcy *et al* ., 1960)

Granuloma pouch model in rats

A pouch on the back of the rats was created by injecting 20 mL of air followed by 1 mL of sterilized 1% croton oil in olive oil. The extracts and the standard drugs were fed from day 1 to day 13 of the experiment. On day 14, percent inhibition of granuloma formation along with other parameters was calculated (Selye, 1953).

Biochemical examination

Total Protein estimation was done by Biuret method using reagent kit (Siemens). Estimation of albumin, SGOT, SGPT and alkaline phosphatase (ALKP) in the serum samples were measured using reagent kits (Siemens). Various haematological parameters like Total RBC count, DLC. Their absorbance was read in the UV-Vis spectrophotometer using specified wavelengths.

Statistical analysis

Results are expressed as Mean ± S.E.M. The difference between experimental groups was compared by One-way Analysis of Variance (ANOVA) followed by Dunnett's test. The results were considered statistically significant when $P< 0.05$.

RESULTS

In carrageenan induced paw edema, reduction of paw volume was significant ($P<0.05$) in the extract treated group than the positive control group dose dependently. Likewise, standard drug indomethacin treated group also showed inhibitory activity, superior to the extract treated group. The biochemical and blood parameters are presented in Fig. 1. The result signifies that the polyherbal formulation could significantly alter the serum biochemical along with haematological parameters as a result of treatment.

In formaldehyde induced arthritis model, which is a sub acute model, inhibition of paw edema was significant ($P<0.05$). Enzyme activities SGOT, SGPT which were elevated during inflammation were reduced significantly in the extract treated and standard group. ESR values were reduced, Hb count was increased and DLC count was reduced in these groups significantly, which can be evident from the graph shown in Fig. 2.

Cotton pellet granuloma is another sub acute model for inflammation in our study. In this group also there was does dependent inhibition of granuloma formation in the treated rats on 8th day as compared to the positive control group on the basis of dry weight. Indomethacin treated standard group showed similar pattern but superior activity as it is a synthetic compound and our extract was a polyherbal formulation. The results were represented in Fig. 3.

Granuloma pouch model is another sub acute model from inflammation, where reduction of dry weight of the granuloma was more than the control group and maximum inhibition was with 200mg/kg oral dose. The standard drug treated group also shows reduction of granuloma tissue weight indicating anti inflammatory activity. In Fig. 4 the enzyme levels, viz. SGOT, SGPT , AKLP levels were reduced significantly in comparison to the control group, so also the haematological parameters like ESR, DLC count etc with subsequent increase in RBC and Hb content.

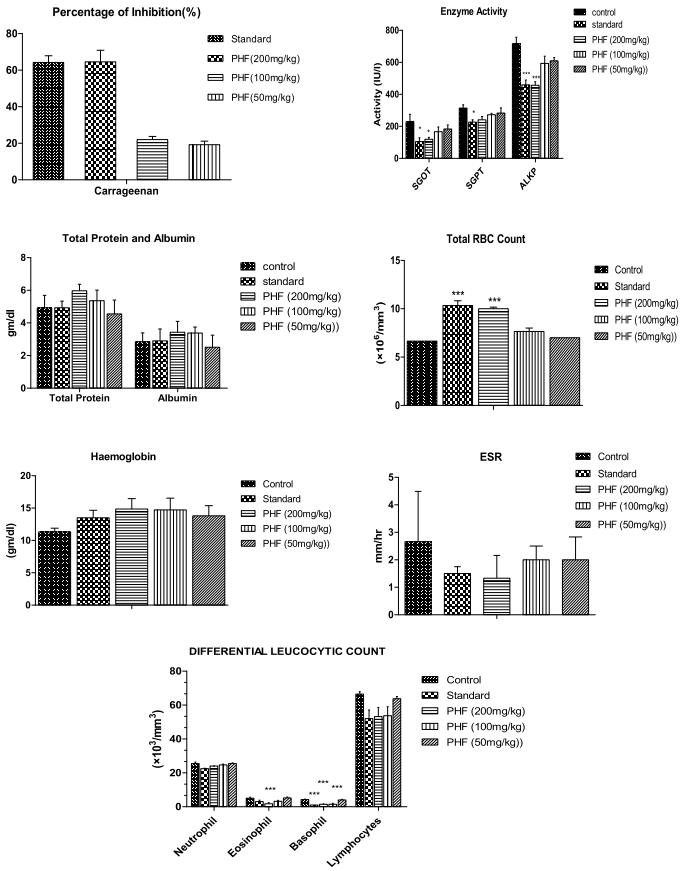

Fig. 1.
Graphical representation of the enzymatic and haematological parameters in carrageenan induced inflammation.*Denotes significant difference of the group in comparison to control (P<0.05).

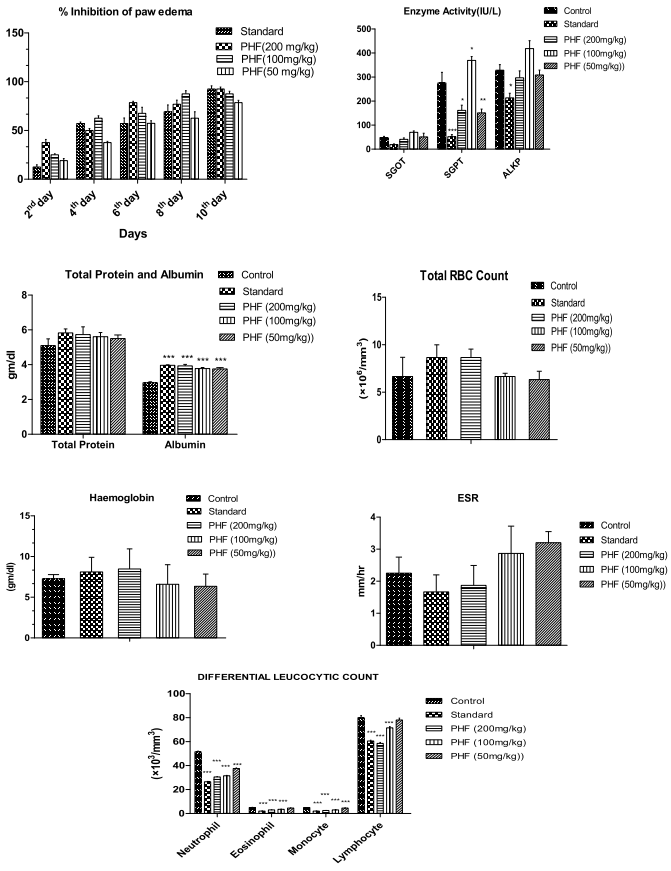

Fig. 2.
Graphical representation of the enzymatic and haematological parameters in formaldehyde induced arthritis on day 10th. *Denotes significant difference of the group in comparison to control (P<0.05).

DISCUSSION

In the present study, the rats were selected to induce inflammation because rats develop a chronic swelling in multiple joints with the influence of inflammatory cells, erosion of joint cartilage and bone destruction. It has close similarities to human rheumatoid disease (Singh & Majumdar, 1996).

Clerodendrum indicum, Acorus calamus and *Piper longum* were known for their anti-inflammatory properties since ages. We have combined these three plant extracts in different ratios like 1:1:1, 1:1:2 and 1:1:3 for a polyherbal formulation. Eventually, they showed the best anti-inflammatory activity with 1:1:3 ratios. *Piper longum* is combined with various drugs since it is known to increase the bioavailability of the drugs mentioned earlier. We have used the polyherbal formulation for studying its synergistic activity and also to study the bioavailability of the other two plants.

The carrageenan induced paw inflammation is used as a model for investigating systemic anti-inflammatory agent (Mujumdar and Misra, 2004). Local injection of carrageenan into rat hind paw induces acute inflammatory responses such as edema (Zhang *et al.,* 2008). The development of the edema induced by carrageenan has been described as a biphasic event. A rapid early phase (up to 2 h) is triggered by the concerted release of histamine, bradykinin, 5-hydroxytryptamine or cyclooxygenase products. And a more sustained late phase (2 to 5 h) is regulated by neutrophil infiltration prostaglandins, protease and lysosome (Crunkhon and Meacock, 1971).

The determination of rat paw swelling is apparently simple, sensitive and one of the quick procedures for evaluating the degree of inflammation and the therapeutic effects of drugs (Begum, 1988). There was a significant reduction in the paw volume in all the models in the PHF treated groups (200 mg/kg) and standard drug treated rats compared to the arthritic rats. The cardinal signs of the chronic inflammatory reactions like redness, swelling, arthralgia and immobility of affected joints were significantly less in the drug treated animal than those of the control.

Anaemic condition is a common feature in

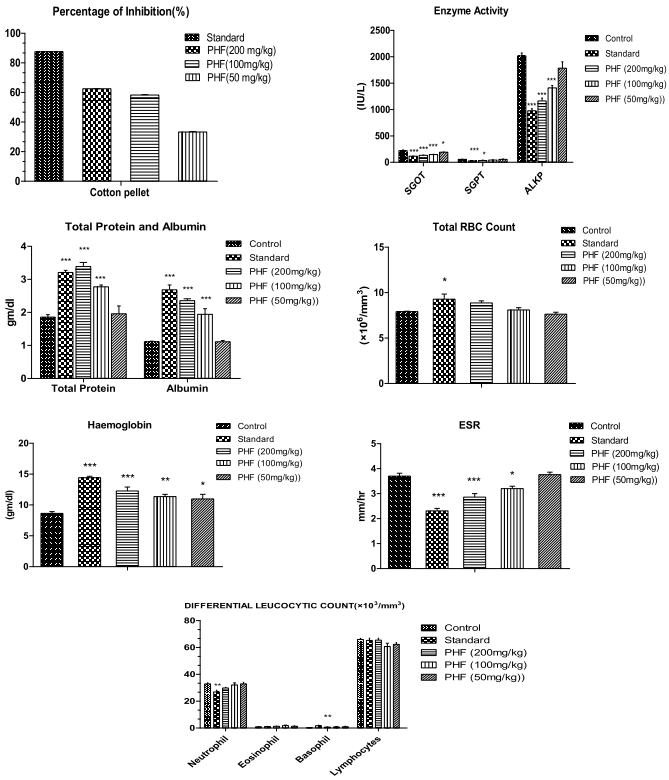

Fig. 3.
Graphical representation of the enzymatic and haematological parameters in cotton pellet granuloma model of arthritis on day 8th. * denotes significant difference of the group in comparison to control (P<0.05).

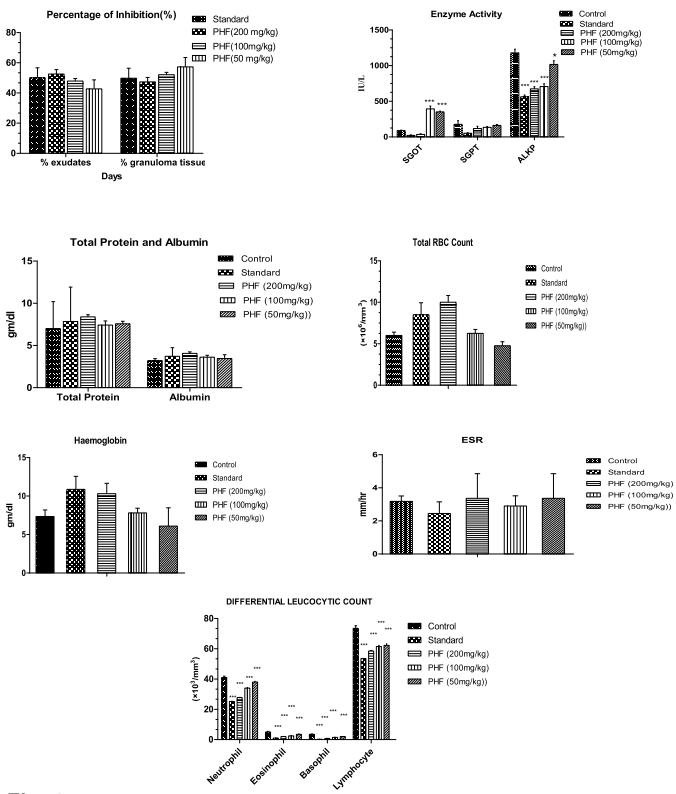

Fig. 4:
Graphical representation of the enzymatic and haematological parameters in Granuloma pouch model of arthritis on day 14th.*Denotes significant difference of the group in comparison to control (P<0.05).

patients with chronic arthritis (Allar, 1977). PHF and standard drug treatment showed a recovery from the anaemic condition evident from the HB content and RBC count as was seen in the arthritic control rats.

The erythrocyte sedimentation rate is a test that measures inflammation in the body indirectly. It measures the rate of settling or sedimentation of red blood cells in a capillary tube. Proteins produced during inflammation cause erythrocytes to move closer and stack up in a group. When this happens, they become denser and settle faster. The closer and faster the erythrocytes settle, the higher the value of the erythrocyte sedimentation rate (Van den Hoogen *et al.,* 1995). The PHF treated and the standard drug treated groups showed significant lower ESR values as compared to the arthritic control rats.

Further, low levels of white blood cells indicate that the PHF is a potent anti arthritic treatment, given that elevated white blood cell levels are associated with active inflammation.In our study the PHF exhibited a significant anti-inflammatory activity in a dose dependent manner. In the present study, we showed that the PHF could significantly inhibit the progression of the inflammation in treated animals. Chronic inflammatory conditions reduce body's antioxidant capacity by affecting a variety of endogenous ROS scavenging proteins, enzymes, and chemical compounds. This leads to oxidative stress, which damages other tissues and organs (Halliwell and Gutteridge, 1990). Therefore, it was assumed that the reported and well established antioxidant properties of each of the plants individually owing to its phytoconstituents particularly flavonoids and its ability to block the COX-2 pathway during the progression of inflammation justify the usage of the plant extract in the treatment of inflammation. Since all the three plants were known for their anti-inflammatory property, their combination with *Piper longum* showed synergistic activity at a lower dose, which justifies that *Piper longum* increases the bioavailability of the other two plants, thereby improving their anti inflammatory potential in the polyherbal formulation at a much lower dose rather than a single extract. This has shown additive property of the individual extracts, thereby reducing the side effect at higher dose.

From the present experimental findings of both pharmacological and biochemical parameters observed from the current investigation, it is concluded that at the doses of 200 mg/kg body weight in the ratio of 1:1:3 (*Clerodendrum indicum: Acorus calamus: Piper longum)* could exert its anti inflammatory potential in different models

of inflammation in our study. Thus, the formulation could be a promising anti-inflammatory agent of plant origin in the treatment of inflammatory disorders.

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REFERENCES

Allar, S., O'Driscoll, J. and Laurie, A. (1977). Salmonella osteomyelitis in aplastic anaemia after antilymphocytic globulin and steroid treatment. *J. Clin. Pathol.* **2:** 174-175**.**

Barua, C.C., Das, A.S., Sen, S., Talukdar, A., Barua, A.G., Baishya, G. and Nath, S.C. (2014a) *Clerodendrum indicum*: A repertoire of phytochemicals and its antioxidant activity. *Int. J. Phytopharmacol.* 5: 252-260.

Barua, C.C., Sen, S., Das, A.S., Talukdar, A., Hazarika, N.J., Barua, A.G., Barua, A.M. and Barua, I.C. (2014b) A comparative study of the *in vitro* antioxidant property of different extracts of *Acorus calamus* Linn. *J. Nat. Prod. Plant Res.* **4**: 8-18.

Begum, V.H. and Sadique, J. (1988) Long term effect of herbal drug *Withania somnifera* on adjuvant induced arthritis in rats. *Indian J. Exp. Biol.* **26:** 877-882.

Crunkhon, P. and Meacock, S.E.R. (1971) Mediators of the inflammation induced in the rat paw by carrageenan. *Brit. J. Pharmacol*. **42:** 392-402.

Dastur, J.F. (1951) *Medicinal plants of India and Pakistan*, D.B. Taraporevala Sons Co.Ltd. Bombay, India.

D'Arcy, P.F., Haward, E.M., Muggleton, P.W. and Townseed, S.B. (1960) The anti-inflammatory action of griseofulvin in experimental animals. *J. Pharm. Pharmacol*. **12**: 659-665.

Elaya, R.A., Vijayalakshmi, M. and Devalarao, G. (2009) *Acorus calamus* Linn. : Chemistry and Biology. *Res.J. Pharm. Tech.* **2:** 256-261.

Halliwell, B. and Gutteridge, J.M.C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Meth. Enzymol.* **186:** 1-85.

Jin, Z., Borjihan, G., Zhao, R., Sun, Z., Hammond, G. B. and Uryu, T. (2009) Antihyperlipidemic compounds from the fruit of *Piper longum* L. *Phytother. Res.* **23:** 1194-1196.

Mujumdar, A.M. and Misra, A.V. (2004) Anti-inflammatory activity of *Jatropha curcas* roots in mice and rats .*J. Ethnopharmacol*. **90:** 11-15.

Pal, A., Zobaer, A.M., Akter, N., Islam, M.S. and Bachar, S.C. (2012) Evaluation of antinociceptive, anti-diarrheal and antimicrobial Activities of Leaf Extracts of *Clerodendrum indicum*, *Pharmacog. J.* **4:** 41-46.

Raihan, S.Z, Biswas, P., Monir, M.M., Biswas, S.K., Chowdhury, A. and Sahidur Rahman, A.K.M. (2012) Phytochemical investigation and *in vitro* antinociceptive activity of *Clerodendrum indicum* leaves. *Pak. J. Biol. Sci.*, **15**: 152-155.

Ratner, L., Vander, H.N. and Dedera, D. (1991) Inhibition of HIV and SIV infectivity by blockade of alpha glucosidase activity. *Virol.,* **181:** 180-192.

Selye, H. (1953) Use of granuloma air pouch technique in the study of antiphlogistic corticoids. *Proc. Soc. Exp. Biol.* **82**:28-33

Singh, S., and Majumdar, D.K. (1996) Effect of fixed oils of *Ocimum sanctum* against experimentally induced arthritis and joint edema in laboratory animals. *J. Pharmacogn*. **34:** 218-222.

Turner, R. (1965) Screening Methods in Pharmacology, Anti inflammatory agent. Academic Press New York, London, pp. 158.

Van den Hoogen, H.M.M., Koes, B.W., van Eijk, J.T.M. and Bouter, L.M. (1995) On the accuracy of history, physical examination, and erythrocyte sedimentation rate in diagnosing low back pain in general practice. *Spine*. **3:** 318-327.

Winter, C.A., Risley, E.A., and Nuss, C.W (1962) Carrageenan induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. Exp. Biol. Med.* **111**:544-547.

Zhang, G.Q., Huang, X.D., Wang, H., Leung, A.K.N., Chan, C.L., Fong, D.W.F. and Yu, Z.L. (2008) Anti-inflammatory and analgesic effects of the ethanol extract of *Rosa multiflora* Thunb hips. *J. Ethnopharmacol.* **118** 290-294.

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Research Article

DETERMINATION OF MTD AND EFFECT OF SUBACUTE EXPOSURE OF IMIDACLOPRID AND ITS AMELIORATION BY RESVERATROL IN MALE RATS

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ABSTRACT

Imidacloprid is one of the most important neonicotinoid insecticides known to target the nicotinic acetylcholine receptor (nAChR) in insects, and potentially in mammals. The aim of present study was to ascertain the maximum tolerated dose (MTD) and to investigate the effects of sub-acute toxicity of imidacloprid on body weight gain and relative organ weight and its amelioration by resveratrol in male Wistar rats. The MTD of imidacloprid was determined to be 1850 mg/kg orally in male rats in a pilot dose range finding study. Animals were observed for toxic signs and symptoms after oral administration of imidacloprid in single dose. Wistar rats were randomly allocated into six groups (n = 6 rats /each group). Imidacloprid was administered orally by gavage once daily for 14 days to 6 rats and for 28 days to remaining 12 rats at doses of MTD/10 and MTD/20. Resveratrol co-treatment was also given in imidacloprid administered groups. Body weight of each rat was recorded on day 0 and at an interval of two days till the completion of experiment and changes in weight of various body organs (liver, heart, spleen, kidney and testes) were examined after sacrifice. The sign and symptoms of toxicity were ataxia, rigidity and fasciculation of muscles, protrusion of eye ball and tremors of head. Imidacloprid treatment resulted in decreased body weight gain as compared to the naïve. A significant change was also observed in relative organ weight in imidacloprid administered groups as compared to naïve which was restored by resveratrol co-treatment. The study revealed a mild to moderate toxic effect of imidacloprid on body weight gain and relative organs weight.

Keywords: imidacloprid, resveratrol, MTD, body weight gain, relative organ weight

INTRODUCTION

The neonicotinoids are a new major class of highly potent insecticides used to control sucking insects, some chewing insects including termites, soil insects and fleas on dogs and cats. Currently the best known neonicotinoid is imidacloprid [1-(6-chloro-3-pyridinyl) methyl-N-nitro-2-imidazolidinimine] which is an active substance in number of commercial insecticide preparations. Imidacloprid and its analogs are remarkably potent neurotoxic insecticides, which act as nicotinic acetylcholine receptor agonists (nAChRs). nAChRs play a central role in rapid cholinergic synaptic transmission and are important targets of insecticides (Matsuda *et al.,* 2005). Most neonicotinoids are partial agonists of native and recombinant nAChRs in both mammals and insects with differential selectivity conferred by only minor structural changes (Shimomura *et al.,* 2006; Ihara *et al.,* 2006). Recently imidacloprid has raised concerns because of its ability to cause egg shell thinning, reduced egg production, hatching time (Berny *et al.*, 1999) and honey bee colony collapse disorder, the decline of honey bee colonies in Europe and North America observed since 2006 (Carrington, 2012).

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin produced naturally by several plants in response to injury or when the plant is under attack by pathogens such as bacteria or fungi (Fremont, 2014). The major dietary sources of stilbenes include grapes, wine, soy, peanuts, and peanut products (Cassidy *et al.,* 2000).

The determination of maximum tolerated dose (MTD) is imperative for toxicity study of insecticides. The information is lacking on MTD of technical grade imidacloprid by oral route and ameliorating effect of resveratrol on body weight gain and relative organ weight parameters following its exposure in adult male Wistar rats. Therefore, the present study was undertaken to determine MTD of imidacloprid orally and assess the effect of subacute toxicity of imidacloprid on body weight gain and relative weight gains of liver, heart, spleen, kidney and testes and its amelioration by resveratrol in male rats following oral administration.

MATERIALS AND METHODS

Imidacloprid technical grade (>98% purity) was procured from Indofil Chemicals Company, Mumbai, India and resveratrol from Sigma-Aldrich Company. Adult male Wistar rats weighing between 120 to 140 g were procured from disease free small animal house, LUVAS, Hisar. The animals were acclimatized to laboratory conditions for 7 days before start of experiment. Animal house temperature varied between 22 to 27º C throughout the study. The prior approval of instituional animal ethics committee was obtained for the use of laboratory animals.

Determination of MTD

MTD of technical grade imidacloprid was determined in male rats by oral route following the method as described by Moser and Padilla (1997). The pilot dose range finding studies were conducted in small groups of rats (n=3) using several doses including few lethal doses. Single dose of imidacloprid was administered in a group of 3 animals and observations were made at various time intervals. Thereafter, several iterations were conducted to determine MTD. Out of these doses, a maximum dose was selected that produced clear signs of toxicity but not resulting in lethality i.e . maximum tolerated dose (MTD).

Effect on body weight gain and relative organ weight

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

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

Group 2: Resveratrol (2 mg/kg) in 3% gum acacia was administered once daily orally for 14 days to 6 rats and for 28 days to remaining 12 rats.

Group 3: Imidacloprid suspension (MTD/10) in 3% gum acacia was administered once daily orally for 14 days to 6 rats and for 28 days to remaining 12 rats.

Group 4: Imidacloprid suspension (MTD/20) in 3% gum acacia was administered once daily orally for 14 days to 6 rats and for 28 days remaining 12 rats.

Group 5: Imidacloprid suspension (MTD/10) and resveratrol (2 mg/kg) in 3% gum acacia was administered once daily orally for 14 days to 6 rats and for 28 days to remaining 12 rats.

Group 6: Imidacloprid **s**uspension (MTD/20) and resveratrol (2 mg/kg) in 3% gum acacia was administered once daily orally for 14 days to 6 rats and for 28 days to remaining 12 rats.

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

Statistical Analysis

Data were expressed as Mean±SEM. Statistical analysis of data was performed using Graph pad prism 6.05 and Microsoft Excel. Data were analyzed by ANOVA and means of various parameters were compared with Tukey's multiple comparison post-hoc test. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Following the pilot dose range finding experiment, MTD of technical grade imidacloprid in adult male Wistar rats was found to be 1850 mg/kg b.wt. by oral route (Table 1). Effect of various doses used for determination of MTD on gross observable behavior was noted, in which toxic symptoms started in 10-15 min after imidacloprid administration and were found to be dose dependent in onset and severity of effects. Head tremors started after 22-25 min (lasting for 5 sec to 30 sec) and peak tremors were noted after 38- 40 min of administration. Tremors were episodic in nature and as time passed on, whole body tremors and intermittent head convulsions were noted. Body posture was abnormal with hind limbs abducted from body. Dyspnea and difficulty in maintaining the complete prostrate position were observed just before death. Animal showed ataxia, rigidity and fasciculation of muscles, head drop and protrusion of eye ball. The rapid appearance (within 22 -25 min of administration) and disappearance (within 24 hours of administration) of toxic signs and symptoms correlate with earlier reports of prompt absorption (92–95% within 1 hour) and excretion of more than 90% within 24 hours of administration of imidacloprid (Klein and Karl, 1990; Broznic *et al.,* 2008). The absence of toxic sign and symptoms after 24 hours indicated no delayed toxic effects of imidacloprid.

Following administration of imidacloprid and resveratrol, body weights of rats were recorded on alternate day till completion of experiment and are presented in Table 2. Imidacloprid treatment alone at both the dose levels significantly reduced ($p<0.05$) the body weight gain in dose dependent manner as compared to naïve group animals in 28 and 42 days trial. Resveratrol co-treatment in imidacloprid administered animals significantly restored ($p<0.05$) body weight gain in IMD/10 + RT group as compared to IMD/10 group in 28 days exposure. Resveratrol treatment alone did not produce any alteration in body weights and relative body weight gain in 14 days exposure as compared to control animals but a statistically significant decrease ($p<0.05$) was observed in weight gain following 28 and 42 days exposure.

The weight gain in animals serves as index of growth rate (Palani *et al.,* 1999). A significant reduction in body weight gain (g) was observed in present study in groups treated with resveratrol and imidacloprid alone in 28 and 42 days exposure. Resveratrol co-treatment in imidacloprid treated group significantly increased the weight gain in 28 days trial. This may be because of the hepato-protective effect of resveratrol or by reduction in protein catabolism caused by imidacloprid.

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

In present study, reduced body weight gain in resveratrol administered groups may be because of anti-obesity effects of resveratrol on adipocytes in body storage tissues. Resveratrol also decreases adipogenesis and viability in maturing preadipocytes, mediated through down-regulating adipocyte specific transcription factors and enzymes and also by genes that modulate mitochondrial

Table 1:
Maximum tolerated dose (MTD) of imidacloprid administered orally in male Wistar rats

Dose (mg/kg)	Number of rats died/ number of rats administered	Percent mortality
2000	3/3	100
1950	2/3	66.6
1925	1/3	33.3
1900	1/3	33.3
1850	0/3	0
1800	0/3	0

MTD of imidacloprid: 1850 mg/kg b.wt. Orally

Table 2:
Effect of subacute toxicity of imidacloprid on body weight gain (g) and its amelioration by resveratrol in male rats

Treatment	14 days	28 days	42 days
Naïve	12.83±0.703	27.66±1.08	43.16±1.85
RT	12.66±0.802	21.33±1.08**	34.5±2.14**
IMD/10	10.66±0.881	14.66±0.714***	26.58±1.58***
IMD/20	11.00±0.687	18.5±0.846***	29.66±1.14***
IMD/10 + RT	11.83±1.10	20.00±1.07†††	32.66±1.20
IMD/20 + RT	12.00±1.00	22.50±1.05	35.16±1.51

n=6, Values are Mean±S.E.M., **p<0.01, ***p<0.001 in comparison to naïve; †††p<0.001 in comparison to IMD/10; in Tukey's multiple comparison post hoc test.

Table 3:
Effect of imidacloprid on relative organ weights (g/100 g b.wt.) and its amelioration by resveratrol in male rats in 14 days exposure

Treatment	Organs				
	Liver	Heart	Spleen	Kidneys	Testes
Naïve	3.21±0.11	0.35±0.007	0.23±0.01	0.59±0.01	1.066±0.07
RT	3.01±0.13	0.4±0.02	0.21±0.01	0.65±0.03	1.083±0.07
IMD/10	3.82±0.28*	0.28±0.01	0.23±0.02	1.02±0.11***	0.88±0.03
IMD/20	3.32±0.19	0.38±0.02	0.23±0.009	0.79±0.05	1.15±0.12
IMD/10 + RT	3.1±0.1††	0.33±0.02	0.25±0.01	0.95±0.06	1.26±0.06†
IMD/20 + RT	3.05±0.09	0.41±0.02	0.33±0.02###	0.62±0.08	1.31±0.11

n=6, Values are Mean±S.E.M., * p<0.05, ***p<0.001 in comparison to naïve; †p<0.05, ††p<0.01 in comparison to IMD/10; ###p<0.001 in comparison to IMD/20 in Tukey's multiple comparison post hoc test.

Table 4:
Effect of imidacloprid on relative organ weights (g/ 100 g b.wt.) and amelioration by resveratrol in male rats in 28 days exposure

Treatment	Organs				
	Liver	Heart	Spleen	Kidneys	Testes
Naïve	3.07±0.15	0.28±0.01	0.4±0.02	0.55±0.01	1.47±0.04
RT	2.97±0.19	0.26±0.01	0.38±0.01	0.63±0.03	1.3±0.02
IMD/10	4.01±0.14*	0.34±0.02	0.23±0.01***	1.22±0.14***	0.97±0.06***
IMD/20	3.3±0.08	0.33±0.03	0.26±0.01***	0.86±0.03*	1.11±0.08**
IMD/10 + RT	2.90±0.33††	0.28±0.04	0.3±0.03	0.93±0.23†	1.32±0.17††
IMD/20 + RT	3.09±0.23	0.36±0.03	0.35±0.03#	0.61±0.03	1.42±0.2##

n=6, Values are Mean±S.E.M.
*p<0.05, **p<0.01, ***p<0.001 in comparison to naïve; †p<0.05, †† p<0.01, in comparison to IMD/10; #p<0.05, ##p<0.01in comparison to IMD/20 in Tukey's multiple comparison post hoc test.

Table 5:
Effect of imidacloprid on relative organ weights (g/ 100 g b.wt.) and amelioration by resveratrol in male rats in 42 days exposure

Treatment	Organs				
	Liver	Heart	Spleen	Kidneys	Testes
Naïve	3.21±0.11	0.35±0.01	0.41±0.02	0.7±0.02	1.48±0.08
RT	3.01±0.13	0.31±0.008	0.33±0.02	0.72±0.01	1.4±0.107
IMD/10	3.62±0.15	0.39±.007	0.32±0.01	0.97±0.04***	1.29±0.12
IMD/20	3.32±0.19	0.3±0.007	0.31±0.01	0.88±0.01***	1.33±0.08
IMD/10 + RT	3.1±0.05	0.26±0.01	0.31±0.04	0.76±0.02†††	1.35±0.03
IMD/20 + RT	3.01±0.09	0.27±0.01	0.35±0.03	0.76±0.04#	1.41±0.08

n=6, Values are Mean±S.E.M., ***p<0.001 in comparison to naïve; †††p<0.001 in comparison to IMD/10; #p<0.05 in comparison to IMD/20 in Tukey's multiple comparison post hoc test.

function. Resveratrol also increased lipolysis and reduced lipogenesis in mature adipocytes (Baile *et al.,* 2011).

The relative organ weight of vital organs of male rats in all treatment groups is expressed as g/100 g b. wt. and presented in Tables 3, 4 and 5 respectively. A statistically significant increase ($p<0.05$) was observed in liver weight in IMD/10 group as compared to naïve group in 14 and 28 days exposure but not in 42 days exposure. Resveratrol treatment significantly decreased ($p<0.05$) the liver weight in IMD/10 + RT group as compared to IMD/10 group in 14 and 28 days trial. No significant changes were observed in weight of heart in all groups. A statistically significant increase ($p<0.05$) was observed in spleen weight in 14 days study in IMD/20 + RT group as compared to IMD/20 group. Imidacloprid treatment alone at both the dose levels significantly reduced ($p<0.05$) the spleen weight in dose dependent manner as compared to naïve group animals in 28 days trial. Resveratrol co-treatment significantly ($p<0.05$) restored the spleen weight in IMD/20 + RT group as compared to IMD/20 group in 28 days study. No significant changes were observed in spleen weight in 42 days exposure. A statistically significant change in kidney weight ($p<0.05$) was observed in IMD/10 group as compared to naïve group in 14, 28 and 42 days trial and likewise in IMD/20 in 28 and 42 days exposure. Resveratrol co-treatment significantly reduced ($p<0.05$) the kidney weight in IMD/10 + RT group as compared to IMD/10 group in 28 and 42 days exposure and in IMD/20 + RT group as compared to IMD/20 group in 42 days exposure. Imidacloprid treatment alone at both the dose levels significantly reduced ($p<0.05$) the testes weight in dose dependent manner as compared to naïve group animals in 28 days trial. Resveratrol co-treatment significantly increased ($p<0.05$) the weight in IMD/10 + RT group as compared to IMD/10 group in 14 and 28 days study and in IMD/20 + RT as compared to IMD/20 in 28 days study.

In toxicological studies, relative organ weights are important criteria for evaluation of organ toxicity (Timbrell, 2000; Crissman *et al.,* 2004). Generally in sub-acute toxicity studies relative weight of affected organ increases.

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

In present study, no significant changes were observed in weight of heart. It indicates imidacloprid and resveratrol have no significant effect on heart.

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

Imidacloprid treatment significantly increased the relative kidney weight in comparison to naïve. Our findings are in agreement with the study of Srivastava *et al.* (2006) where a significant increase was observed in the weight of kidney after exposure to liquid mosquito repellent containing allethrin (3.6% w/w). In male rats, long term feeding studies with cypermethrin have shown an increase in kidney weights (Elbeitha *et al.,* 2001). Cypermethrin resulted in alteration in the distribution pattern of oxidoreductase in the kidney of rats. The general loss of oxidoreductases in kidney of rabbits because of cypermethrin toxicity may be suggestive of decreased metabolism of physiological processes due to degenerative changes in various segments of nephron (Bansal *et al.,* 2007). Resveratrol co-treatment decreased the relative kidney weight in the present study. Our results are in accordance with Palsamy *et al.* (2011) where resveratrol treatment revealed improvement in kidney weight showing its renoprotective nature. This is possibly through the anti-oxidant potential of resveratrol.

In the present study, a statistically significant decrease in testes weight was observed. The reduction in the weight of testes may be due to the decreased number of germinal cells and elongated spermatids in the testes.The decrease in the testicular weight in imidacloprid treated rats may be due to reduced tubule size, spermatogenic arrest and inhibition of steroid biosynthesis of leydig cells, a site of steroid biosynthesis. In our study, resveratrol co-treatment restored the testicular weight in imidacloprid treated rats. Similar results were obtained in the study of Sharma *et al.* (2014), in which resveratrol treatment in cypermethrin toxicity restored the decreased testicular weight.

REFERENCES

Baile. CA., Yang. JY., Rayalam. S., Hartzell. DL., Lai. CY., Andersen. C. and Della-Fera MA. (2011).

Effect of resveratrol on fat mobilization. *Ann N Y Acad Sci.* **1215:**40-7.

Bansal, N., Uppal, V., Roy, K. S. and Singh, O. (2007). Histoenzymatic observations on the kidneys of rabbits due to cypermethrin toxicity. *Indian J. Anim. Sci.* **77:**148-150.

Berny P.J., Florence, Buronfoss, F., Videmann, B. and Buronfosset, T., (1999). Evaluation of the toxicity of imidacloprid in wild birds. A new high performance thin layer chromatography method for the analysis of liver and crop samples in suspected poisoning cases. *Liq. Chrom. Rel. Technol*. **22:** 1547-1559.

Bhardwaj S., Srivastava, M.K., Kapoor, U. and Srivastava, L.P. (2010). A 90 days oral toxicity of imidacloprid in female rats: Morphological, biochemical and histopathological evaluations. *Food and Chemical Toxicology.* **48:** 1185-1190.

Broznic, D., Marinic, J., Tota, M., Juresic, G.C. and Milin, C. Kinetic evaluation of imidacloprid degradation in mice organs treated with olive oil polyphenol extract. *Croat. and Chem. Acta.* **81:** 203-209.

Carrington, Damian (2012). "Pesticides linked to honeybee decline". The Guardian.

Cassidy, A.; Hanley, B. and Lamuela-Raventó´s, R. M. (2000). Isoflavone, lignans and stilbenes-origins, metabolism and potential importance to human health. *Sci. Food Agric.* **80:** 1044- 1062.

Crissman, J. W., Goodman, D. G., Hildebrandt, P. K., Maronpot, R.R., Prater D. A. and Riley, J.H. (2004). Best practice guideline. *Toxicol. Pathol.* **32:** 126-131.

Elbeitha, A., Daas, S. I., Khamas, W. and Darmani, H. (2001). Evaluation of toxic potential of cypermethrin pesticide on some reproductive and fertility parameters in the male rats. *Arch. Environ. Contam. Toxicol.* **41:** 522-528.

Fremont, Lucie. (2014). Biological Effects of Resveratrol. *Life Sciences* **66:** 663–673.

Gatne, M.M., Ramesh, Bhoir, P.S., Deore, M.D. (2006). Immunotoxicity studies of imidacloprid in rats. *Toxicol. International*. **13:** 89-92.

Gómez-Zorita. S., Fernández-Quintela, A., Macarulla. MT., Aguirre, L., Hijona E., Bujanda. L., Milagro. F., Martínez. JA., Portillo MP. (2012). Resveratrol attenuates steatosis in obese Zucker rats by decreasing fatty acid availability and reducing oxidative stress. *Br J Nutr*. **107(2):**202–210

Israa S. Al-Dabbagh and Layla J. M. Al-Bahadyli. (2015). Study the behavioral changes and gravimetric changes for weight organs in liver, kidney and spleen exposure to insecticide imidacloprid in the white mice. *World Journal of Pharmaceutical Research*. **4:**114-122.

Karabulut AB, Kirimlioglu V, Kirimlioglu H, Yilmaz S, Isik B, Isikgil O.(2006). Protective effects of resveratrol on spleen and ileum in rats subjected to ischemia-reperfusion. *J Transplant Proc*. **38(2):**375-7.

Klein, O. and Karl, W. (1990) Methylene [^{14}C] imidacloprid: Metabolism part of the general metabolism study in rat, Unpublished report from Bayer A.G., report of no. PF 3316, GLP. Submitted to WHO by Bayer AG, under Mannheim, Germany.

Ihara. M., Brown. L.A., Ishida. C., Okuda. H., Sattelle. D.B., Matsuda. K. (2006). Actions of imidacloprid, clothianidin and related neonicotinoids on nicotinic acetylcholine receptors of American cockroach neurons and their relationships with insecticidal potency. *Pestic. Sci.* **31 (1):** 35–40.

Shimomura. M., Yokota. M., Ihara. M., Akamatsu. M., Sattelle. D.B., Matsuda. K.(2006). Role in the selectivity of neonicotinoids of insect-specific basic residues in loop D of the nicotinic acetylcholine receptor agonist binding site. *Mol. Pharmacol*. **70:** 1255–1263.

Matsuda, K.; Shimomura, M.; Ihara, M.; Akamatsu, M.; Sattelle, D.B. (2005). Neonicotinoids show selective and diverse actions on their nicotinic receptor targets: electrophysiology, molecular biology, and receptor modeling studies. *Biosci. Biotechnol. Biochem.* **69:** 1442–1452.

Moser, V.C. and Padilla, S. (1997). Age and gender related differences in the time course of behavioural and biochemical effects produced by oral chloripyrifos in rats. *Toxicol. Appl. Pharmacol.* **49:** 161-170

Palani, V., Senthikumaran, R.K., Govindawamy, S. (1999). Biochemical evaluation of antitumor effect of Muthu marunthu (herbal formulation) on experimental fibrosarcoma in rats. *Ethnopharmacol*. **65:** 257-265.

Palsamy P. and S. Subramanian, (2011). Resveratrol protects diabetic kidney by attenuating hyperglycemia mediated oxidative stress and renal inflammatory cytokines via Nrf2-Keap1 signaling. *Biochim. Biophys. Acta*., **1812:**719-731.

Sharma, P., Ul Huq. A., and Singh. R. (2014). Cypermethrin-induced reproductive toxicity in the rat is prevented by resveratrol. *Hum Reprod Sci.* **7(2):** 99–106.

Srivastava, A., Srivastava, M.K, and Raizada R.B. 2006. A ninety day toxicity and one generation reproduction study in rats exposed to allethrin based LMR. *J. Toxicol. Sci.* **31:**1-7

Timbrell, J. A., 2000. Biomarkers of organ toxicity. *Arch. Ind. Hyg. Toxicology.* **33:** 295-302 (Alves, 1990; Houk, 1992).

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Research Article

EVALUATION OF HEPTAOPROTECTIVE EFFICACY OF *SYZYGIUM CUMINI* IN CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY IN CHICKS

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ABSTRACT

The present study was designed to evaluate the heptaoprotective efficacy of hydroethanolic extract (1:1) of *Syzygium cumini* leaves (HESC) in carbon tetrachloride (CTC) induced hepatotoxicity in chicks. Thirty male Rhode Island Red (RIR) chicks were equally and randomly divided into five groups. Group I served as healthy control., group II as negative control, group III as positive control, group IV and V were kept as treatment groups. A sample dose as carbon tetrachloride was administered @ 1 ml/kg b. wt., i.p. to groups II, III, IV and V. Group III was administered silymarin @ 100 mg/kg body weight, p.o. once daily and HESC @ 300 and 600 mg/kg body weight p.o. orally daily for 21 days in groups IV and V, respectively. Liver damage was manifested by a significant ($P<0.05$) increase in serum enzyme activities of AST, ALT and ALP in the negative control group II as compared to the healthy control. After CCl_4 administration, AST, ALT and ALP levels were also increased in groups III, IV and V as compared to healthy control but significantly ($P<0.05$) decreased by the administration of the HESC at a dose level of 600 mg/kg body weight compared to group II. ALT and ALP also significantly ($P<0.05$) decreased in HESC treatment groups. Microscopic examination of liver showed degeneration and necrosis of hepatocytes in group II confirming liver damage. Group IV showed moderate degeneration while group III and V showed high regeneration of hepatocytes suggesting HESC potential of reversing CCl_4 induced the hepatotoxicity. It is concluded from this study that single i.p. dose of CTC @ 1 ml/kg, i.p produced hepatotoxicity which was ameliorated by HESC given @ 600 mg/kg, for 21 days in chicks.

Key words: Carbontetra chloride, chicks, heptaoprotective, *Syzygium cumini,* transferases enzymes.

INTRODUCTION

Liver, the main organ of biliary system, one of the vital organs involved in various functions including metabolism and homeostasis. Excessive exposure of liver to xenobiotics most often results in liver damage that may lead to depletion of glutathione and subsequently result in degeneration, necrosis and acidosis.

The phytochemical analysis of the seeds of *Synzigium cumini* has revealed the presence of alkaloids, flavonoids, glycosides, phytosterols, saponins, tannins and triterpenoids (Kumar *et al.,* 2009). Review of literature indicated that very few studies on the hepatoprotective potential of this plant on experimentally induced hepatic damage, have so far been undertaken. In view of the importance of herbal therapy in hepatic disorders, present study was undertaken to study the protective efficacy of the hydroethanolic extract of the *Syzygium cumini* leaves against carbon tetrachloride-induced hepatotoxicity in chicks.

MATERIALS AND METHODS

Chemicals and drugs

All the chemicals were procured from Hi Media. Autospan diagnostics kits were used for biochemical analysis of serum transferases and alkaline phosphatase.

Collection of plant material and preparation of HESC

The *Syzygium cumini* leaves were collected from Medicinal Plant Research and Developmental Centre (MRDC), G.B.P.U.A & T, Pantnagar. Leaves were chopped and dried in a dryer under hot circulating air at 40ºC for 72 to 96 hours and powdered, grinding in a grinding mill having stainless steel blades. Ground powder was filled in air tight containers (plastic/glass) and used for preparation of hydroethanolic extract water. Ethanol was added in equal proportions and dry powder mixed gently after closing the mouth with parafilm. This solution was stirred with the help of magnetic stirrer for 1 hour and kept in incubator shaker at 37ºC with gentle swirling at 120 rpm per minute for overnight. The solution was then filtered with muslin cloth and doubly filtered with whatman filter paper No. 1. The filtrate was then left in incubator for 2 week for drying to cofeet HESC.

Experimental animals

Thirty Rhode Island Red male chicks of 4-5 weeks age, weighing between 300-400 gm were procured from Instructional Poultry Farm, Nagla and housed in the poultry house of the Poultry farm under standard managemental conditions. Standard poultry feed and water was provided *ad libitum* throughout the experimental period. Chicks were kept under constant observation during entire period of study.

Experimental design

Thirty chicks were divided randomly and equally into five groups. All the chicks were fasted overnight, then

body weight was recorded. Carbon tetrachloride was injected i.p. to the chicks of all groups except control group as a single dose @ 1 ml/ kg bwt only on 1st day of the experiment. Group I was kept as control. Group II was kept as negative control and given carbon tetrachloride alone. Group III was kept as positive control given CTC with silymarin (100 mg/ml) @ 100 mg/kg bwt p.o. Group IV and group V were kept as treatment groups treated with HESC @300 and 600 mg/kg b wt, respectively. After three weeks of treatment chicks were sacrificed humanely. Liver samples were collected for histopathological exchanged and blood was collected in heparinized tube for different parameters and without heparin for serum collection for the analysis of enzymes.

Biochemical examination

Blood samples were collected from the wing vien and transferred to heparinized tubes for whole blood collection, and in non-heparinized tubes for serum separation. Serum was used for the estimation of serum enzymes viz. aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) by diagnostic kits.

Histopathological examination

The tissue pieces from liver were collected in 10% formal saline solution. Formalin fixed tissues were dehydrated in increasing strength of alcohol, cleared in xylene and embedded in paraffin blocks. Paraffin embedded sections were cut at 4-5 micrometer thickness and stained with hematoxylin and eosin.

Statistical analysis

Statistical analysis of data was done by using SPSS software, one way ANOVA technique. Comparisons were made with help of Tukey's multiple comparison test at 5 percent level by the method of Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

Hydroethanolic extract of *Syzygium cumini* leaves was prepared in the ratio of 1:1. Percent yield of extract obtained was 5.5%. A significant ($P<0.05$) increase in AST activity in group II as compared to group I indicated liver damage after CTC administration as shown in Table 1. A significant ($P<0.05$) decline was observed in group III and group V as compared to group II revealing that silymarin and HESC reversed liver damage caused by CTC administration. There was no decline in group IV which indicated that low dose level (300 mg/kg bwt.) of HESC wasn't effective in ameliorating AST activity compared to high dose group V (600 mg/kg bwt.).

The AST, a cytoplasmic enzyme, catalyzes the reductive transfer of an amino group from aspartate to α-ketoglutarate to yield oxaloacetate and glutamate. Besides liver, it is also found in other organs like heart, muscle, brain and kidney. Injury to any of these tissues can cause an elevated blood level (Nathwani *et al.,* 2005). It can also signify abnormalities in heart, muscle, brain or kidney (Dufour *et al.,* 2001). Restoration of activity of serum AST occurs with the healing of hepatic parenchyma and regeneration of hepatocytes (Jia *et al.,* 2014). Thus, its decrease caused by hyderoethanolic extract of *Syzygium cumini* in the present study suggests its protective effect against liver damage caused by CTC administration as also suggested by Sharma *et al.* (2013).

The ALT activity in group II was significantly ($P<0.05$) increased as compared to control indicating liver damage after CTC administration. There was a significant ($P<0.05$) decline of ALT levels in groups III, IV and V as compared to negative control group. No significant change was observed in ALT activity in groups III, IV and V in comparison to control and groups IV and V in comparison to group III which indicates that both dose levels of HESC produce a hepatoprotective effect like silymarin and restores the liver to its normal healthy state. Decline in ALT activity in treatment groups suggests *Syzygium cumini* extract (HESC) mediated recovery in CTC induced liver damage at both dose levels along with silymarin

Estimation of ALT activity is the most frequently relied biomarker of hepatotoxicity. It is predominantly found in hepatocytes and other tissues and plays an important role in amino acid metabolism and gluconeogenesis. It catalyzes the reductive transfer of an amino group from alanine to α-ketoglutarate to yield glutamate and pyruvate. Elevation in level of this enzyme in blood occurs due to leakage after liver damage. The estimation of this enzyme is a more specific test for detecting liver abnormalities since it is primarily found in the liver (Dufour *et al.,* 2001; Amacher 2002). In the present study we found out that *Syzygium cumini* extract decreased ALT activity suggesting hepatoprotective effect HESC in CTC induced hepatotoxicity in chicks. Our findings are in agreement with the findings of the study in which Jamun fruit extract treatment (100 mg/kg, po) for 10 days reversed elevated serum ALT levels and protected against hepatocellular injury in mice (Donepudi *et al.*, 2012).

Liver damage in this study was also indicated by a significant ($P<0.05$) increase in alkaline phosphatase (ALP) levels in CTC treated group II as compared to control. A significant ($P<0.05$) diminution in ALP level in group III and group V reveals that silymarin and HESC reversed the liver damage caused by CTC in group II. A decline in ALP values in group IV revealed lower effect of HESC with regard to its hepatoprotective efficacy than higher dose group.

Alkaline phosphatase is a hydrolase enzyme that is secreted into the bile. It hydrolyzes monophosphates at an alkaline pH. It is particularly present in the cells which line the biliary ducts of the liver. It is also found in other organs including bone, placenta, kidney and

Table 1:
Effect of daily oral administration of hydroethanolic extract (1:1) of *Syzygium cumini* leaves (HESC) for 21 days on serum enzyme parameters (Mean ± SE, n=6) following carbon tetrachloride (CTC) induced hepatotoxicity in chicks.

Groups	Treatments	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
I	Control	131.67±10.24	18.36±1.12	251.81±13.92
II	CTC (1 ml/kg bwt.) only	281.39±18.9 [a]	53.14±7.27[a]	442.71±10.18 [a]
III	CTC + Silymarin(100 mg/kg bwt)	165.75±21.68[b]	26.77±3.96 [b]	304.26±24.64 [b]
IV	CTC + HESC(300 mg/kg bwt.)	223.81±12.32[a]	34.03±4.25[b]	360.20±20.62[ab]
V	CTC + HESC(600 mg/kg bwt.)	195.19±19.08 [b]	30.19±2.33[b]	310.55±21.62[b]

a= Significant difference (P<0.05) as compared to group I within same column.;b= Significant difference (P<0.05) as compared to group II within same column; HESC: Hydroethanolic extract of *Syzygium cumini* leaves.

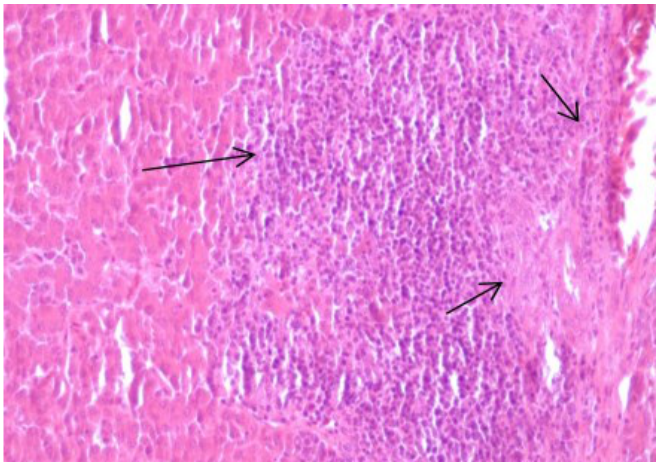

Plate 1: Photomicrograph of liver showing significant degeneration,necrosis and lymphoid aggregation in group II (H&E, 20X).

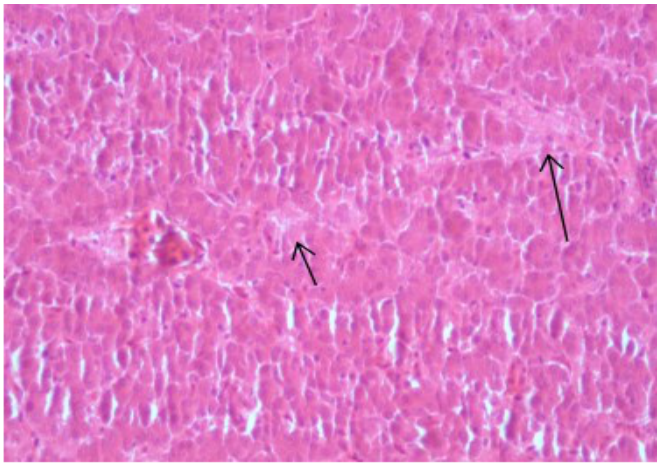

Plate2: Photomicrograph of liver showing liver mild degeneration of hepatocytes in group III (H&E, 20X).

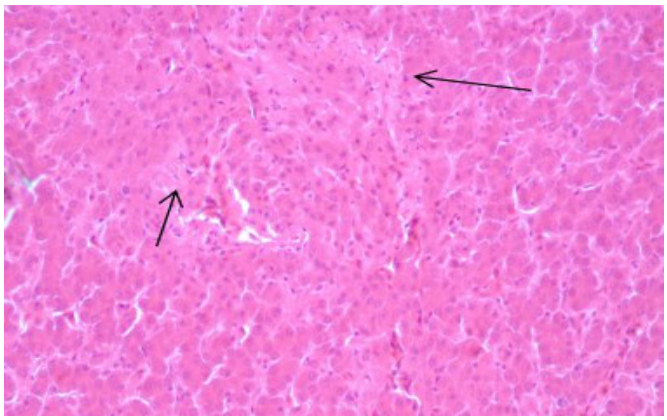

Plate 3: Photomicrograph of liver showing moderate degeneration of hepatocytes and lymphoid aggregation in group IV (H&E, 20X).

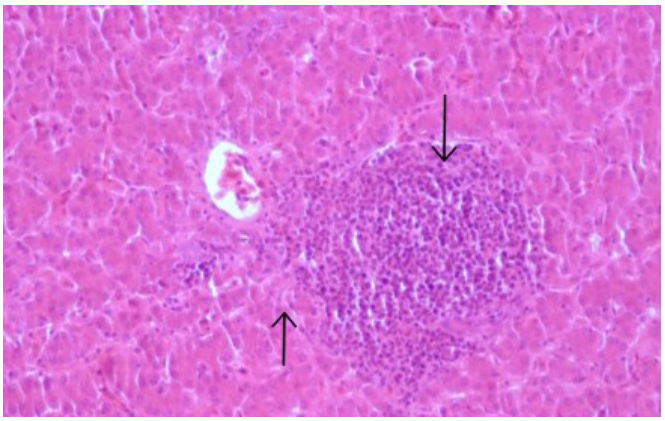

Plate 4: Photomicrograph of liver showing mild significant egeneration of hepatocytes in group V (H&E, 20X).

intestine. The serum ALP increased during primary biliary cirrhosis, a condition of disorganized hepatic architecture (cirrhosis) and in diseases characterized by inflammation, regeneration, and obstruction of intrahepatic bile ductules (Witthawaskul *et al.,* 2003). High levels of ALP also exist in cells that are rapidly dividing or are otherwise metabolically active. Decrease in ALP level caused by HESC in this study reveals its hepatoprotective action against CTC induced hepatotoxicity. Our study is in agreement with the study of Behera *et al.* (2014) who also reported a significant decrease in liver enzymes ALT, AST and ALP after the treatment with agneous *S. Cumini* 500 mg/kg p/o indicating hepatoprotective effect in streptozotocin (STZ)–induced diabetic rats.

Augmentation in enzyme activities of serum AST, ALT and ALP are considered as indicators of hepatic damage. The rise in activity of serum AST, ALT and ALP enzymes in our study might have occurred due to the altered structural integrity of the liver following administration of CTC which lead to formation of various free radicals that are responsible for damage to hepatic cellular mass and organelles. Being cytoplasmic in

location, these enzymes are released into systemic blood circulation after cellular damage of hepatocytes (Ahmed and Khater, 2001). In a study carried out by Sisodia and Bhatnagar (2009) to evaluate the hepatoprotective effects of the methanolic seed extract of *Eugenia jambolana* Lam. (*Syzygium cumini*), in rats *Eugenia jambolana* seed extract significantly decreased the level of serum marker enzymes AST, ALT and ALP than CTC treated groups which indicated the hepatoprotective effect of the extract. The flavonoids present in *Syzygium cumini* may be attributed for removing free radicals that help in restoring structural integrity of liver tissue and subsequently decrease the levels of liver enzymes showing a hepatoprotective effect in this study.

Hepatoprotection was also evident by histopathological examination of liver which revealed less centriolobular necrosis and hepatocytes showed regeneration activity as compared to carbon tetrachloride treated animals with large ares of centrilobular necrosis.

In group II, histopathological examination revealed degeneration with lymphoid aggregation and necrosis in some hepatic cells (Plate 1) which confirmed liver damage as was suggested by an increase in AST, ALT and ALP in the serum enzyme assay. In group III mild degeneration of hepatocytes was observed with no other noticeable changes (Plate 2). Group IV showed lymphoid aggregation with moderate degeneration of hepatocytes (Plate 3). HESC treatment group V showed only mild to moderate degeneration, observed in hepatocytes showing massive regenerated areas of hepatocytes as compared to CTC induced hepatotoxic negative control (Plate 4). Thus, regeneration of hepatocytes in dose dependent manner suggested ameliorating effect of HESC in CTC intoxicated chicks. It is concluded from this investigation that single dose of carbon terachloride @ 1ml/kg body weight produced hepatotoxicity and hydroethanolic extract of *Syzygium cumini* leaves @ 300 mg/kg and 600 mg/kg daily for 21 days produced hepatoprotective effect in dose dependent manner in carbon tetrachloride induced hepatotoxicity in chicks.

REFERENCES

Ahmed, M.B. and Khater, M.R. (2001). Evaluation of the protective potential of *Ambrosia maritima* extract on acetaminophen induced liver damage. *J. Ethnopharmacol.* **75**: 169-174.

Amacher, D.E. (2002). A toxicologist's guide to biomarkers of hepatic response. *Hum. Exp. Toxicol.* **21:** 253-262.

Behera, S. R., Sekkizhar, M. and Babu, K. (2014). Hepatoprotactive activity of Aqueous Extract of *Syzygium cumini* Seed on Streptozotocin Induced Diabetes in Rats. *International Journal of Ayurvedic and Herbal Medicine.* **4(2):**1470-1477.

Donepudi, A. C., Aleksunes, L.M., Driscoll, M. V., Seeram, N. P.and Slitt, A. L. (2012). The traditional ayurvedic medicine, *Eugenia jambolana* (Jamun fruit), decreases liver inflammation, injury and fibrosis during cholestasis. *Liver Int.* **32**(4): 560-73.

Dufour, D.R., Lott, J.A., Nolte, F.S., Gretch, D.R. and Koff, R.S. (2001). Diagnosis and monitoring of hepatic injury. II. Recommendations for use of laboratory tests in screening, diagnosis and monitoring. *Clin. Chem.* **47:** 1133-1135.

Jain, A., Soni, M., Deb, L., Jain, A, Rout, S.P., Gupta, V.B. and Krishna. K.L. (2008). Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Momordica dioica* Roxb. Leaves. *J. Ethnopharmacol*. **115:** 61-66.

Kumar, P., Prasad, R., Singh, K. K. and Roy, B. K. (2009). Hepatoprotective effect of *Centella asiatica* against paracetamol induced liver damage in broiler chicken. *Indian J. Poult. Sc.* **44(1):**101-104.

Nathwani, R.A., Pais, S., Reynolds, T.B. and Kaplowitz, N. (2005). Serum alanine aminotransferase in skeletal muscle diseases. *Hepatology.* **41:** 380-382.

Sharma, B., Siddiqui, M., Kumar, S. S., Ram, G. and Chaudhary, M. (2013). Liver protective effects of aqueous extract of *Syzygium cumini* in Swiss albino mice on alloxan induced diabetes mellitus. *Journal of Pharmacy Res*. **6:** 853-858.

Sisodia, S.S. and Bhatnagar, M (2009). Hepatoprotective activity of *Eugenia jambolana* Lam. in carbon tetrachloride treated rats. *Indian J. Pharmacol*. **41(1):** 23-27.

Snedecor and Cochran (1967)

Witthawaskul, P., Panthong, A., Kanjanapothi, D., Taesothikul, T., Lertprasertsuke, N. (2003). Acute and subacute toxicities of the saponin mixture isolated from *Schefflera leucantha* Viguier. *J. Ethnopharmacol.* **89(1):** 115-121.

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Research Article

EFFECT OF KETOPROFEN CO-ADMINISTRATION AND FEBRILE STATE ON PHARMACOKINETIC OF LEVOFLOXACIN IN GOATS

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ABSTRACT

The pharmacokinetic of levofloxacin (4 mg/kg) was studied following subcutaneous administration in ketoprofen (3 mg/kg) treated and febrile goats. The concentration of levofloxacin in plasma was detected by using high performance liquid chromatography (HPLC). No significant changes were reported in pharmacokinetic parameters following co-administration of levofloxacin and ketoprofen. While under febrile state, significant increase in elimination half life and decrease in volume of distribution and total body clearance were observed. Integrating the pooled pharmacokinetic data generated from the present study, levofloxacin via subcutaneous administration (4 mg/kg) repeated at 12 h interval is sufficient to maintain MIC in plasma above 0.05 μg/mL for most of the gram-positive and gram-negative microorganisms.

Key words: Pharmacokinetic, Levofloxacin, Ketoprofen, Fever, Goat

INTRODUCTION

Levofloxacin is a third-generation fluoroquinolone with a wide spectrum of bactericidal activity. The drug is active against gram-negative, gram-positive and anaerobic bacteria including *Pseudomonas species.* It has enhanced activity against *Streptococcus pneumoniae, Staphylococcus aureus and Enterococcus species*, besides having good activity against *Mycoplasma* and *Chlamydia* (Martinez *et al*., 2006). Ketoprofen is routinely used as non-steroidal anti-inflammatory, analgesic and antipyretic agent in veterinary practice. Pharmacokinetics of levofloxacin has been investigated earlier in animals (Goudah, 2009; Goudah and Hasabelnaby, 2010; Patel *et al*., 2012 & 2013). As there is no information available on the influence of co-administration of ketoprofen and febrile state on the pharmacokinetics of levofloxacin in goats, this study was undertaken to determine effect of ketoprofen and febrile condition on pharmacokinetics of levofloxacin in goat.

MATERIALS AND METHODS

Experimental Animals

The experiment was conducted on six healthy adult (2-3 years of age) Surti goat, weighing 28-32 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 the possibility of any diseases. The experimental protocol was approved by IAEC.

Drug and Chemical

Levofloxacin infusion (500 mg/100 mL; Tavanic®, Aventis Pharmaceutical Ltd, Bangalore) was procured from local pharmacy. Levofloxacin technical grade powder was procured from Moxi Laboratory Pvt. Ltd., Gujarat, India. Acetonitrile, Triethylamine, Perchloric acid (70%) and Ortho-phosphoric acid (min. 58%) (Analytical grade) were purchased from Merck Limited, Mumbai.

Drug Administration and sample collection

All six animals were randomly allocated to receive injection of levofloxacin at the dose rate of 4 mg/kg. A washout period of 2 weeks was observed between treatments. The s.c. injection (4 mg/kg) was administered in the neck region, while ketoprofen was administrated via. deep i.m. (3 mg/kg) in gluteal muscle. Blood samples (3 mL) were collected, before administration and at 5, 10, 15, 30 min and 1, 2, 4, 8, 12, 18, 24 and 36 h after concurrent subcutaneous and i.m. administration of levofloxacin and ketoprofen, respectively. Febrile state in goat was induced by injecting lipopolysaccharide (LPS) of *Escherichia coli* (O55:B5) at the dose rate of 0.2 µg/kg b wt i.v. (Verma and Roy, 2006). LPS was repeated at dose rate of 0.1 and 0.05 µg/kg at 12 h and 24 h, respectively, to maintain the febrile state up to 36 h. Blood samples (3 mL) were collected before administration and at 5, 10, 15, 30 min and 1, 2, 4, 8, 12, 18, 24 and 36 h after s.c. administration of levofloxacin in febrile goats. Goats were monitored for any adverse reactions during the entire study period. Blood samples were subjected to centrifugation at 3000g for 10min and plasma samples collected and preserved -20 °C and analyzed within 48 h for determination of levofloxacin concentration.

Analytical assay of levofloxacin and pharmacokinetic analysis

Levofloxacin concentration in plasma samples

was determined by reverse-phase (HPLC) after extraction, using a reported assay (Varia *et al*., 2009) with minor modifications. The HPLC apparatus (Laballiance, USA) comprised of quaternary gradient delivery pump (model AIS 2000), UV detector (model 500) and C18 column (Thermo ODS: 250 x 4.6 mm ID) were used. Pharmacokinetic data integration was done by software "Clarity" (Version 2.4.0.190). Pharmacokinetic data of levofloxacin following subcutaneous injection in ketoprofen treated and in febrile goats were compared to subcutaneous injection of levofloxacin alone in goats (Patel *et al*., 2013)

Solution of pure enrofloxacin powder (40 µl of 0.5 mg/mL concentration) was utilized as an internal standard (IS). After adding internal standard in each plasma samples (500 µL), it was deproteinized by addition of perchloric acid (50 µl) and vortexed for one minute. This was followed by centrifugation at 3000 g for 10 minutes. An aliquot of supernatant was collected in clean vial and 20 µL was injected into loop of HPLC system using 25 µL glass syringe (Hamilton Bonaduz AG, Switzerland). The mobile phase consisted of a mixture of 1% triethylamine in water and acetonitrile (85:15 v/v) adjusted to pH 3.0 with ortho-phosphoric acid. Mobile phase was filtered by 0.45 µ size filter (Ultipor N66Nylone 6,6 membrane, PALL Pharmalab filtration Pvt., Ltd., Mumbai) and degassed by ultra-sonication. Thereafter mobile phase was pumped into column at a flow rate of 1.5 mL/min at ambient temperature. The effluent was monitored at 290 nm wavelength.

Calibration curve was prepared daily for drug concentration ranging from 0.01 to 50 µg/mL. The assay was sensitive (LLOD: 0.01 µg/mL), reproducible and linearity was observed from 0.01 to 50 µg/mL (r^2 = 0.99). The lower limit of quantification of the drug with a coefficient of variation was less than 8.36% for 0.01 µg/mL concentration. The mean extraction recovery from plasma was >82.81 ± 3.83% at the spiked concentrations between 0.01 and 50 µg/mL. Precision and accuracy were determined using quality control (QC) samples at concentrations 0.05, 1, 2.5, 10 and 50 µg/mL (5 replicates each per day). The intraday and inter day coefficients of variation for 5 QC samples were satisfactory with the relative deviations (RSD) of less than 9.77 %.

The bioavailability (F) was calculated using following formula:

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

PK/PD integration

The peak plasma drug concentration (C_{max}) and area under the curve ($AUC_{(0-\infty)}$) were applied in the calculation of the predictors of efficacy (C_{max}/MIC and $AUC_{(0-\infty)}$/MIC) for levofloxacin following subcutaneous administration in ketoprofen treated and febrile goat. MIC_{90} of 0.05 µg/mL of levofloxacin has been taken into consideration to determine dosage of levofloxacin.

Statistical Analysis

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

RESULTS

Plasma levofloxacin concentrations at different time intervals following subcutaneous injection under febrile state and ketoprofen co-administered intramuscularly in goats is presented as semi logarithmic plot in Figure 1.

On concurrent administration of levofloxacin (4 mg/kg, SC) and ketoprofen (3 mg/kg, IM), the initial plasma concentration of levofloxacin at 5 min was 0.03 ± 0.004 ìg/mL, which increased to attain the peak plasma concentration at 1 h (2.67 ± 0.073 ìg/mL). In febrile condition, plasma levofloxacin concentration following subcutaneous injection was 0.038 ± 0.004 µg/mL at 5 min, which attained peak concentration at 1 h (2.90 ± 0.355 µg/mL). Levofloxacin levels above the minimum inhibitory concentration (MIC: 0.05 µg/mL) were detected in plasma up to 12 h following co-administrated with ketoprofen and in febrile state. Various levofloxacin pharmacokinetic determinants that describe the absorption and elimination pattern after concurrent levofloxacin subcutaneous administration and ketoprofen intramuscular administration and under febrile state in goats are presented in Table 1.

DISCUSSION

No adverse effects or toxic manifestations were observed in goats following subcutaneous administration levofloxacin (4 mg/kg) in concurrent administration with

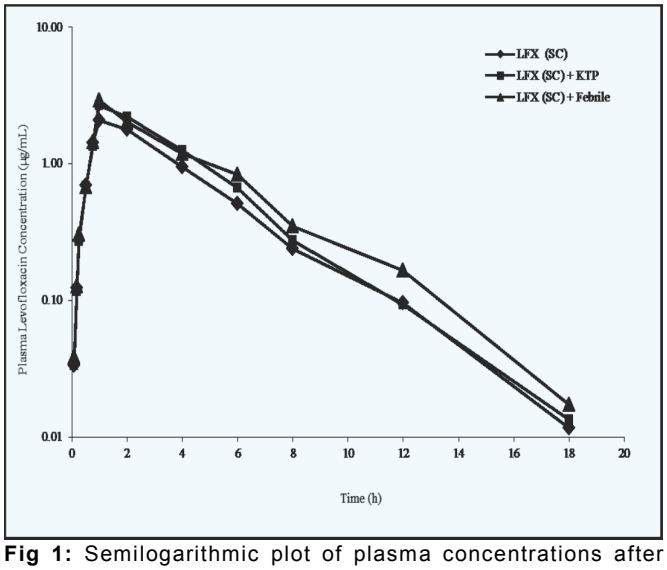

Fig 1: Semilogarithmic plot of plasma concentrations after subcutaneous administration of levofloxacin (4 mg/kg) in Ketoprofen-treated (3 mg/kg) and febrile goats. Each point represents mean of six animals.

Table 1:
Levofloxacin (4 mg/kg) pharmacokinetic parameters following subcutaneous administration in ketoprofen treated (3 mg/kg) and febrile goats (Mean ± SE, n=6).

Pharmacokinetic Parameter	Unit	Levofloxacin (SC)	Levofloxacin (SC) andKetoprofen (IM)	Levofloxacin (SC) and Febrile State
K_a	/h	0.98 ± 0.08	0.92 ± 0.04	0.77 ± 0.039*
â	/h	0.32 ± 0.003	0.32 ± 0.003	0.31 ± 0.003*
$t_{1/2ká}$	H	0.72 ± 0.05	0.76 ± 0.033	0.91 ± 0.04**
$t_{1/2â}$	H	2.13 ± 0.023	2.16 ± 0.022	2.22 ± 0.02*
C_{max}	ìg/mL	2.77 ± 0.10	2.67 ± 0.07	2.90 ± 0.04
T_{max}	H	1.00 ± 0.00	1.0 ± 0.00	1.00 ± 0.00
$AUC_{0-"}$	ìg.h/mL	9.47 ± 0.13	9.69 ± 0.25	11.37 ± 0.23**
AUMC	ìg.h^2/mL	35.94 ± 1.03	41.81 ± 3.34	47.81 ± 1.24**
Vd_{area}	L/kg	1.27 ± 0.03	1.26 ± 0.04	1.10 ± 0.04**
Cl_B	L/h/kg	0.41 ± 0.007	0.40 ± 0.014	0.34 ± 0.01**
MRT	H	3.79 ± 0.08	3.87 ± 0.04	4.20 ± 0.07*
	H	1.25 ± 0.25	1.34 ± 0.20	1.66 ± 0.20*
F	%	82.47 ± 0.05	83.23 ± 2.50	85.02 ± 4.00

*Significant difference at p<0.05 when compared with s.c. levofloxacin administered alone in goats .
K_a: Absorption rate constant, Â: Zero-time intercept of elimination phase, $t_{1/2ka}$: Absorption half life, $t_{1/2â}$: Elimination half life, C_{max}: Maximum drug concentration, T_{max}: Time of maximum observed concentration in plasma, $AUC_{0-á}$: Area under curve, AUMC: Area under first moment of curve, Vd_{area}: Apparent volume of distribution, Cl_B: Total body clearance, MRT: Mean residence time, MAT: Mean absorption time and F: Bioavailabilty.

ketoprofen (3 mg/kg, IM). In endotoxin induced febrile state, symptoms viz., increased respiration and pulse rate, decrease in feed intake, dryness of mouth and muzzle, and incoordination in movements were observed. The peak plasma drug concentration observed at 1 h post-subcutaneous administration of levofloxacin in ketoprofen-treated goats (C_{max} : 2.67 ± 0.07 µg/mL) and in febrile goat (C_{max} : 2.90 ± 0.04 µg/mL) was not altered significantly (p<0.05) in comparison to C_{max} (2.77 ± 0.10) observed in normal goats (Patel *et al*., 2013). However, significant higher (p<0.05) plasma drug concentrations were observed in febrile goats between 4 to 18 h compared to respective values in normal goats (Patel *et al*., 2013). Following subcutaneous administration of levofloxacin in ketoprofen-treated goats, significant changes in pharmacokinetic parameters were not observed as compared to pharmacokinetic parameters of levofloxacin in normal goats (Patel *et al*., 2013).

Following subcutaneous administration of levofloxacin in febrile goats, the decrease in the volume of distribution could be related decrease in systemic vascular pressure, central venous pressure, cardiac output (Salam Abdullah and Baggot, 1986), the peripheral blood flow (due increases in body temperature and to counter act loss of heat), gastrointestinal and hepatic blood flow, which has been reported in ruminants given E. coli endotoxin (Waxman *et al*., 2003). In addition to this the acute phase response induced by febrile sate includes synthesis of acute phase hepatic proteins, including α_1-acid glycoprotein which binds drugs and may decrease their volume of distribution. However, it was observed that plasma protein binding contribute only to a minor degree to the decrease volume of distribution of levofloxacin because drug has low degree of plasma protein binding i.e 22% (Goudah and Hasabelnaby, 2010).

Significant decrease in total body clearance (Cl_B: 0.34 ± 0.01 L/h/kg) of levofloxacin following subcutaneous injection was observed in febrile goats. The mean residence time (4.20 ± 0.07 h) observed in febrile goats was significantly higher than respective value (3.79 ± 0.08 h) observed in normal goats following subcutaneous administration (Patel *et al*., 2013). The elimination half-life was also significantly increased from 2.13 ± 0.02 to 2.22 ± 0.02 h in febrile goats. However, bioavailability of the drug was not altered compared to normal goats (Patel *et al*., 2013). Similar results for total body clearance mean residence time and elimination half life were also observed in sheep following subcutaneously route in febrile sheep (Patel *et al*., 2012). The findings clearly indicate that administration of lipopolysaccharide reduces the elimination of the drug which could be endotoxin induced toxic and adverse effects on the kidneys, including direct vascular damage to the endothelium and platelet aggregation in renal glomerular capillaries. It also produces some functional changes including decrease in the renal blood ûow and glomerular ûltration rate and changes in the intra-renal hemodynamics (Hasegawa *et al*., 1999). Endotoxin could produce a metabolic acidosis which would cause a decrease in urinary pH in febrile animals (Vann Miert, 1990) and may favour reabsorption of drug from renal tubules. It is probable that the decrease in glomerular filtration rate induced by endotoxin plays an important role in the decrease of body clearance of drugs which are eliminated by the renal route including levofloxacin.

It is suggested that the critical breakpoints determining the efficacy of fluoroquinolones are C_{max}/MIC_{90} e"8–10, and $AUC_{0"24}/MIC_{90}$ e"100 to avoid bacterial resistance emergence (Walker, 2000; Toutain *et al*., 2002).

Calculation of surrogate parameter following subcutaneous administration of levofloxacin (4 mg/kg b wt) in ketoprofen-treated and febrile goats resulted in Cp^0/MIC ratio (MIC: 0.05 μg/ml) of 53.40 and 58.00, respectively, which exceeds the recommended ratio of Cp^0/MIC ratio. For AUC/MIC ratio at MIC: 0.05 μg/mL was found higher than 100 in ketoprofen-treated (193.80) and febrile condition (227.40) but in case of MIC (0.1 μg/mL) ketoprofen treated goats, AUC/MIC ratio (96.90) proximate to 100 and in febrile goats AUC/MIC ratio (113.70) found higher than 100.

Following subcutaneous administration, average minimum inhibitory concentration i.e 0.05 μg/ml of levofloxacin maintained in plasma up to 12 h in ketoprofen treated goats and in febrile goats. Considering the values of C_{max}/MIC and AUC/MIC ratios obtained in the present study, it can be concluded that levofloxacin administered subcutaneously at the dose rate of 4 mg/kg at 12 hr interval may be efficacious against bacteria with MIC values under 0.05 μg/mL, satisfactorily.

Levofloxacin can successfully co-administrated with ketoprofen for combating inflammatory conditions without alteration of dosage regimen of levofloxacin. Moreover, integrating the pooled pharmacokinetic data generated from the present study, levofloxacin via subcutaneous administration (4 mg/kg) repeated at 12 h interval was sufficient to maintain plasma concentration above the 0.05 μg/mL MIC for most of the gram-positive and gram-negative microorganisms.

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REFERENCES

Goudah, A. (2009). Pharmacokinetics of levofloxacin in male camels (*Camelusdromedarius*). J. Vet. Pharmacol. Therap., **2**: 296-299.

Goudah, A. and Hasabelnaby, S. (2010). Disposition kinetics of levofloxacin in sheep after intravenous and intramuscular administration. Vet. Med. Int., 2010: 1-6.

Hasegawa, T., Takagi, K., Kitaichi, K. (1999). Effects of bacterial endotoxin on drugs pharmacokinetics. *Nagoya J Med. Sci.* **62:** 11–28.

Martinez, M., McDermott, P and Walker, R. (2006). Pharmacology of the ûuoroquinolones: A perspective for the use in domestic animals. *Vet. J.* **172**: 10-28.

Patel J. H., Vihol P. D., Patel U. D., Bhavsar S. K. and Thaker A. M. (2013). Pharmacokinetics of levofloxacin following subcutaneous administration in goat. *J. Vet. Pharmacol Toxicol.* **12(1-2)**: 35-38.

Patel, U.D., Patel, J.H., Varia, R.D., Patel, H.B, Bhavsar, S.K., and Thaker, A.M. (2012). Disposition kinetic of levofloxacin in experimentally induced febrile model of sheep. *J. Pharmacol Toxicol.* 7: 11-19.

Salam Abdullah, A. and Baggot, J.D. (1986). Influence of induced disease states on the disposition kinetics of imidocarb in goats. *J. Vet. Pharmacol Ther.* **9**:192-197.

Toutain, P.L.; DelCastillo, J.R.E. and Bousquet M´ elou A. (2002). The pharmacokinetic pharmacodynamic approach to a rational dosage regimen for antibiotics. *Res. Vet. Sci.* **73**(2):105-114.

Vann Miert, A. (1990). Influence of febrile disease on the pharmacokinetics of veterinary drugs. *Ann. Recher. Vet.* **21**:11–28.

Varia, R.D., Patel, J.H., Patel, U.D., Bhavsar, S.K. and Thaker, A.M. (2009). Disposition of levofloxacin following oral administration in broiler chickens. *Israel J. Vet. Med.* **64**: 118-121.

Verma, D.K. and Roy, B.K. (2006). Milk kinetics of gatifloxacin after single dose intravenous administration in healthy and febrile goats. Indian Journal Pharmacology **38**, 366-367.

Walker, R.D. (2000). The use of fluoroquinolones for companion animal antimicrobial therapy. *Aust. Vet. J.* **78**(2): 84-90.

Waxman, S.; SanAndres, M.D.; Gonzalez, F.; DeLucas, J.J.; SanAndres, M.I. and Rodrýguez, C.(2003). Inûuence of *Escherichia coli* endotoxin-induced fever on the pharmacokinetic behavior of marboûoxacin after intravenous administration in goats. *J. Vet. Pharmacol. Ther.* **26**:65-69.

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Research Article

PHYTOCONSTITUENTS FOR *IN VITRO* ANTIOXIDANT ACTIVITY OF *MURRAYA KOENIGII* AND *PHYLLANTHUS NIRURI*

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ABSTRACT

The present study was undertaken to quantify phytoconstituents phyllanthin and tannic acid in alcoholic and water extracts of *P. niruri* and *M. koenigii* leaves by high pressure liquid chromatography (HPLC) and to evaluate their antioxidant potential by DPPH radical scavenging assay method. The percent yields of alcoholic and water extract of *M. koenigii* (AEMK and WEMK) were found to be 7.9 and 9.8 %, respectively, whereas the percent yields of alcoholic and water extract of *P.niruri* (AEPN and WEPN) were found to be 16.5 and 7%, respectively. The extracts exhibited significant antioxidant activity in the DPPH radical- scavenging assay. The IC_{50} values obtained for DPPH-scavenging establish that *P. niruri* extracts possessed higher antioxidant activity in comparison to *M. koenigii.* Tannic acid (TA), a known antioxidant was quantified using HPLC in *M. koenigii* extracts with AEMK showing the highest content of TA whereas HPLC quantification of phyllanthin, a lignan known for its hepatoprotective potential was done for all *P. niruri* extracts with WEPN showing the highest content.

Key words: Antioxidant, DPPH radical, *M. koenigii*, Phyllanthin, Tannic acid, *P. niruri*

INTRODUCTION

Herbal plant extracts have played a crucial role in traditional medicine for the treatment of different ailments as described in ancient scriptures of Ayurveda. *Phyllanthus niruri* commonly known as Bhui amlaki a member of the family Euphorbiaceae and *Murraya koenigii* commonly known as the curry leaf tree a member of the family Rutaceae, have long been used to treat liver ailments in traditional medicine since time immemorial. The plants have been reported to possess many medicinal properties and their therapeutic aspects have been validated in recent scientific works carried out around the world (Paithankar *et al.,* 2011, Ajay *et al.,* 2011).

The leaf extracts of the plant *Phyllanthus niruri* have been reported to be rich in alkaloids, flavonoids, tannins, lignans, polyphenols, triterpenes, sterols and volatile oils. Phyllanthin and hypophyllanthin are two major lignans present in the plant extracts obtained from *P. niruri* and have been reported to possess hepatoprotective potential. The leaves of *M. koenigii* have been reported to possess essential oils, coumarins, terpenoids and carbazole alkaloids, well established for their antioxidant properties (Rajeshkumar *et al.,* 2002, Nayak *et al.,* 2010). In the present study phytochemical screening for different leaf extracts of *P. niruri* and *M. koenigii* was performed to quantify phytoconstituents phyllanthin and tannic acid and evaluate their antioxidant potential by DPPH radical scavenging assay method.

MATERIALS AND METHODS

Chemicals and plant material

The HPLC standards of phyllanthin was procured from Natural Remedies, Bangalore whereas tannic acid and other chemicals used in this study were procured from Sigma (St. Louis, MO, USA). The leaf samples for *Murraya koenigii* and *Phyllanthus niruri* were obtained from Medicinal and Aromatic Plant Research Development Centre (MRDC) of G.B. Pant University of Agriculture and Technology, Pantnagar, India.

Preparation of extracts

The leaves were shade dried and on complete drying were grinded to make a fine powder. Leaf extracts were prepared by the method previously described by Ningappa *et al.* (2008) with slight modifications. One gram of shade dried powder was added to 50 ml of double distilled water and ethyl alcohol, respectively. The solution was homogenized and the resultant suspension was centrifuged at 11,000 rpm for 10 min at 4°C. The supernatants were filtered using Whatman No.1 filter paper. The water extract (WE) of the leaves was lyophilized while the alcoholic extract (AE) of the leaves was concentrated in a hot air oven at 37°C. The percentage yield of different plant extracts was also determined.

Determination of flavanoids and phenolic content

The total flavonoid content was measured by using aluminum chloride colorimetric assay as given by Kumar *et al*. (2008) whereas the total phenolic content was determined according to the method of Folin-Ciocalteu (Kujala *et al.,* 2000) with minor modifications.

Determination of phyllanthin and tannic acid

High pressure liquid chromatography was carried out for determination of phyllanthin in WEPN (Water extract of *P. niruri* leaves) and AEPN (Alcoholic extract of *P. niruri* leaves) by the method given by Annamalai and Lakshmi (2009). Determination of tannic acid in *M. koenigii* leaf extracts i.e. WEMK (Water extract) and AEMK (Alcoholic

extract) was carried out by the method given by Pegg *et al*. (2008) with some minor modifications.

***In vitro* DPPH assay**

In vitro DPPH (2, 2-diphenyl-1-picrylhydrazyl), free radical scavenging assay to determine the antioxidant potential of plant extracts was performed by the method described by Kano *et al.* (2005).

RESULTS AND DISCUSSION

As shown in Table 1, the percent yields of WEMK and AEMK were found to be 7.9 and 9.8 % respectively whereas the percent yields of the WEPN and AEPN were found to be 16.5 and 7% respectively. The total phenol and flavonoid content in different leaf extracts were also determined (Table 1). WEMK possessed higher phenol and flavonoid content in comparison to AEMK whereas in case of *P. niruri* extracts AEPN possessed greater phenol and flavonoid concentration when compared to WEPN. Phenolic and flavonoid content in plant extracts have often been associated with their antioxidant potential.

Several herbs and spices have been reported to exhibit antioxidant activity. Antioxidants are considered important nutraceuticals on account of many health benefits (Valko *et al.,* 2007). The leaf extracts exhibited significant antioxidant activity in the DPPH radical-scavenging assay (Fig. 1). The IC_{50} values obtained for DPPH-scavenging establish that *Phyllanthus niruri* extracts possess higher antioxidant activity in comparison to *Murraya koenigii* extracts (Table 1). This can be attributed to higher concentration of phenols in *P. niruri* extracts.

The polyphenolic compounds most commonly found in plant extracts are the phenolic acids, flavonoids and tannins (Sati *et al.,* 2010) and are responsible for the antioxidant potential of plant extracts. Several of these compounds have been analysed by use of HPLC and other analytical techniques such as NMR and GC MS. HPLC was used to determine the content of phyllanthin and tannic acid in different leaf extracts of *P. niruri* and *M. koenigii*, respectively. The peak for phyllanthin was observed at a retention time of 7.98 minutes in standard solution as shown in Fig. 2 (A). The prominent peak of phyllanthin was observed at a retention time of 7.92 and 7.96 in AEPN and WEPN with a concentration of 157ppm and 223ppm respectively (Table 2). The lignans phyllanthin and hypophyllanthin found in *Phyllanthus niruri* extracts have been reported to possess immense hepatoprotective potential (Chirdchupunseree and Pramyothin, 2010). The peak of tannic acid has been identified to be at a retention time of 3.18 minutes in standard solution as shown in Fig.2 (B). The prominent peak of tannic acid was observed at a retention time of 3.21 and 3.20 minutes for AEMK and WEMK and had a concentration of 288ppm and 122ppm, respectively (Table 2). Tannic acid is a polyphenolic compound found in many plants and is known

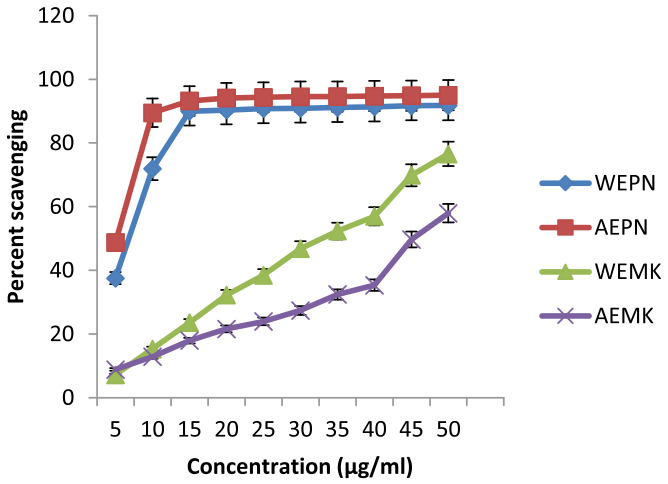


Fig 1:
Scavenging of DPPH radical by different extracts of *M.koenigii* and *P. niruri* leaves. Values are given as Mean ± S.D. (n=3).

Table 1:
Phytochemical analysis of different extracts of *Murraya koenigii* and *Phyllanthus niruri* leaves.

S.No.	Phytochemical analysis	*Murraya koenigii*		*Phyllanthus niruri*	
		WEMK	AEMK	WEPN	AEPN
1.	% Yield	7.9 ± 0.3	9.8 ± 0.2	16.5 ± 0.9	7 ± 1.6
2.	Total polyphenolic content (mgGAE*/g leaf extract)	103.7 ± 1.8	76.3 ± 1.6	190.5 ± 4.4	222.2 ± 5.6
3.	Total flavonoid content (mgCE$^+$/g of leaf extract)	47.1 ± 0.5	21.6 ± 1.2	40.9 ± 0.3	54.4 ± 1.2
4.	IC_{50} values for DPPH radical scavenging (µg/ml)	35 ± 0.3	45 ± 0.05	7 ± 0.1	5 ± 0.2

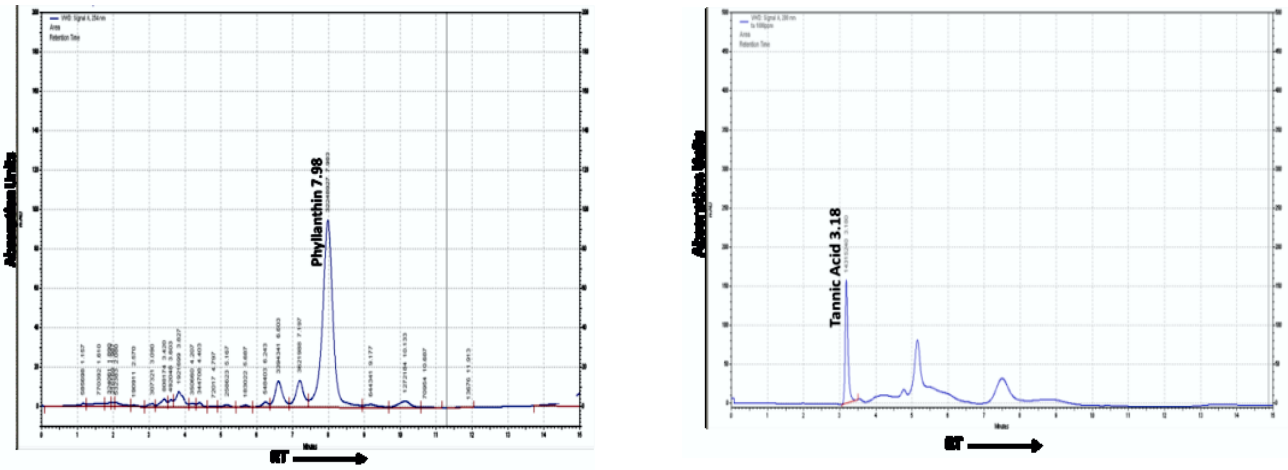
All values are expressed as Mean ± S.D. (n=3). GAE* Gallic Acid Equivalents, CE$^+$ Catechin Equivalents

Table 2:

Amount of phyllanthin in *P.niruri* and tannic acid in *M. koenigii* .

S.No.	Component/ Extract	RT (min)	Quantity (ppm)	Component/ Extract	RT (min)	Quantity (ppm)
		Amount of phyllanthin in *P. niruri*		Amount of tannic acid in *M. koenigii*		
1.	AEPN	7.92±0.01	157.59 ± 1.6	AEMK	3.21 ±0.03	288.8 ± 0.5
2.	WEPN	7.96 ±0.02	223.47 ± 1.5	WEMK	3.20 ±0.002	122.18 ± 0.8

RT: retention time, All values are expressed as Mean ± S.D. (n=3)



(A) (B)

Fig 2:
HPLC chromatogram depicting peak of standards: phyllanthin (A) and tannic acid (B) using diode array detector.

to possess antioxidant properties. Presence of noticeable amounts of tannic acid in *Murraya koenigii* leaf extracts validates their antioxidant nature (Sathaye *et al.,* 2011).

It is thus concluded that agous and alcohole exceeding of *P. niruri* and *M. koenigii* leaf passed antioxident properties.

REFERENCES

Ajay, S., Rahul, S., Sumit, G., Paras, M., Mishra, A., Gaurav, A. (2011). Comprehensive review: *Murraya koenigii Linn. Asian J. Pharm. Life Sci*. **4**: 417-425.

Annamalai, A. and Lakshmi, P.T.V. (2009). HPTLC and HPLC analysis of bioactive phyllanthin from different organs of *Phyllanthus amarus*. *Asian J. of Biotech.* **1(4):** 154-162.

Chirdchupunseree, H. and Pramyothin, P. (2010). Protective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes. *J. Ethnopharmacol*. **128**: 172-176.

Kano, M., Takayanagi, T., Harada, K., Makino, K. and Ishikawa F. (2005) Antioxidative activity of anthocyanins from purple sweet potato *Ipomoera batatas* cultivar Ayamurasaki. *Biosci. Biotechnol. Biochem.* **69**: 979–988

Kujala, T.S., Loponen, J.M., Klika, K.D., Pihlaja, K. (2000). Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: Distribution and effect of cold storage on the content of total phenolics and three individual compounds. *J. Agric. Food Chem.* **48:** 5338–5342.

Kumar, S., Kumar, D., Saroha, K., Singh, N. and Vashishta, B. (2008). Antioxidant and free radical scavenging potential of *Citrullus Colocynthis* (L.) Schrad. methanolic fruit extract. *Acta. Pharma.* **58:** 215-220.

Nayak, A., Mandal, S., Banerji, A., Banerji, J. (2010). Review on chemistry and pharmacology of *Murraya koenigii* Spreng (Rutaceae). *J. Chem. Pharm. Res.* **2(2):** 286-299.

Ningappa, M. B., Dinesha, R. and Srinivas, L. (2008). Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (*Murraya koenigii* L.) extracts. *Food Chem*. **106**: 720–728.

Paithankar, V.V., Raut, K.S., Charde, R.M. and Vyas, J.V. (2011). *Phyllanthus niruri*: A magic Herb. *Res Pharm*. **1**: 1-9.

Pegg, R.B., Rybarczyk, A. and Amarowicz, R. (2008). Chromatographic separation of tannin fractions from a bearberry-leaf (*Arctostaphylos uva-ursi* L. Sprengel) extract by SE –HPLC a short report. Pol. J. Food Nutr. Sci. **58**:485-490.

Rajeshkumar, N.V., Joy, K. L., Girija, K., Ramsewak, R.S., Nair, M.G., Ramadasan, K. (2002). Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. *J Ethnopharmacol* **81**: 17–22.

Sathaye, S., Bagul, Y., Gupta, S., Kaur, H. and Redkar, R. (2011) Hepatoprotective effects of aqueous leaf extract and crude isolates of *Murraya koenigii* against in vitro ethanol-induced hepatotoxicity model. *Exp. Toxicol. Pathol.* **63:** 587-591.

Sati, S.C., Sati, N., Rawat, U. and Sati, O. P. (2010). Medicinal plants as a source of antioxidants. *Res. J. Phytochem*. **4:** 213-224.

Valko, M., Leibfritz, D., Moncol, J., Cronin , M.T., Mazur, M. and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol*. **39(1):**44-84.

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Research Article

COMBINED EFFECT OF ANAESTHETICS KETAMINE AND XYLAZINE ON MEAN ARTERIAL PRESSURE OF DOCA HYPERTENSIVE RATS

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ABSTRACT

Present study was conducted with the aim to see the effect of anaesthetics ketamine and xylazine together after intramuscular administration on mean arterial pressure (MAP) of hypertensive rats. Uni-nephrectomised male Wistar rats were divided into two groups, namely DOCA hypertensive and Sham control groups. Hypertension in male rats was induced after administering deoxycorticosterone acetate salt in soyabean oil @ 25mg/kg b. wt. subcutaneously twice in a week for 4-weeks provided *ad lib* drinking water containing sodium chloride (1%). In Sham control unilateral nephrectomised rats were treated with vehicle only providing normal tap water for drinking. After completion of four weeks, rats of both the groups were anaesthetized to ketamine and xylazine together to analyse its effect on mean arterial pressure ($P<0.05$). It is concluded from this study that intra-muscular administration of ketamine and xylazine in combination significantly decreased the mean arterial pressure of DOCA hypertensive rats.

Key words: Ketamine & xylazine, combination, MAP, DOCA hypertension, male rats.

INTRODUCTION

Hypertension is a most common medical disorder. It is designated as 'silent killer' as the disease often asymptomatic and produces mortality without prior intimation. A remarkable fact of hypertension is that sufferers are at higher risk of cardiovascular diseases which includes stroke, coronary disease, heart failure and renal dysfunction (Cebova *et al*., 2006; Pechanova *et al*., 2006; Simko, 2007). Lifestyle and heredity are the important determinant of hypertension (Meyer *et al*., 2009). Preventive measures viz salt restriction, exercise, control the obesity, modification in lifestyle and antihypertensive drugs all help to restrict the consequences of hypertension. Anaesthetics influencing blood pressure of normal and hypertensive patients and usually lower the blood pressure (Goldman and Caldera, 1979). As stability is prime requisite while performing the surgery in animals. Keeping the importance of anaesthesia on mean arterial pressure (MAP) the present study was planned with the target to analyse the effect of anaesthetics on MAP.

Anaesthetics such as ketamine and xylazine were frequently used to restrain the animals for various purposes. It has been found that anaesthetics usually influence the blood pressure of normal and hypertensive patients. Although less scientific literature were available pertaining to report showing the impact of ketamine and xylazine on Mean Arterial Pressure of animals when used alone or in combination (Wixson *et al*., 1987; Afshari *et al*., 2005). Therefore, to generate more scientific data, the present study was planned to conduct the combined effect of ketamine and xylazine on mean arterial pressure of normal and hypertensive rats.

MATERIALS AND METHODS

Experimental animals

Healthy adult male wistar rats weighing 150-200g were housed with an alternate period of 12 hour light and dark cycle. The experimental animals were procured from Laboratory Animal Resource Section, Indian Veterinary Research Institute, Izatnagar (UP), India. Research work was conducted after approval by the Institute Post-Graduate study committee and as per Animal Ethics Committee guidelines. After acclimatization, rats were divided into two groups namely Sham control and DOCA salt treated hypertensive rats.

Induction of hypertension and experimental design

Left kidney of the male wistar rats were removed using anaesthesia ketamine (75mg/kg, i.m.) and xylazine (10mg/kg, i.m.). After three days of nephrectomy, mineralocorticoid deoxycorticosterone acetate (DOCA) salt was dispersed in soyabean oil and administered subcutaneously @ 25mg/kg body weight twice in a week for four weeks (Chan *et al*., 2006). Drinking water of DOCA treated rats were containing sodium chloride (1%) and potassium chloride (0.2%). Whereas unilateral nephrectomised sham control rats were treated with soyabean oil (vehicle) provided normal drinking water. After four weeks of DOCA salt administration, rats were anaesthetized with ketamine (75mg/kg) and xylazine (10mg/kg) in combination and pentobarbital sodium (60mg/kg, intra-peritoneally) alone. Mean Arterial Pressure of anaesthetised rats were measured by connecting to data

acquisition system possess transducer (Model MLT0380/ D, AD Instrument) to analyse the effect of anaesthetics on MAP.

Statistical analysis

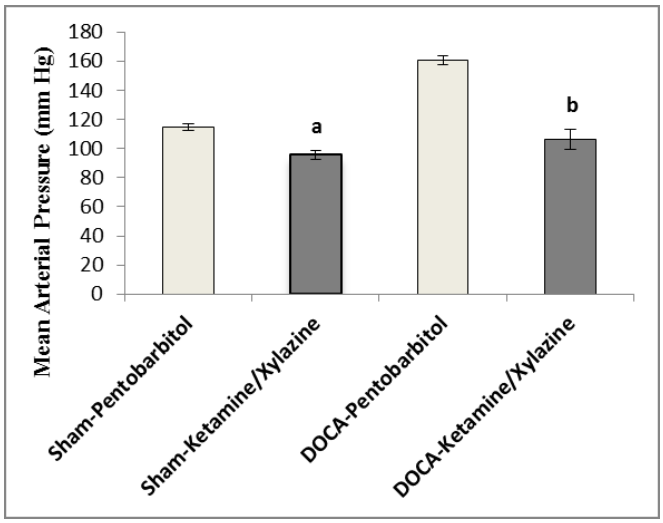
Experimental data was expressed as Mean±SD and analyzed by Student Newman Keuls method (Snedecor& Cochran, 1989). Differences in values were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Combined effects of ketamine and xylazine on MAP of DOCA hypertensive rats

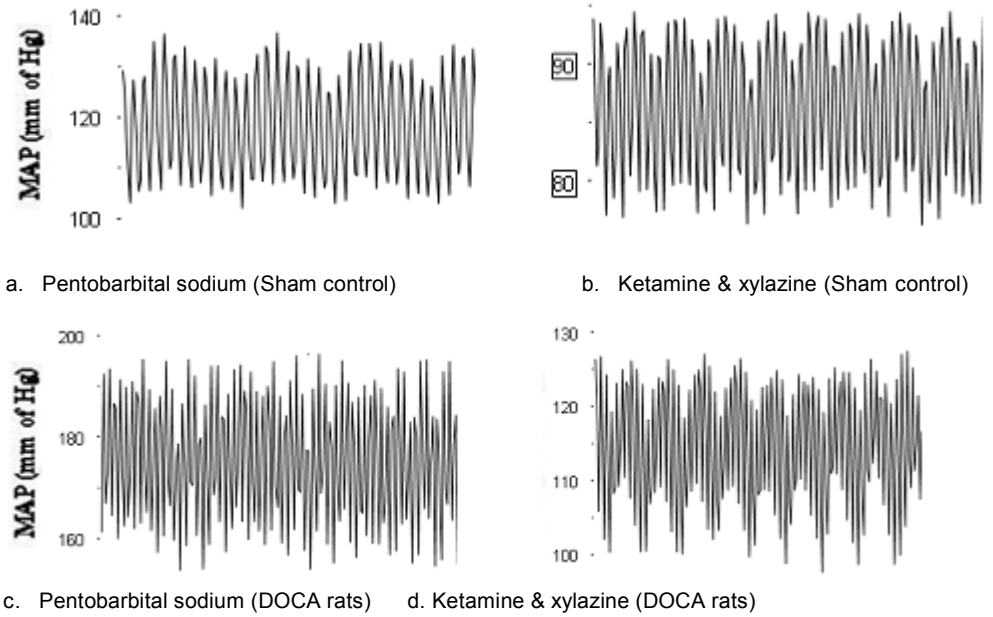
A significant increase of MAP (160.3±3.02mm of Hg, n=9) was found in DOCA salt treated uninephrectomised rats measured under anaesthetic pentobarbital sodium (Fig.2). In contrary, a significant decrease of MAP of DOCA hypertensive rats (106.0±6.83mm of Hg, n=5) was observed under anaesthetics ketamine and xylazine in combination as compared to MAP of same group under pentobarbital sodium (Fig.1.a-d & fig.2). Similarly, ketamine and xylazine also significantly decreases the MAP of uninephrectomised sham control (95.52±2.93, n=6) in comparison to MAP of same group using pentobarbital sodium (114.6±2.34mm of Hg, n=5).

Several anaesthetics are showing their impact over the Mean Arterial Pressure of normal and disease patients. Therefore, while performing surgery it is very much required that anaesthetics have not to distract the blood pressure of patients under surgery. An important finding of the present study was that anaesthetics ketamine and xylazine used together significantly decreases the Mean Arterial Pressure of DOCA hypertensive rats and also of sham control rats. The decrease in MAP of DOCA hypertensive rats was noted to the level that is comparable to sham control rats. Similarly, Wixson *et al*. (1987) and Saha *et al*. (2007) were reported that ketamine and xylazine significantly decreases the MAP in male Sprague Dawley rats. The reason for decrease in MAP may because of central and peripheral stimulatory effect of xylazine which produces hypotensive effects (Wixson *et al*., 1987; Hall *et al*., 2001; Afshari *et al*., 2005). Also ketamine and xylazine



At $p^a<0.05$ level of significance in comparison to sham control (Pentobarbital sodium), At $p^b<0.05$ level of significance in comparison to DOCA hypertensive rats (Pentobarbital sodium),

Fig.2:
Showing the combined effects of ketamine and xylazine on MAP of DOCA hypertensive rats and Sham control male wistar rats.



a. Pentobarbital sodium (Sham control)

b. Ketamine & xylazine (Sham control)

c. Pentobarbital sodium (DOCA rats)

d. Ketamine & xylazine (DOCA rats)

Fig.1:
Tracing depicting the combined effect of ketamine and xylazine on MAP of Sham control and DOCA hypertensive male wistar rats.

together may decreases the MAP through sympatholytic action (Wixson *et al*., 1987; Hall *et al*., 2001).

It is concluded from the present study was that Intra-muscular administration of ketamine and xylazine in combination significantly decreases the MAP of DOCA hypertensive rats that overshadowed the real reading of MAP measured by invasive method.

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REFERENCES

Afshari, F.S., Baniadam, A. and Marashipour, S.P. (2005). Effect of xylazine-ketamine on arterial blood pressure, arterial blood pH, blood gases, rectal temperature, heart and respiratory rates in goats. *Bull. Vet. Inst. Pulawy.* **49:**481-484.

Cebova, M., Kristek, F. and Kunes, J. (2006). Differential remodelling of carotid artery in spontaneously hypertensive and hereditary hypertriglyceridemic rats. *Physiol. Res.,* **55:**S81-S87.

Chan, V., Hoey, A. and Brown, L. (2006). Improved cardiovascular function with aminoguanidine in DOCA-salt hypertensive rats. *Br. J. Pharmacol.* **148:**902-908.

Goldman L. and Caldera D.L. (1979). Risk of general anaesthesia and elective operation in the hypertensive patient. *Anesthesiology*. **50**:285-292.

Hall, L.W., Clark, K.W. and Trim, C.M. (2001). In: Veterinary anaesthesia. 10th ed. W. B. Saunder's Co, London, Pp. 84-87, 352-353.

Meyer, T.E., Shiffman, D., Morrison, A.C., Rowland, C.M., Louie, J.Z., Bare, L.A., Ross, D.A., Arellano, A.R., Chasman, D.J., Ridker, P.M., Pankow, J.S., Coresh, J., Malloy, M.J., Kane, J.P., Ellis, S.G., Devlin, J. and Boerwinkle, E. (2009). GOSR2 Lys67Arg is associated with hypertension in whites. *Am. J. Hypertens.* **22**:163-168.

Pechanova, O., Rezzani, R., Babal, P., Bernatova, I. and Riantsitohaina, R. (2006). Beneficial effects of ProvinolsTM: cardiovascular system and kidney. *Physiol. Res.* **55**:S17-S30.

Saha, D.C., Saha, A.C., Malik, G., Astiz, M.E. and Rackow, E.C. (2007). Comparison of cardiovascular effects of tiletamine-zolazepam, pentobarbital, and ketamine-xylazine in male rats. *J. Am. Assoc. Lab. Anim. Sci.* **46:**74-80.

Simko, F. (2007). Is NO the king? Pathophysiological benefit with uncertain clinical impact. *Physiol. Res.* **56**:S1-S6.

Snedecor, G.W. and Cochran, W.G. (1989). Statistical Methods (8th edition). The Iowa State University Press.

Wixson, S.K., White, W.J., Hughes, H.C., Jr. Lang C.M. and Marshall, W.K. (1987). The effects of pentobarbital, fentanyl-droperidol, ketamine-xylazine and ketamine-diazepam on arterial blood pH, blood gases, mean arterial blood pressure and heart rate in adult male rats. *Lab. Anim. Sci.* **37:**736-742.

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Research Article

EFFECT OF SUPPLEMENTATION OF ENZYMES ON HAEMATO-BIOCHEMICAL PARAMETERS IN BROILER CHICKENS*

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ABSTRACT

This study was conducted to evaluate the effect of enzymes supplementation through feed for six weeks on haematological and biochemical parameters in broilers. One hundred eighty chicks were divided into four treatment groups (T_1, T_2, T_3 and T_4 respectively) with 3 replicates of 15 chicks in each group and mixture of eight enzymes was supplemented at graded levels @ 0, 0.25, 0.50 and 0.75 g/ kg of feed to each treatment. At the interval of three and six weeks, two birds from each replicate (6 birds/ treatment) were randomly selected and blood was collected for haemato-biochemical study. The results of present study revealed that at three and six weeks age broilers of T_2, T_3 and T_4 groups showed significantly ($P<0.05$) higher values of total erythrocyte count (TEC), total leukocyte count (TLC), packed cell volume (PCV) and haemoglobin (Hb) as compared to control (T_1) group. There was no significant difference in erythrocyte sedimentation rate (ESR) and mean corpuscular volume (MCV) values at three weeks of age where as a significant ($P<0.05$) decrease in ESR and increase in MCV were observed at six weeks of age in enzymes supplemented groups compared to control group. At six weeks of age, total serum protein and serum HDL cholesterol values in T_2, T_3 and T_4 groups were significantly ($P<0.05$) higher than T_1 (control) group, however, serum albumin and serum globulin values in T_3 and T_4 groups were significantly ($P<0.05$) higher than T_1 and T_2 groups. Serum glucose, serum total cholesterol, serum LDL cholesterol and serum triglyceride values in T_2, T_3 and T_4 groups were significantly ($P<0.05$) lower than T_1 (control) group. The study therefore revealed that enzymes supplementation through feed at 0.50 g/ kg of feed is beneficial in improving haemato-biochemical values in broilers.

Key words: Broilers, haematological parameters, biochemical parameters, enzymes

INTRODUCTION

Feed supplementation is very important for poultry nutrition, because it increases the efficiency of feed utilization (Craig *et al.,* 2008). Various feed additives and growth promoters have been developed to improve growth rate and haemato-biochemical parameters. In order to prevent nutrients and performance losses several enzymes have been used. Paul *et al.* (1995) defined enzymes as proteins that facilitate specific chemical reactions. Enzymes as feed additives have begun to play a great role in poultry industry. They not only improve the utilization of diets containing cereals such as maize, soybean, wheat and byproducts like rice bran and deoiled rice bran, but have a positive impact on the quality of the environment through reduced output of excreta and pollutants such as phosphate and nitrogen including ammonia. The non starch polysaccharides and phytates are anti-nutritive factors in poultry feed. The ingestion of soluble non starch polysaccharides increases the digester viscosity (Annison *et al.,* 1991) in broilers. Phytate or phytic acid is a naturally occurring organic complex found in cereal grains and oilseeds. Supplementation of feed enzymes has become important domestically and internationally due to economic considerations, as they increase the feeding value of raw feed ingredients. Many reports have demonstrated performance benefits of enzymes when added to wheat and maize based diets (Wyatt *et al.,* 1999). Therefore, present study was carried out to study effect of dietary supplementation of enzymes on haemato-biochemical parameters in the broilers.

MATERIALS AND METHODS

Experimental animals

The study was conducted on one hundred eighty day-old straight run commercial broiler chicks at Instructional Poultry Farm, College of Veterinary and Animal Sciences, GBPUA&T, Pantnagar. All the chicks were wing banded, individually weighed and randomly allocated into 4 different treatment groups with 3 replicates of 15 chicks each. The first treatment was considered as control (T_1) in which no supplement was added to basal feed, while in treatments T_2, T_3 and T_4 mixture of eight enzymes (Enzymex supplied by Exotic Biosolutions Pvt. Ltd., Mumbai) comprising alpha amylase, protease, xylanase, betagluconase, cellulase, phytase, pectinase and lipase were provided @ 0.25, 0.50 and 0.75 g/ kg of feed, respectively. The chicks of all the groups were housed in deep litter system under similar housing and management conditions. Feed and water were provided *ad libitum*

throughout the study. Standard feeds for starter and finisher period having isocaloric and isonitrogenous composition were provided to the chicks.

Haemato-Biochemical examination

At the end of three and six weeks, two birds from each replicate (6 birds/ treatment) were randomly selected and approximately 5.0 ml blood samples were collected from each bird in EDTA coated tubes for determining hematological and in plane tube for biochemical parameters in aseptic manner and the serum was stored at -20^{0}C until the day of assay. Out of 5.0 ml blood, 3.0 ml of blood was transferred to the tubes containing ethylene diamene tetra acetate (EDTA) and it was used for estimation of haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC) and erythrocyte sedimentation rate (ESR) (Jain, 1986). Haemoglobin (Hb) concentration was estimated spectrophotometrically at 540 nm by cyanomethemoglobin method, using Drabkin's solution of Span Diagnostics (Fudge, 2000). RBC counts were determined using a Neubauer's counting chamber as described by Natt and Herrick (1952). The packed cell volume (PCV) was determined using the capillary microhaematocrit method. At the end of six weeks 2.0 ml of the blood collected was used for serum separation to evaluate total serum protein, serum albumin, serum globulin, glucose, total cholesterol, HDL cholesterol, LDL cholesterol and triglyceride which were measured using a biochemical analyzer (Erba chem.-5-plus).

Statistical analysis

All the data pertaining to various parameters were analyzed statistically by ANOVA using SPSS 16 statistical software. The significant mean differences between the treatments were determined at P<0.05 using Duncan's Multiple Range Test (DMRT) as modified by Kramer (1957).

RESULTS AND DISCUSSION

The results of effect of enzymes supplementation on haematological parameters in broilers are presented in Table 1. The results showed that at the end of three and six weeks of age, significant (P<0.05) increase in the mean values of TEC, TLC, PCV and Hb was noted in T_2, T_3 and T_4 groups as compared to T_1 (control) group, however, there were no significant differences in the TEC, TLC, PCV and Hb values between T_3 and T_4 groups of broilers. TEC, TLC, PCV and Hb values were maximum in T_3 and minimum in T_1 groups. A significant increase in TEC, PCV and Hb were also reported by Rahman *et al.* (2013) in broilers supplemented with enzymes (mixture of cellulase, xylanas, protease, amylase, phytase, pactinase, invertase, hemicellulase, lipase and α-galactosidase). However, Chuka (2014) did not report any significant difference in TEC, PCV and Hb in broilers supplemented with enzymes (mixture of protease, amylase, glucoamylase, phytase, hemicellulase, lipase, lactase, glucanase and α-galactosidase). An Increase in TEC, TLC, PCV and Hb in enzymes supplemented groups might have been due to an increase absorption of iron and copper minerals required for synthesis of haem pigment resulting in improved TE proliferation, formation of Hb and maturation of blood cells. There was no significant difference in the ESR at three weeks of age whereas significantly (P<0.05) lower at six weeks of age in enzymes supplemented groups in broilers. Similar finding were reported by Rahman *et al.* (2013) who found significant decrease in ESR in broilers supplemented with enzymes up to six weeks of age. However, Yadava *et al.* (2009) did not report any significant difference in ESR of broilers supplemented with multi-enzymes. There was no significant difference in the MCV at three weeks of age whereas significantly (P<0.05) higher at six weeks of age in enzymes supplemented groups in broilers. However, there were no significant differences in the MCV values between T_3 and T_4 groups of broilers. MCH values were significantly (P<0.05) lower in T_3 and T_4 groups at three weeks of age and T_2, T_3 and T_4 groups at six weeks of age in enzymes supplemented groups of broilers in comparison to control. At three weeks of age MCHC values in T_2, T_3 and T_4 groups were significantly (P<0.05) higher than T_1 (control) group, however, there were no significant differences in the MCHC values between T_2, T_3 and T_4 groups of broilers. MCHC values at six weeks of age in T_2, T_3 and T_4 groups were significantly (P<0.05) lower than T_1 (control) group, however, there were no significant differences in the MCHC values between T_3 and T_4 groups of broilers. The results are in contrast with the findings of Chuka (2014) who reported that there were no significant differences in MCV, MCH and MCHC in broilers supplemented with enzymes (mixture of protease, amylase, glucoamylase, phytase, hemicellulase, lipase, lactase, glucanase and α-galactosidase).

The results of effect of enzymes supplementation on biochemical parameters in broilers are presented in Table 2. The results showed that serum total protein values in T_2, T_3 and T_4 groups were significantly (P<0.05) higher than control group, however, there were no significant differences in the serum total protein values between T_3 and T_4 groups of broilers. Maximum and significantly (P<0.05) higher serum total protein compared to the control was found in broilers of group T_4 which was statistically similar to group T_3. Minimum and significantly (P<0.05) lower serum total protein was observed in the broilers of control group. Serum albumin and globulin values in T_3 and T_4 groups were significantly (P<0.05) higher than T_1 and T_2 groups, however, there was no significant differences in the serum albumin and globulin values between T_1 and T_2 groups of broilers. Maximum and significantly (P<0.05) higher serum albumin and globulin values compared to the control was found in broiler of group T_4 which was

Table 1:
Haematological profile (mean ± S.E., n=24) of broilers fed diets supplemented with enzymes

Parameters	TEC (10^6/µl)		TLC (10^3/ µl)		PCV (%)		Haemoglobin (g/dl)		ESR (mm/hr)		MCV (fl)		MCH (pg)		MCHC (%)	
Treatments	21d	42d	21d	42d	21d	42d	21d	42d	21d	42d	21d	42d	21d	42d	21d	42d
T_1	2.32^c ± 0.01	2.32^c ± 0.01	24.00^c ± 0.06	24.12^c ± 0.03	28.02^c ± 0.02	28.03^c ± 0.01	8.87^c ± 0.04	9.88^c ± 0.04	1.66 ± 0.03	2.34^a ± 0.02	168.99 ± 2.58	119.82^c ± 0.87	38.23^a ± 0.12	42.59^a ± 0.25	31.66^a ± 0.15	35.25^a ± 0.16
T_2	2.43^b ± 0.02	2.59^b ± 0.01	24.43^b ± 0.01	24.58^b ± 0.03	28.61^b ± 0.02	29.96^b ± 0.03	9.22^b ± 0.03	10.22^b ± 0.04	1.65 ± 0.02	2.04^b ± 0.01	173.51 ± 2.01	146.89^b ± 0.84	37.96^a ± 0.48	39.46^b ± 0.17	32.23^a ± 0.11	34.11^b ± 0.14
T_3	2.58^a ± 0.02	2.82^a ± 0.01	24.71^a ± 0.03	24.98^a ± 0.01	29.18^a ± 0.01	30.87^a ± 0.01	9.40^a ± 0.03	10.41^a ± 0.03	1.64 ± 0.03	1.84^c ± 0.01	178.34 ± 3.86	167.79^a ± 0.89	36.44^b ± 0.26	36.92^c ± 0.06	32.21^a ± 0.11	33.72^a ± 0.09
T_4	2.56^a ± 0.02	2.81^a ± 0.02	24.64^a ± 0.03	24.96^a ± 0.10	29.15^a ± 0.02	30.86^a ± 0.02	9.38^a ± 0.02	10.39^a ± 0.03	1.64 ± 0.05	1.86^c ± 0.01	178.45 ± 5.01	165.92^a ± 0.24	36.65^b ± 0.23	36.98^c ± 0.17	32.18^a ± 0.10	33.67^a ± 0.10

Values bearing different superscripts in a column differ significantly (P<0.05);
T_1=Basal diet; T_2=Basal diet + Cocktail of enzymes @ 0.25 g/kg of feed;
T_3=Basal diet + Cocktail of enzymes @ 0.50 g/kg of feed; T_4=Basal diet + Cocktail of enzymes @ 0.75 g/kg of feed

Table 2:
Biochemical profile (mean ± S.E., n=24) of broilers fed diets supplemented with enzymes (42nd day)

Parameters / Treatments	Total Serum Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A/G ratio	Serum glucose* (mg/dl)	Serum total cholesterol* (mg/dl)	Serum HDL-cholesterol* (mg/dl)	Serum LDL-cholesterol* (mg/dl)	Serum triglyceride* (mg/dl)
T_1	3.11^c ± 0.04	1.01^b ± 0.01	2.10^b ± 0.03	0.48 ± 0.00	257.12^a ± 0.93	175.71^a ± 1.30	21.26^c ± 0.49	135.16^a ± 1.72	96.43^a ± 0.77
T_2	3.21^b ± 0.01	1.04^b ± 0.03	2.17^b ± 0.04	0.48 ± 0.02	249.01^b ± 0.48	160.94^b ± 0.18	26.17^b ± 0.23	116.56^b ± 0.18	91.03^b ± 0.25
T_3	3.52^a ± 0.03	1.15^a ± 0.01	2.37^a ± 0.04	0.49 ± 0.01	229.68^c ± 0.91	136.64^c ± 0.64	31.89^a ± 0.32	87.67^c ± 0.79	85.41^c ± 0.41
T_4	3.55^a ± 0.03	1.16^a ± 0.02	2.39^a ± 0.01	0.49 ± 0.00	230.03^c ± 0.50	138.01^c ± 0.54	31.14^a ± 0.29	89.61^c ± 0.49	86.31^c ± 0.66

Values bearing different superscripts in a column differ significantly (P<0.05);
T_1=Basal diet; T_2= Basal diet + Cocktail of enzymes @ 0.25 g/kg of feed;
T_3=Basal diet + Cocktail of enzymes @ 0.50 g/kg of feed; T_4=Basal diet + Cocktail of enzymes @ 0.75 g/kg of feed

statistically (P<0.05) similar to group T_3. Minimum and significantly (P<0.05) lower serum albumin and globulin values were observed in the broiler of control group which was statistically (P<0.05) similar to group T_2. There was no significant (P<0.05) difference in the serum albumin - globulin ratio among different treatment groups. The albumin-globulin ratio was maximum in groups T_3 and T_4 and minimum in T_1 groups of broilers. Similar findings were reported by Hassen and Chauhan (2003) who found significant increase in serum total protein, serum albumin and globulin levels of broilers supplemented with phytase. However, Chuka (2014) noted non-significant increase in serum total protein, serum albumin, globulin levels and albumin-globulin ratio of broilers supplemented with enzymes. Increased total protein level in enzymes supplemented groups of broilers in the present experiment may be attributed to the stimulating effect of enzymes on the synthesis of protein in the liver. Serum glucose, cholesterol, LDL cholesterol and triglyceride values in T_2, T_3 and T_4 groups were significantly (P<0.05) lower than T_1 (control) group, however, there were no significant differences in the serum glucose, cholesterol, LDL cholesterol and triglyceride values between T_3 and T_4 groups of broilers. The serum glucose, cholesterol, LDL cholesterol and triglyceride were maximum in group T_1 and minimum in T_3 group. Serum HDL cholesterol values in T_2, T_3 and T_4 groups were significantly (P<0.05) higher than T_1 (control) group of broilers. The serum HDL cholesterol was maximum in group T_3 and minimum in T_1 group. Rama Rao *et al.* (2004) observed that addition of non starch polysaccharides (NSPs) hydrolyzing enzyme to diet of broiler led to significant decrease in glucose and LDL-cholesterol. However, Shehab *et al.* (2012) could not find effect of enzymes (phytase and kemzyme) supplementation on serum glucose and triglyceride in broilers. Reduction in the serum glucose level of enzymes supplemented groups may be due to gluconeogenesis which lead to increase in the body weight gain of broilers. This reduction of serum cholesterol concentration may be attributed to the role of enzymes in ameliorating hyperlipidemia. The results of the study indicated that increase in TEC, TLC, PCV and Hb in enzymes supplemented groups of broilers. Serum glucose, cholesterol, LDL cholesterol and triglyceride values in enzymes supplemented groups were lower than control. It can be concluded that enzyme supplementation in broilers through feed can be used to improve haematological and biochemical profile and enzyme supplementation through feed at 0.50 g/ kg of feed is beneficial in improving haemato-biochemical values in broilers.

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REFERENCES

Annison, G. and Choct, M. (1991). Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimizing their effects. *World's poult. Sci. J.* **47**: 232-242.

Chuka, Ezema (2014). Comparative study of the effects of probiotic and commercial enzyme on growth rate, haematology and serum biochemistry of broiler chicken. *Food Process and Techno.* **5**: 9 http://dx.doi.org/10.4172/2157-7110.1000367.

Craig, L., Wyatt, T. and Mike. B. (2008). Mechanism of action for supplemental NSP and phytate enzymes in poultry diet. AB Vista Feed Ingredients Ltd. in their paper to the Carolina, Poultry Nutrition Conference.

Fudge, A. M. (2000). Avian complete blood count, in Laboratory Medicine: Avian and Exotic pets, 1st Ed., Saunders Company, U.S.A. 9-18.

Hassen, B. H. and Chauhan, S. S. (2003). Phytase supplementation on biochemical blood parameters of maize-based-diets-fed broilers. *Agri Topica*. **18**: 2-3.

Jain, N. C. (1986). Schalm's Veterinary Haematology. Lea and Febiger, Philadelphia, USA.

Kramer, C. Y. (1957). Extension of multiple range tests to group correlated adjusted means. *Biometrics.* **13**: 13-17.

Natt, M. P. and Herrick, C. A. (1952). A new blood diluent for conducting the erythrocytes and leucocytes of the chicken. *Poult. Sci.* **31**(1): 735-738.

Paul, J. F. (1995). Biochemistry-an illustrated review with question and explanation. Clinical Division of Auckland University School of Medicine Hamilton, New Zealand.

Rahman, M. S., Mustari, A., Salauddin, M. and Rahman, M. M. (2013). Effects of probiotics and enzymes on growth performance and haematobiochemical parameters in broilers. *J. Bangladesh Agril. Univ.* **11**(1): 111–118.

Rama Rao, S. V., Raju, M. V. L. N., Panda, A. K. and Sharma, R.P. (2004). Replacement of maize with pearl millet (*Pennisetum typhoides*) on weight and nutrient basis broiler chickens diet. *Indian J. Poult. Sci.* **39**: 15-24.

Shehab, A. E., Kamelia, M. Z., Khedr N. E., Tahia E. A. and Esmaeil F. A. (2012). Effect of dietary enzyme supplementation on some biochemical and hematological parameters of Japanese quails. *J. Anim. Sci. Adv.* **2**(9): 734-739.

Wyatt, C. L., Bedford, M. R. and Waldron, L. A. (1999). Role of enzymes in reducing the variability in nutritive value of maize using the ileal digestibility method. *In Proc. Aust. Poult. Sci. Symp.* **11**: 108-111.

Yadava, P. K., Niranjan, P. S., Udeybir, Koley, S. and Verma, D. N. (2009). Performance of broiler chicken as affected by varying levels of multi enzyme supplementation. *Anim. Nutri. and Feed Tech.* 9(1): 103-108.

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Research Article

EFFICACY OF DIETARY SUPPLEMENTATION OF *NIGELLA SATIVA*, SELENIUM AND VITAMIN E ON EGG LIPID PROFILE

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ABSRACT

In the present study, *Nigella sativa* alone and in combination with selenium and vitamin E was used to identify its hypocholesterolemic activity in egg yolk lipid profile of laying hens. The reduction in concentration of cholesterol in egg yolk of layers supplemented with *Nigella sativa* alone and combination of *Nigella sativa*, Selenium and vitamin E was calculated to be 8.94 and 14.92 percent, respectively. Similarly, reduction in egg yolk triglycerides was also observed with 4.28 and 6.53 percent, respectively in *Nigella sativa* and combination of *Nigella sativa*, selenium and vitamin E treated group.

Keywords: hypocholesterolemic, *Nigella sativa,* Selenium, Vitamin E

INTRODUCTION

Diet plays an important role in maintaining health. Among the different products delivering essential nutrients to the body, an egg has possibly a special place, being a rich and balanced source of essential amino and fatty acids, some minerals and vitamins. However, in addition to these essential dietary components; egg contains about 200-250 mg of cholesterol. Concern regarding the cholesterol content of the human diet is growing, because of the probable importance of cholesterol and cholesterol oxide products in the development of atherosclerosis (Blankenship *et al*., 1991). Thus, egg processors are interested in finding technologies to reduce the cholesterol content of their products and use of nutritional strategies to reduce egg cholesterol concentrations is an attractive alternative.

Medicinal plants have been a major source of therapeutic agents since ancient times. *Nigella sativa* is also known as Kalongi, black cumin or black seed which grows in Asian and Mediterranean countries. The seed of *Nigella sativa* has been used for centuries in the Middle East, Northern Africa, Far East and Asia for the treatment of asthma (El-Tahir *et al.,* 1993) and as an antitumor agent (El-Daly, 1998). The seed has been reported to have many biological properties including antiparasitic (Mahmoud *et al*., 2002), antidiabetic (Al-Hader *et al.,* 1993) and diuretic effects (Zaoui *et al.,* 2000). Kalongi seeds have also been reported to possess a favorable effect on serum lipid profile by decreasing its total cholesterol, low density lipoprotein (LDL), triglycerides and elevating the high density lipoprotein (HDL) level (El-Dakhakhny *et al*., 2000).

The immune system benefits greatly from proper nutrition of the bird. In this respect, antioxidants play an important role in maintaining bird health, productive and reproductive performance. Some researchers have reported that antioxidant supplementation causes significant improvement in blood lipid parameters of humans. Selenium and vitamin E are inter-related; hence complete protection of living cells requires both vitamin E and selenium in the diet (Kanchana and Jeyanthi, 2010). In the light of lack of information on the influence of vitamin E and selenium on egg lipid profile, this study was planned to investigate the efficacy *Nigella sativa* alone and in combination with Selenium and Vitamin E on egg lipid profile.

MATERIALS AND METHODS

The study was conducted on a total of thirty six healthy Jabalpur colour birds of twenty eight weeks age. Seeds of *Nigella sativa* were obtained from the Department of Aromatic and Medicinal Plants, Agriculture College, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur and used as such for supplementation of the diet of birds. Selenium in the form of L (+) - selenomethionine was obtained from Fisher Scientific, U.S.A. and vitamin E in the form of α-Tocopherol from vegetable oil was obtained from Sigma- Aldrich, U.S.A.

Experimental design

Thirty six birds (28 weeks age) were randomly divided into three groups with twelve birds in each group. The birds of different groups were kept separately in individual cages and maintained under similar hygienic conditions. Group T_1 was kept as control.

Egg yolk lipid profile

Six eggs were collected from each dietary treatment group of birds on day 0, 28, 56 and 84 of the experiment. Yolk was collected from each egg for the extraction of lipids.

Extraction of Lipids from egg yolk

Lipid was extracted from the egg yolk by the method of Folch *et al.* (1957) using chloroform: methanol mixture (2:1 v/v).

Determination of Cholesterol and Triglycerides

Quantitative estimation of cholesterol and triglycerides in egg yolk was done using kit from Transasia Bio-Medicals Ltd., Mumbai in fully automated Biochemical analyzer (ERBA Manheim-EM 200, Germany). The levels of cholesterol and triglycerides were expressed as mg/ gm.

Statistical analysis

Statistical analysis of the data was done by using Hierarchial method described by Snedecor and Cochran (1994). Differences among the treatments and intervals were tested for significance by Duncan's Multiple Range Test (Duncan, 1955).

RESULTS

The efficacy of *Nigella sativa* alone and in combination with selenium and vitamin E on egg cholesterol in layers have been summarized in Table 2. Egg cholesterol was calculated in terms of mg/gm of egg yolk on day 0, 28, 56 and 84 of the experimental period in different treatment groups of birds. Untreated control (Group T_1) did not show any significant change in egg cholesterol and was 18.0±0.12 mg/gm on day 84 of the study. However, layers supplemented with *Nigella sativa*, selenium and vitamin E revealed significant ($p<0.05$) reduction in egg cholesterol from day 28 post treatment. The mean values of egg cholesterol in layers supplemented with 2 percent *Nigella sativa* (group T_2) and 2 percent *Nigella sativa*, Selenium @ 0.4 mg/kg feed and Vitamin E @ 100 mg/kg feed (group T_3), were 16.3±0.26 and 15.4±0.21 mg/gm of egg yolk, respectively, on day 84 of the study. While the percent reduction was calculated to be 8.94 and 14.92 percent, respectively on day 84 post treatment.

The mean values of egg triglycerides of layers as influenced by dietary supplementation of *Nigella sativa*, selenium and vitamin E have been presented in the Table 2. Untreated control group did not reveal any significant variation on day 0, 28, 56 and 84 of the experimentation and was 189.2±1.40 mg/gm yolk on day 84. Inclusion of *Nigella sativa* in feed of layers significantly ($p<0.05$) reduced the egg triglycerides level from 186.7±0.93 mg/ gm on day 0 to 178.7±1.33 mg/gm on day 84 post treatment. Supplementation of Selenium and Vitamin E along with *Nigella sativa* further reduced egg triglycerides level from 189.8±1.33 mg/gm on day 0 to 177.4±1.04 mg/gm on day 84 post treatment with 6.53 percent reduction.

DISCUSSION

In the present study, *Nigella sativa* alone and in combination with selenium and vitamin E was used to identify their hypocholesterolemic activity in laying hens in egg yolk lipid profile. *Nigella sativa* seeds (2 percent in feed) significantly ($p<0.05$) reduced the concentration of cholesterol and triglycerides in egg yolk of layers. The reduction in egg yolk cholesterol was found to be 8.94 percent as compared to control. This hypocholesterolemic effect of seeds of *Nigella sativa* has also been demonstrated by other researchers and supports our findings. Akhtar *et al*. (2003) demonstrated the cholesterol lowering effect of *Nigella sativa* seeds (1.5 percent in feed) in White-Leghorn layers with significant decrease in egg yolk cholesterol from 227.63 mg/egg to 199.72 mg/egg and serum triglyceride level from 941.4 to 896.6 mg/dl ($p<0.05$). Similarly, Aydin *et al*. (2008) reported that the

Table 1:
Design of experiment

Groups	No. of birds per treatment	Treatment	Sample collection
T_1	12	Standard Diet	Eggs were collected on day 0, 28, 56 and 84 of the study
T_2	12	Standard Diet+ *Nigella sativa* (20 gm/kg feed)	Same as T_1
T_3	12	Standard Diet+ *Nigella sativa* (20 gm/kg feed) + Selenium (0.4 mg/kg feed) + Vitamin E (100 mg/kg feed)	Same as T_1

Table 2:
Efficacy of *N. sativa,* selenium and vitamin E on egg cholesterol of Jabalpur colour bird

Treatment	Egg cholesterol (mg/gm yolk) Mean±SE				Percent reduction		
	Pre treatment	Post treatment			Day 28	Day 56	Day 84
	Day 0	Day 28	Day 56	Day 84			
T1	18.3^{A}±0.65	18.6^{A}±0.43	18.4^{A}±0.28	18.0^{A}±0.12	-	-	-
T2	17.9^{A}±0.64	17.3^{AB}±0.33	16.7^{B}±0.18	16.3^{B}±0.26	3.35	6.70	8.94
T3	18.1^{A}±0.46	16.9^{B}±0.33	16.0^{BC}±0.16	15.4^{C}±0.21	6.63	11.60	14.92
			Egg triglycerides (mg/gm)				
T1	189.2^{A}±1.15	189.4^{A}±1.14	189.2^{A}±1.66	189.2^{A}±1.40	-	-	-
T2	186.7^{A}±0.93	183.9^{AB}±1.43	180.4^{BC}±1.65	178.7^{C}±1.33	1.50	3.37	4.28
T3	189.8^{A}±1.33	183.4^{B}±1.67	179.9^{BC}±1.42	177.4^{C}±1.04	3.37	5.22	6.53

- Values are mean of six observations.
- Values in columns with different superscripts differ significantly ($p<0.05$)

inclusion of 2 percent black cumin in the rations significantly ($p<0.05$) decreased the concentration of cholesterol in egg yolk.

Researchers have reported that antioxidant supplementation causes significant improvement in blood lipid parameters of humans (Jain *et al*., 1996). Several animal studies have also shown that vitamin E and selenium supplementation affect lipoprotein metabolism by reducing serum triglycerides and total cholesterol and increasing HDL-cholesterol levels (Kanchana and Jeyanthi, 2010 and Skrivan *et al*., 2010). In the present experiment the indigenous herb *Nigella sativa* was included in the diet of layers along with vitamin E and Selenium and this combination further reduced the concentration of cholesterol and triglycerides in egg yolk as compare to indigenous herb alone.

It is concluded from this study that supplementation of indigenous herb *Nigella sativa* (20 gm/kg) and antioxidants viz. Selenium (0.4 mg/kg) and Vitamin E (100 mg/kg) in the feed of layers significantly reduced the concentration of cholesterol and triglycerides in egg yolk.

REFERENCES

Akhtar, M. S., Nasir, Z. and Abid, A. R. (2003). Effect of feeding powdered N*igella sativa* L. (kalongi) seeds on poultry egg production and their suitability for human consumption. *Veterinarski Arhiv*. **73**: 181-190.

Al-Hader, A., Aqel, M. and Hasan, Z. A. (1993). Hypoglycemic effects of the volatile oil of *Nigella sativa* seeds. *International J. Pharmacognosy.* **31**:96–100.

Aydin, R., Karaman, M., Cicek, T. and Yardibi, H. (2008). Black cumin (*Nigella sativa* L.) Supplementation into the diet of the laying hen positively influences egg yield parameters, shell quality, and decreases egg cholesterol. *Poultry Sc.* **87**:2590–2595.

Blankenship, J. W., Vangent, C. M., Sandberg, L. B., Roos, P. J. and Scarffenberg, J. A. (1991). Oxysterol incorporation into rat aorta resulting in elastin compositional changes. *Lipids.* **26**:381-386.

Duncan, D. B. (1955). Multiple range and multiple F tests. *Biometrics,* **11**: 1-42.

El-Dakhakhny, M., Mady, N. I. and Halim, M. A. (2000). *Nigella sativa* oil protects against induced hepatotoxicity and improves serum lipid profile in rats. *Arzenim Forsch Drug Res.* **50**: 832-836.

El-Daly, E. S. (1998). Protective effect of cysteine and vitamin E, *Crocus sativus* and *Nigella sativa* extracts on cisplatin-induced toxicity in rats. *Journal de Pharmacie de Belgique*. **53**:87–95.

El-Tahir, K. E. H., Ashour, M. M. and Al-Harbi, M. M. (1993). The respiratory effects of the volatile oil of the black seed (*Nigella sativa*) in guinea-pigs: Elucidation of the mechanism(s) of action. *General Pharmacology.* **24**:1115–1122.

Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957). A simple method for isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*. **226**: 497-509.

Jain, S. K., McVie, R., Jaramillo, J. J., Palmer, M., Smith, T., Meachum, Z. D. and Little, R. L. (1996). The effect of modest vitamin E supplementation on lipid peroxidation products and other cardiovascular risk factors in diabetic patients. *Lipids*. **31**: 87-90.

Kanchana, G. and Jeyanthi, G. P. (2010). The effect of supplementation of diet with vitamin-E and selenium and their combinations on the performance and lipid profiles of layer chickens. *International J. Pharma. Bio Sc.* **1**: 6-11.

Mahmoud, M. R., El-Ahbar, H. S. and Saleh. S. (2002). The effect of *Nigella sativa* oil against the liver damage induced by *Schistosoma mansoni* infection in mice. *J. Ethnopharmacol.* **79**:1–11.

Skrivan, M., Bubancova, I., Marounek, M. and Dlouha, G. (2010). Selenium and α-tocopherol content in eggs produced by hens that were fed diets supplemented with selenomethionine, sodium selenite and vitamin E. *Czech Journal of Animal Sc.* **55**: 388–397.

Snedecor, G. W. and Cochran, W. G. (1994). Statistical Methods, 7th Edn., Oxford and IBH Publishing Co., New Delhi, 350 p.

Zaoui, A., Cherrah, Y., Lacaille-Dubois, M. A., Settaf, A., Amarouch, H. and Hassar. M. (2000). Diuretic and hypotensive effects of *Nigella sativa* in the spontaneously hypertensive rat. *Therapie.* **55**:379–382.

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Research Article

PHARMACOKINETICS OF LEVOFLOXACIN IN POULTRY FOLLOWING SINGLE DOSE INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION

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ABSTRACT

The present study was undertaken to investigate the pharmacokinetics of levofloxacin @ 10.0 mg.kg^{-1} in poultry following single dose i.v. and i.m. administration. The concentration of levofloxacin in plasma of these animals were analysed by HPLC with a UV detector. The initial peak plasma concentration of 2.83 µg.ml^{-1} and 1.46µg.ml^{-1} of levofloxacin was observed in plasma of poultry following single dose i.v. and i.m. administration respectively. The volume of distribution, clearance, mean area under curve (AUC) and elimination half life calculated were 5758.4 ml.kg^{-1}, 1603.95ml.h^{-1}.kg^{-1}, 6.45h.µg.mL^{-1} and 2.85h following single dose i.v and 5719.04 ml.kg^{-1}, 1395.05ml.h^{-1}.kg^{-1}, 7.3 h.µg.ml^{-1} and 2.89, following single dose i.m. administration respectively. A priming dose of 7.0 mg.kg^{-1} and 10 mg.kg^{-1} followed by a maintenance dose of 6.4 mg.kg^{-1} and 9.5 mg.kg^{-1} was calculated and recommended after single dose i.v. and i.m. administration, respectively.

Key words: HPLC, intravenous, levofloxacin, pharmacokinetics, poultry, HPLC

INTRODUCTION

Levofloxacin is a new third generation fluoroquinolone effective against gram negative and gram positive bacteria (Martinez *et al*., 2006). Compared to other fluoroquinolones, levofloxacin has more pronounced bactericidal activity against organisms such as Pseudomonas and Enterobacteriaceae (Klesel *et al*., 1995). The bactericidal effect of levofloxacin is achieved through reversible binding to DNA gyrase and subsequent inhibition of bacterial DNA replication and transcription (Albarellos *et al*., 2005). The drug distributes well to target body tissues and fluids in the respiratory tract, skin, urine and prostrate and its uptake by cells makes it suitable for use against intracellular pathogens (Langtry and Lamb, 1998). However, it penetrates poorly into the CNS. Levofloxacin is metabolized in the liver to demethyl-levofloxacin and levofloxacin *N*-oxide. The pharmacokinetics of levofloxacin has been investigated in humans (Verho *et al*., 1996; Amsden *et al*., 1999; Chulavatnatol *et al*., 1999; Gascon *et al*., 2000), rabbits (Mochizuki*et al*., 1994; Destache *et al*., 2001), rats (Ito *et al*., 1999), guinea pigs (Edelstein *et al*., 1996) and in calves (Dumka and Srivastava, 2007). Fluoroquinolones act by a concentration dependent killing mechanism, whereby the optimal effect is attained by the administration of high doses over a short period of time. This concentration-dependent killing profile is associated with a relatively prolonged post antibiotic effect. In view of the marked species variation in the kinetic data of antimicrobial drugs, the present study was undertaken to determine the pharmacokinetics, and an appropriate dosage regimen of levofloxacin in poultry following single dose i.v. and i.m. administration.

MATERIALS AND METHODS

The pharmacokinetic study of levofloxacin were conducted in four poultry birds with an average weight of 1.0± 0.5kg. Levomac® (Levofloxacin hemihydrate infusion 0.5% obtained from Macleods Pharm. Ltd.) was injected as single dose 10.0mg.kg^{-1} i.v. and i.m. in poultry birds. A washout period of 14 days was followed in between single dose i.v. and i.m. The blood samples following single dose i.v. and i.m. administration from four birds were collected in heparinized tubes through an i.v. cannula placed in the contralateral jugular veins or wing vein at 0, 5, 10, 15, 30 min. and 1, 2, 4, 8, 12, 24, 48 and 72h after drug administration. Plasma was separated and stored at -20º C till analysis. The plasma proteins were removed via methanol precipitation; 200 µl plasma were mixed with 400 µl methanol and vigorously shaken. The precipitated proteins were removed via centrifugation at 12000×g for 5min. Subsequently, 20 µl of the supernatant were injected into the column. The mobile phase consisted of water (80%) and acetonitrile (20%) with 0.3% of triethylamine and the pH was adjusted to 3.3 with phosphoric acid, with a flow rate of 0.7 ml /min to be detected at UV wavelength of 295 nm. The calibration curves of plasma were prepared with different concentrations between 0.025 and 10 µg/ml using blank poultry plasma. Pharmacokinetic analysis of plasma levofloxacin concentration versus time data was conducted by using WinNonLin Professional version 5.3 software. The plasma concentration-time relationship was best estimated as a two-compartment open model for single dose i.v. $Cp = Ae^{-\alpha t} + Be^{-\beta t}$, and a one compartment model for i.m. $Cp^{(t)} = Be^{-\beta t}$.

Table 1:
Pharmacokinetic parameters of levofloxacin in poultry (n = 4) following single dose IV administration at a dose rate of 10mg.kg^{-1} .

Parameters	Units	Mean± S.E.	
		I.V.	I.M.
V1	ml.kg^{-1}	3359.82±334.06	-
V F	ml.kg^{-1}	-	5719.04±232.62
K_{10}	h^{-1}	0.49±0.08	0.24±0.02
K_{01}	h^{-1}	-	4.14±0.39
K_{12}	h^{-1}	1.14±0.52	-
K_{21}	h^{-1}	1.30±0.34	-
AUC	h.µg.ml^{-1}	6.45±0.65	7.30±0.54
K_{10}_HL	h	1.51±0.25	2.89±0.23
K_{01} _ HL	h	-	0.17±0.02
A	h^{-1}	2.71±0.92	-
	h^{-1}	0.25±0.02	-
Alpha HL	H	0.35±0.11	-
Beta HL	H	2.85±0.26	-
A	µg.ml^{-1}	1.69±0.29	-
B	µg.ml^{-1}	1.38±0.08	-
Cmax	µg.ml^{-1}	3.07±0.32	1.46±0.04
CL	ml.h^{-1}.kg^{1}	1603.95±182.28	-
CL_F	ml.h^{-1}.kg^{1}	-	1395.05±115.51
AUMC	h^{2}.µg.ml^{-1}	24.59±4.57	-
MRT	H	3.69±0.36	-
V_{ss}	ml. kg^{-1}	5758.40±131.49	-
V2	ml. kg^{-1}	2398.58±355.63	-
CLD2	ml.h^{-1}.kg^{1}	3402.03±1275.03	-

β (kel): elimination rate constant; **α (ka):** distribution (absorption) rate constant; **Alpha HL** : distribution half-life; **K_{10}_HL**: elimination half life (for i.m.) and distribution half life (for i.v.); **Beta HL**: elimination half-life; **V(ss):** volume of distribution; **V1**: volume of distribution in central compartment ; **V2**: volume of distribution in peripheral compartment ; **V_F**: volume of distribution where the absorption of drug is not known; **CL**: total body clearance; CL_F: total body clearance where the absorption of drug is not known**; AUC**: area under the curve from zero to infinity by the trapezoidal integral; **MRT**: mean residence time; **Cmax**: maximum plasma concentration; **Tmax**: time to peak concentration; **K_{10}** : rate constant for elimination of drug ; **K_{12}** : transfer of drug from central to peripheral compartment; **K_{21}**: transfer of drug from peripheral to central compartment; **K_{01}**: first order absorption rate constant.

RESULTS AND DISCUSSION

The mean plasma concentration-time profiles of levofloxacin following single dose i.v. and i.m. administrations of 10mg/kg b.wt are presented graphically in Figure 1. Mean± SE values of pharmacokinetic parameters estimated from the curve fitting are shown in Table 1. In the present study, the mean plasma concentrations of levofloxacin ranged from 0.042±0.002 mg.mL^{-1} to 2.83±0.14m g.mL^{-1}, the elimination half-life was 2.85±0.26 h following single dose (10mg.kg^{-1}) i.v. administration. Our findings could be corroborated with the findings of Varia *et al*. (2009) who calculated elimination half-life of 3.18h. However, lower elimination half-life (1.61h, (Dumka and Srivastava, 2007) and greater elimination half-life (3.29h, Goudah and Hasabelnaby, 2010) have been reported in calves and sheep, respectively. The results obtained in the present study suggested that levofloxacin is widely distributed in the tissues of birds as reflected by its volume of distribution (5758.40±131.49ml.kg^{-1}). Clearance (CL) of levofloxacin in chicken was 1603.95±182.28mL.h^{-1}.kg^{-1}, which is comparable to that obtained (1471 mL.h^{-1}.kg^{-1}) by Varia *et al*. (2009) following single dose i.v. administration. However, low clearance has been reported in calves (317 mL.hr^{-1}.kg^{-1}; Dumka and Srivastava, 2007) and in sheep (200 mL.h^{-1}.kg^{-1}, Goudah and Hasabelnaby, 2010). The mean area under curve (AUC) calculated was 6.45±0.65h.g.mL^{-1} which is lower than 12.7 and 21.61h.g.mL^{-1} observed in crossbred calves (Dumka and Srivastava, 2007) and sheep (Goudah and Hasabelnaby, 2010) respectively. On the basis of the present study, the priming and maintenance doses of levofloxacin, at a convenient dosage interval of 12 h, were calculated to be 7.0 and 6.4 mg.kg-1, respectively.

Following single dose i.m. administration the peak plasma concentration 1.46±0.04 g.ml^{-1} of the drug was attained at 1h post administration which decreased slowly to a minimum of 0.05±0.01 g.ml^{-1} at 48 h. An average plasma concentration of 0.032–0.5 µg/ml has been reported to be the minimum therapeutic concentration (MIC_{90}) of levofloxacin against most gram-positive, gram-negative and atypical bacteria (Chulavatnatol *et al*., 1999). In the present study, an average MIC_{90} of 0.1 µg.mL^{-1} of levofloxacin has been considered. The hybrid rate constant of absorption phase

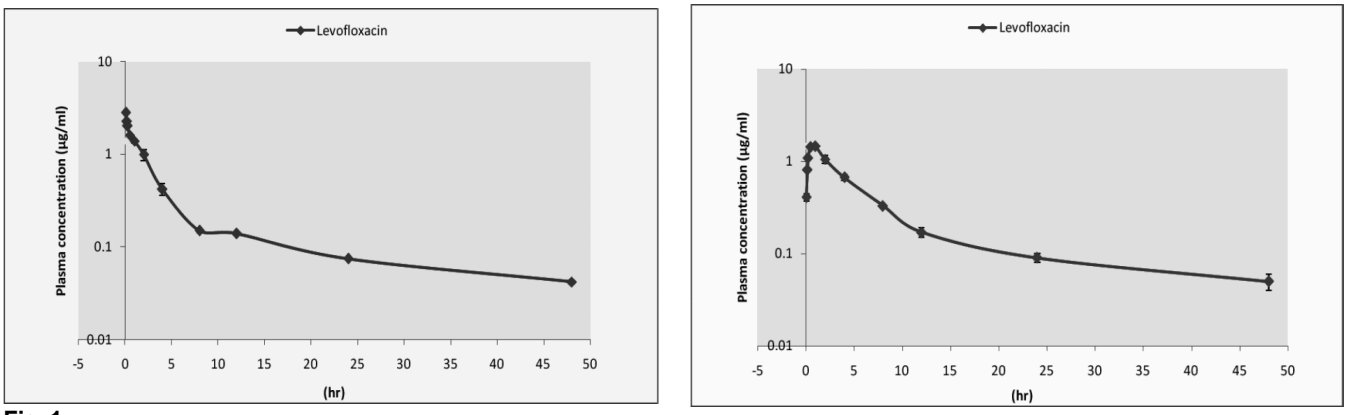

Fig. 1:
Semi logarithmic plot of levofloxacin concentrations (mean±SE) in plasma versus time following single dose i.v. and i.m. administration of levofloxacin (10 mg.kg^{-1}) in poultry (n=4)

(K_{01}) was 4.14±0.39 h^{-1} with absorption half-life (K_{01}_HL) of 0.17±0.02 h. The elimination rate constant (K_{10}) and elimination half-life (K_{10}_HL) were calculated to be 0.24±0.02 h^{-1} and 2.89±0.23 h, respectively in the poultry birds following single dose (10mg.kg^{-1}) i.m. administration. The elimination half- life in the present study (2.89±0.23h) could be compared with that reported for stallion (2.94h, Goudah *et al*., 2008). However, a high elimination half-life was reported in sheep (3.58h, Goudah and Hasabelnaby, 2010). In the present study, the volume of distribution and clearance were 5719.04±232.62mL.kg^{-1} and 1395.05±115.51 ml.h^{-1}.kg^{-1}, respectively. These values are higher than volume of distribution (1020 mL.kg^{-1}) and clearance (190 ml.h^{-1}.kg^{-1}) reported in sheep (Goudah and Hasabelnaby, 2010). AUC reported in the present study was 7.3±0.54h.µg.mL^{-1} which is lower than 17.21 h.µg.mL^{-1} observed in stallion (Goudah *et al*., 2008). The bioavailability of levofloxacin after intramuscular administration was calculated as the ratio of $AUC_{i.m.}$ / $AUC_{i.v.}$. In this study, a mean bioavailability of 117±16 percent was observed which is higher than 91.35±6.81percent observed in sheep (Goudah and Hasabelnaby, 2010) suggesting complete absorption of drug after i.m. administration for clinical application.

REFERENCES

Albarellos, G.A., Ambros, L.A. and Landoni, M.F. (2005). Pharmacokinetics of levofloxacin after single intravenous and repeated oral administration to cats. *J. Vet. Pharmacol. Therap.* **28(4)**: 363–369.

Amsden, G.W., Graci, D.M., Cabelus, L.J. and Hejmanowski, L.G. (1999). A randomized, crossover design study of the pharmacology of extended-spectrum fluoroquinolones for pneumococcal infections. *Chest*. 116: 115–119

Chulavatnatol, S., Chindavijak, B., Vibhagool, A., Wananukul, W., Sriapha, C. and Sirisangtragul, C. (1999). Pharmacokinetics of levofloxacin in healthy Thai male volunteers. *J. Med. Assoc. Thai.* **82**: 1127-1135.

Destache, C.J., Pakiz, C.B., Larsen, C., Owens, H. and Dash, A.K. (2001). Cerebrospinal fluid penetration and pharmacokinetics of levofloxacin in an experimental rabbit meningitis model. *J. Antimicrob. Chemother.* **47**: 611.

Dumka, V. K. and Srivastava, A. K. (2007). Disposition kinetics, urinary excretion and dosage regimen of levofloxacin formulation following single intravenous administration in cross bred calves. *Vet. Res. Commun*. **31**: 873-879.

Edelstein, P. H., Edelstein, M. A., Lehr, K. H. and Ren, J. (1996). *In vitro* activity of levofloxacin against clinical isolates of *Legionella* spp., its pharmacokinetics in guinea pigs, and use in experimental *Legionella pneumophila* pneumonia. *J. Antimicrob. Chemother.* **37**: 117-126.

Gascon, A.R. Campo, E. Hernandez, R.M. Calvo, B. Errasti, J. and Pedraz Munoz, J.L. (2000). Pharmacokinetics of ofloxacin enantiomers after intravenous administration for antibiotic prophylaxis in biliary surgery. *J. Clinical Pharmacol*. **40**: 869–874.

Goudah, A. and Hasabelnaby, S. (2010). Disposition kinetics of levofloxacin in sheep after intravenous and intramuscular administration. *Vet. Med. Int*. Article ID 727231 pp. 6.

Goudah, A., Abo El-Sooud, K., Shim, J.H., Shin, H.C. and Abd El-Aty, A.M. (2008). Characterization of the pharmacokinetic disposition of levofloxacin in stallions after intravenous and intramuscular administration. *J. Vet. Pharmacol. Therap.* **31(5)**: 399–405.

Ito, T., Yano, I., Masuda, S., Hashimoto, Y. and Inui, K. (1999). Distribution characteristics of levofloxacin and grepafloxacin in rat kidney. *Pharmacol. Res*. **16**: 534–539

Klesel, N., Geweniger, K. H., Koletzki, P., Isert, D., Limbert, M., Markus, A., Riess, G., Schramm, H. and Iyer, P. (1995). Chemotherapeutic activity of levofloxacin (HR355,DR-3355) against systemic and localized infections in laboratory animals. *J. Antimicrob. Chemother*. **35(6):** 805-819.

Langtry, H. D. and Lamb, H. M. (1998). Levofloxacin: its use in infections of the respiratory tract, skin, soft tissues and urinary tract. *Drugs*. **56**: 487-515.

Martinez, M., McDermott, P. and Walker, R. (2006). Pharmacology of the fluoroquinolones: a prespective for the use in domestic animals. *Vet. J*. **172 (1):** 10-28.

Mochizuki, K., Ohkubo, S., Torisaki, M., Yamashita, Y., Tanahashi, T., Jin, Y., Okamura, Y. and Tanaka, M. (1994). Intraoccular penetration of oral levofloxacin in rabbits. *Nippon Ganka Gakkai Zasshi*. **98**: 1085–1090.

Varia, R.D., Patel, J.H., Patel, U.D., Bhavsar, S.K. and Thaker, A.M. (2009). Disposition of levofloxacin following oral administration in broiler chickens. *Israel J. Vet. Med*. **64(4):** 181-121.

Verho, M., Malerczyk, V., Damm, D. and Lehr, K.H. (1996). Pharmacokinetics of levofloxacin in comparison to the racemic mixture of ofloxacin in man. *Drug Metabol. Drug Int*. **13**: 57–67.

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Research Article

PHARMACOKINETICS OF ENROFLOXACIN IN CATTLE FOLLOWING INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION

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ABSTRACT

The present study was undertaken to evaluate the pharmacokinetics of enrofloxacin in male cattle following intravenous and intramuscular administration at a single dose of 5.0 mg kg^{-1} body weight. Plasma concentrations of enrofloxacin were determined by a high performance liquid chromatographic method. A two compartment model adequately described plasma concentration time profile of enrofloxacin following single dose intravenous administration whereas, the disposition kinetics following intramuscular administration fitted well to one compartment model. The d_{area}, Vd_{ss}, AUC and Cl_B were calculated as 7.524 $L.kg^{-1}$,6.099 $L.kg^{-1}$, 3.614 $\mu g.h.ml^{-1}$ and 1.657 L.kg-1.h^{-1}, respectively, following intravenous administration and 5.585 $L.kg^{-1}$,4.396 L.kg-^{1,} 4.694 $\mu g.h.ml^{-1}$ and 1.282 L.h^{-1}.kg^{-1} respectively, following intramuscular administration. The bioavailability (F) of enrofloxacin following intramuscular administration was 133.619 %. Based on pharmacokinetic data, a priming dose of 9.929 and a maintenance dose of 9.177 mg.kg^{-1} at 12 h interval following intravenous administration and the priming dose of 9.258 and a maintenance dose of 8.699 mg.kg^{-1} at 24 h interval following intramuscular administration was calculated in cattle.

Key words: Ciprofloxacin, cattle, enrofloxacin, HPLC, pharmacokinetics.

INTRODUCTION

Enrofloxacin is a veterinary quinolone antibiotic with actions and uses similar to ciprofloxacin, a counterpart for human use. It belongs to the group of synthetic 6 – fluoroquinolones or 4 – quinolones and has a strong, broad spectrum bactericidal effect (Prescott and Yielding, 1990) related to the deactivation of the enzyme DNA gyrase – a type II topoisomerase (Shen *et al.*, 1989). Because of its high bioavailability following both parenteral and oral administration, the excellent tissue distribution and intracellular penetration, attainment of bactericidal activity in low tissue concentrations (Scheer, 1987) and the relatively slow and limited appearance of resistance (Boothe, 1994), enrofloxacin is approved as a highly effective chemotherapeutic with extensive clinical application. Since, not much work has been reported earlier regarding the pharmacokinetic properties of enrofloxacin in male cattle and no literature is available in context of HPLC analysis, the present study was carried out to generate its pharmacokinetic data in male cattle involving HPLC analysis.

MATERIALS AND METHODS

The present study was conducted in five adult, cross-bred male cattle (2.0-2.5 yrs in age, weighing 170 ± 20 kg) procured from instructional dairy farm of the university. The animals were kept on closed housing system with *ad libitum* stall- feeding of green and dry fodder and supplemented with concentrate ration . A pre-experimental period of one month before the commencement of experiment was given to the animals for their acclimatization to the new environment. During this pre-experimental period all the animals were dewormed with ivermectin (Ivectin®, 1% ivermectin injection) @ 0.2 mg.kg^{1} b.w. and albendazole @ 10mg/kg body weight one month prior to the experiment. Physical and clinical examination was done before the start of experiment.

Gyroflox® (enrofloxacin, 10 % w/v injection, M/S Indian Immunologicals Ltd., New Delhi) was used for the pharmacokinetic study. The drug was injected as a single dose (5 mg kg^{-1}) intravenous in jugular vein and intramuscular in rump region in all the animals. An intervening wash out period of 20 days was given to all the animals before commencement of pharmacokinetic study following i.m. administration. The blood samples were collected from contralateral jugular vein of each animal in marked heparinized microcentrifuge tubes by disposable plastic syringes at time interval of 0, 0.03, 0.08, 0.17, 0.25, 0.50, 0.75, 1, 2, 4, 6, 8, 12, 24, 36, 48 h. The blood samples collected in heparinized tubes following administration of enrofloxacin were centrifuged at 3000 rpm (20 min) for separation of plasma. The plasma thus obtained was collected in microcentrifuge tubes and stored at -20 ˚C till further analysis. Drug extraction from plasma sample was carried out as per the method of Neilson and Gyrd-Hansen (1997) with slight modification.

The analysis of plasma samples for enrofloxacin was done as described by Kung *et al.* (1993) with an isocratic mobile phase consisting of acetonitrile, methanol and deionized water (all HPLC grade) in the ratio of 17:3:80,

v/v/v containing 0.4 percent triethylamine and 0.4 percent orthophosphoric acid (85%, v/v). The pH of mobile phase ranged from 2.5-3.0. Drug estimation in plasma was done by high performance liquid chromatography. Separation was achieved using C_{18} reverse phase column, particle size 5 µm (4x 150 mm,) as a stationary phase. The flow rate was kept at 0.6 ml.min^{-1} and the elution was monitored at 20 ± 2 ^{0}C with UV detection at 278 nm. The chromatogram was analyzed by 'Chromatopak'. Enrofloxacin and ciprofloxacin were quantified from their respective peaks. The standard calibration curve for enrofloxacin and ciprofloxacin was obtained by plotting concentrations *vs* mean of the peak areas obtained for their respective standards. The limit of quantification (LOQ) for enrofloxacin and ciprofloxacin was 0.0125 µg.ml^{-1}.

The pharmacokinetic analysis of the plasma concentrations of drug obtained following i.v. and i.m. administration in this study was carried out by pharmacokinetic software 'Pharmkit'.

RESULTS AND DISCUSSION

A two-compartment model adequately described plasma concentration-time profile of enrofloxacin in adult male cattle following single dose i.v. administration as the drug distributed rapidly into the central compartment and comparatively slowly into the peripheral compartment. The plasma concentration-time profile following single dose (5 mg.kg^{-1}) i.v. administration of enrofloxacin in adult male cattle depicted the mean concentration of enrofloxacin at 0.03 h post as 2.814 ± 0.154 µg.ml^{-1} which declined rapidly to 0.839 ± 0.125 µg.ml^{-1} at 0.50 h and then decreased slowly to a minimum of 0.062 ± 0.013 µg.ml^{-1} in 12 h, whereas the plasma concentration-time profile of ciprofloxacin (as a metabolite of enrofloxacin) evidenced a fast increase in its plasma concentration from an initial value of 0.394 ± 0.056 µg.ml^{-1} at 0.03 h to attain a peak value of 0.606 ± 0.059 µg.ml^{-1} at 0.25 h, after which it declined to a minimum of 0.067 ± 0.018 g.ml^{-1} at 12 h. The initial plasma concentration of enrofloxacin (2.814 ± 0.154 µg.ml^{-1}) obtained in the present study was about double as that reported by Amorena *et al.* (1992) following a dose rate of 2.5 mg.kg^{-1} in buffaloes (1.784 ± 0.346 µg.ml^{-1}) while it was comparable to that obtained by Rahal *et al*., 2006 at same dose rate in sheep. The combined therapeutic concentration (e" 0.1 g.ml^{-1}) was maintained for more than 12 h. Kaartinen *et al.* (1995) have reported detectable antimicrobial activity in lactating cows up to 8 h, whereas in rabbits detectable concentration was observed up to 10 h (Broome et al., 1991). The pharmacokinetic parameters describing the disposition kinetics of enrofloxacin following single dose (5 mg.kg^{-1}) intravenous administration are presented in Table I.

Table 1:
Pharmacokinetic parameters of enrofloxacin in plasma following its single dose (5 mg.kg^{-1}) i.v. administration in adult male cattle (n=5)

Parameters	Unit	Mean ± S.E.
A	ìg.ml^{-1}	2.494 ± 0.122
B	ìg.ml^{-1}	0.602 ± 0.099
á	h^{-1}	3.722 ± 1.046
â	h^{-1}	0.215 ± 0.022
$t_{1/2\ á}$	h	0.152 ± 0.03
$t_{1/2\ â}$	h	3.376 ± 0.385
$AUC_{0-\infty}$	ìg.h.ml^{-1}	3.614 ± 0.754
AUMC	ìg.h^2.ml^{-1}	15.39 ± 4.662
MRT	h	3.969 ± 0.403
$Vd_{(area)}$	L.kg^{-1}	7.524 ± 1.175
Vd_{ss}	L.kg^{-1}	6.099 ± 0.846
Cl_B	L. h^{-1}.kg^{-1}	1.657 ± 0.361

Table 2: Pharmacokinetic parameters of enrofloxacin in plasma following its single dose (5 mg.kg^{-1}) i.m. administration in adult male cattle (n=5).

Parameters	Unit	Mean ± S.E.
B´	ìg.ml^{-1}	1.272 ± 0.125
K_a	h^{-1}	4.667 ± 1.351
K_e	h^{-1}	0.306 ± 0.065
$t_{1/2\ Ka}$	H	0.201 ± 0.046
$t_{1/2\ Ke}$	H	2.587 ± 0.389
C_{max}	ìg.ml^{-1}	1.222 ± 0.179
T_{max}	H	0.650 ± 0.100
$AUC_{0-\infty}$	ìg.h.ml^{-1}	4.694 ± 0.772
AUMC	ìg.h^2.ml^{-1}	19.660 ±4.320
MRT	H	3.921 ± 0.534
Vd/F	L.kg^{-1}	5.585 ± 0.794
Vd_{ss}	L.kg^{-1}	4.396 ± 0.412
Cl_B	L.h^{-1}.kg^{-1}	1.282 ± 0.342
F	%	133.619 ±12.314

The mean values of zero time intercept of distribution phase (A) and elimination phase (B) in the present study were calculated to be 2.494 ± 0.122 µg.ml^{-1} and 0.602 ± 0.099 µg.ml^{-1}, respectively. The mean elimination rate constant (β) was 0.215 ± 0.022 h^{-1} in the present study which was quite similar to the value of 0.283 ± 0.024 h^{-1} obtained after intravenous administration of enrofloxacin in goats (Rao *et al.*, 2002), respectively. The elimination half-life and total plasma clearance in present study were 3.376 ± 0.385 h and 1.657 ± 0.361 L.h^{-1}.kg^{-1} following single dose i.v. administration of enrofloxacin. The half-life indicates the rate of elimination of the drug and total plasma clearance of the drug is the volume of the blood or plasma cleared of drug by metabolism and excretion per unit of time. It is better index of efficiency of drug elimination than half-life as it gives the clearance of the drug from blood per unit of time. The elimination half-life in the present study (3.376 ± 0.385 h) was quite comparable to that obtained in cattle calves (3.53 h) by Garcia *et al*.(1996). However, the slower elimination in sheep has also been determined (Rahal *et al*., 2006). MRT of enrofloxacin in the present study was found to be 3.969 ± 0.403 h suggesting the retention of the drug for quite good duration in the body. The distribution half-life of enrofloxacin in the present study indicated an easy and efficient distribution of enrofloxacin in the body tissues

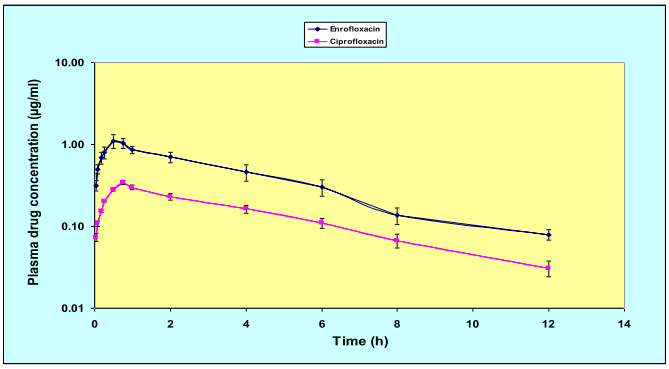

Fig 1:
Semilogarithmic plot of enrofloxacin and ciprofloxacin concentrations (mean± SE) in plasma versus time following i.v. administration of enrofloxacin (5 mg.kg^{-1}) in adult male cattle (n=5).

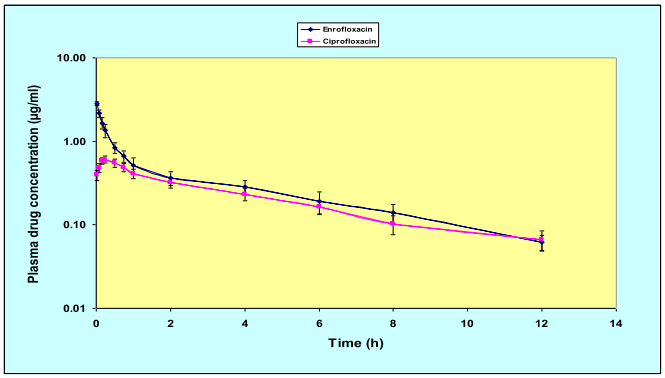

Fig 2:
Semilogarithmic plot of mean enrofloxacin and ciprofloxacin concentrations (mean± SE) in plasma versus time following i.m. administration of enrofloxacin (5 mg.kg^{-1}) in adult male cattle (n=5).

and fluids of cattle as reported in sheep (0.16 ± 0.04 h), Rahal *et al*. (2006). while much shorter distribution half-life has been reported in goats (Rao *et al*., 2002). In the present study, enrofloxacin was widely distributed in the tissues of cattle as reflected by its Vd_{area} (7.524 ± 1.175 L.kg^{-1}) which is much higher than most of the studies carried out till date. The area under curve (AUC) is the parameter that integrates both time and intensity of drug concentration. The AUC characterizes the relative availability of the drug in the body (Dudley, 1991). It is used for calculating drug clearance and other non-compartmental kinetic variables. A value of 3.614 ± 0.754 µg.h.ml^{-1} was observed following single dose i.v. administration of enrofloxacin in present study which was lower than reported earlier by Kaartinen *et al*., (1994) in cattle (7.42 ± 1.02 µg.h.ml^{-1}). Higher levels of AUC have also been reported in sheep (Rahal *et al*., 2006) and goat (Elmas *et al,* 2001).

The plasma concentration-time profile of enrofloxacin following single dose (5 mg.kg^{-1}) i.m. administration in adult male cattle was adequately described by one-compartment model. It depicted the mean peak plasma concentration of 1.104 ± 0.210 µg.ml^{-1} at 0.50 h post administration which decreased slowly to a minimum of 0.079 ± 0.012 µg.ml^{-1} at 12 h. Concentration of ciprofloxacin (as a metabolite of enrofloxacin) paralleled the parent drug and attained a mean plasma concentration peak of 0.344 ± 0.019 µg.ml^{-1} at 0.75 h. The plasma ciprofloxacin concentration was also observed up to 12 h post administration of enrofloxacin intramuscularly. The pharmacokinetic parameters describing the disposition kinetics of enrofloxacin following single dose (5 mg.kg^{-1}) intramuscular administration are presented in Table 2.

The time to reach peak plasma concentration in the present study was in accordance to that reported in buffalo bull (Verma *et al*., 1999). The values of absorption half-life (0.201 ± 0.046 h), distribution rate constant (4.667 ± 1.351 h^{-1}), volume of distribution (5.585 ± 0.794 L.kg^{-1}), AUC (4.694 ± 0.772 µg.h.ml^{-1}) and time required to reach the highest concentration (T_{max}, 0.650 ± 0.100 h) suggested the rapid absorption from the site of administration and wide distribution in the body fluids and tissues. The elimination of the drug indicated by half-life of 2.587 ± 0.389 h suggested fast elimination from the body. C_{max} in the present study (1.222 ± 0.179 µg.ml^{-1}) was comparable to that reported by Varma *et al*. (2006) in non lactating cows whereas further lower level has been reported in lactating cows Kaartinen *et al*., 1995 and mastitic cows (Sharma *et al*., 2005). C_{max} of enrofloxacin in the present study was similar as reported in non lactating cows (Varma *et al*., 2006) at the same dose.

The mean AUC value of 4.694 µg.h.ml^{-1} and MRT of 3.921 ± 0.534 h found along with large volume of distribution (5.585 ± 0.794 L.kg^{-1}) suggested retention of the drug for longer duration which might be due to wide distribution of the drug across the body fluids and tissues after i.m. administration on account of its lipid solubility. The bioavailability of enrofloxacin after intramuscular administration was compared by the ratio of $AUC_{i.m.}$ / $AUC_{i.v.}$. In this study, a mean bioavailability of 133.619 ± 12.314 percent was observed suggesting complete absorption after i.m. administration for clinical application. Kaartinen *et al.* (1997) has reported comparatively less intramuscular bioavailability (82 ± 14 percent) in lactating cows at the same dose. Some other studies have also revealed the intramuscular bioavailability near to 100 percent (Garcia, 1996) which indicated almost complete absorption of the drug after intramuscular administration. It is concluded from the study that enrofloxacin is well absorbed and distributed after i.m. administration.

The principle objective of disposition kinetics is to obtain a suitable dosage regimen and the goal is to achieve a successful clinical outcome. Full knowledge of interrelationship between pharmacodynamics and pharmacokinetics is important in choosing an appropriate

antibiotic and determining its optimal dosage regimens for treatment of various infections. Keeping a therapeutic level of 0.1 $mg.kg^{1}$ of enrofloxacin as is found to be MIC for most of the susceptible micro-organisms, the dosage regimen of 9.929 $mg.kg^{1}$ as priming dose and 9.177 $mg.kg^{1}$ as maintenance dose at 12 h interval following intravenous administration and 9.258 $mg.kg^{1}$ as priming dose and 8.699 $mg.kg^{1}$ as maintenance dose at 24 h interval following intramuscular administration is recommended in adult male cattle.

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REFERENCES

Amorena, M., Olina, G. and Luna, R.D.C. (1992). Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after i.v. and i.m. administration in sheep. *Am. J. Vet. Res.* **57:** 1040-1043.

Boothe, D.M. (1994). Enrofloxacin revisited. *Vet. Med.* **(8):** 744-754.

Broome, R.L., Brooks, D.L., Babish. J.G., Copeland, D.D. and Conzelman, G.M. (1991). Pharmacokinetic properties of enrofloxacin in rabbits. *Am. J. Vet. Res.* **52(11):**1835–1841.

Dudley, M.N. (1991). Pharmacodynamics and pharmacokinetics of antibiotics with special reference to the fluroquinolones. *Am. J. Vet. Med.* **91:** 455-505.

Elmas, M., Tras, B., Kaya, S., Bas, A. L., Yazar, E. and Yarsan E. (2001). Pharmacokinetics of enrofloxacin after intravenous and intramuscular administration in Angora goats. *Can. J. Vet. Res.* **65(1):** 64–67.

Garcia Ovando, H., Errecalde, C., Priesto, G., Luders, G, Puelles, I., Berecochea, C. and Fernandez, M. (1996). Pharmacokinetics of enrofloxacin in calves. *Archivos de Medicina Veterinaria.* **28:** 107-111.

Kaartinen, L., Alli, L. and Pyorala, S. (1994). Pharmacokinetics of enrofloxacin in lactating cows. Proc.XVIII .World Buiatrics Congress, Aug. 29 sept.2, Bologna, Italy.599-601.

Kaartinen, L., Salonen, M., Alli, L. and Pyorala, S. (1995). Pharmacokinetics of enrofloxacin after single intravenous, intramuscular and subcutaneous injections in lactating cows. *J. Vet. Pharmacol. Ther.* **18(5):** 357–362.

Kaartinen, L., Pyorala, S., Moilanen, M. and Raisanen, S. (1997). Pharmacokinetics of enrofloxacin in newborn and one-week-old calves. *J. Vet. Pharmacol. Ther.* **20(6):** 479–482.

Kung, K., Riond, J.L., Wanner, M. (1993). Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after intravenous and oral administration of enrofloxacin in dogs. *J. Vet. Pharmacol. Therap.* **16**: 462-468.

Neilsen, P. and Hansen, N.G. (1997). Bioavailability of enrofloxacin after oral administration to fed and fasted pigs. *Pharmacol. Toxicol.*, **80:** 246-250.

Prescott, J. F. and K. M. Yielding, (1990). *In vitro* susceptibility of selected veterinary bacterial pathogens to ciprofloxacin, enrofloxacin and norfloxacin. *Can. J. Vet. Res.* **54:** 195-197.

Rahal, A., Kumar, A.; Ahmad, A.H., Malik, J.K. and Ahuja, V. (2006). Pharmacokinetics of enrofloxacin in sheep following intravenous and subcutaneous administration. *J. vet. Pharmacol. Therap.* **29:** 321–324.

Rao, G. S. (1999). Pharmacokinetic studies of enrfofloxacin in normal and febrile goats. Ph.D. thesis. Indian Veterinary Research Institute, Izatnagar, India.

Rao, G. S., Ramesh,S., Ahmad, A. H., Tripathi, H. C., Sharma, L. D. and Malik, J. K. (2002). Disposition kinetics of enrofloxacin and ciprofloxacin following intravenous administration of enrofloxacin in goats. *Small Rum. Res.* **44(1):** 9-15.

Scheer, M. (1987a). Studies on the antibacterial activity of Baytril. *Veterinary Medical Rev.* **2:** 90-97.

Sharma, P.K., Ahmad, A.H., Varma, R., Gautam, V., Ahuja, V.,Pradeep, K. and Singh, K.P. (2005). Disposition kinetics of enrofloxacin in mastitic cows following multiple dose intramuscular administration. *Ind. J. Vet. Med.* **25(1):**1-15

Shen, L.L., W. E. Kohlbernner, D. Weigl and J. Baranowski, (1989). Mechanism of quinolone inhibition of DNA gyrase. Appearance of unique norfloxacin binding sites in enzyme-DNA complexes. *J. Biol. Chem.* **264(5):** 2973-2978.

Varma, R., Ahmad, A.H., Ahuja, V., Gautam, V., Pardeep, K. and Singh, K.P. (2006). Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin and their interaction with diclofenac sodium in cows. *Ind. J. Ani. Sci.* **76(1):** 6-9

Verma, H.K.,Pangawkar, G.R., Chaudhary, R.K. and Srivastava, A.K. (1999). Pharmacokinetics and dosage regimen of enrofloxacin in buffalo bulls after intramuscular administration. *Vet. Res. Commun.* **23(8)**: 501-505.

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Research Article

ACUTE TOXICITY STUDY OF BUPARVAQUONE IN RATS

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ABSTRACT

The study was designed to evaluate the acute toxic effects of Theilex® (buparvaquone, 5% w/v injectable solution) in rats. Wistar rats of age 2 to 2.5 months, weighing between 150 to 200 gm, were selected and randomly divided into five groups of 4 rats each. An initial dose of 50 mg/kg b.wt. i.p. was used and then it was serially increased to 100, 150, and 200 mg/kg b. wt. After 14 days, the animals were sacrificed and blood, liver, kidney and brain samples were collected for the evaluation of hematological, biochemical and antioxidative parameters. Haemotoxic effect of buparvaquone was seen at the dose level of 100-150 $mg.kg^{-1}$ b.w. but there was no hepatotoxic effect at this dose. There was no elevation in the level of oxidative stress in erythrocytes except at dose level of 150 $mg.kg^{-1}$b.w where a slight elevation in the oxidative stress level was found to occur in liver.

Keywords: Theilex, buparvaquone, acute toxicity, rats

INTRODUCTION

Buparvaquone is a second-generation hydroxynaphthaquinone antiprotozoal drug which belongs to the group of the naphthaquinones. Naphthaquinone compounds have antimicrobial as well as fungicidal properties (Kayser and Kiderlen, 2003). It is a promising compound for the therapy and prophylaxis of all forms of theileriasis. It has been tested against *T. annulata, T. parva* and *T. sergenti,* both in laboratory studies and in field trials and has undergone various toxicological studies (McHardy, 1988). In an acute oral toxicity study in rat, the observed LD_{50} value for buparvaquone was more than 8000 mg/kg (McHardy, 1988). Atovaquone and buparvaquone are less toxic as compared to other compounds.

MATERIALS AND METHODS

Wistar rats of 2 to 2.5 months age, weighing between 150- 200 gm, were selected for the study. They were kept in plastic cages and acclimatized for two weeks in the experimental animal house of the department under standard managemental conditions. Standard feed and water were provided *ad libitum* throughout the experimental period to the animals and they were kept under constant observation during entire period of study. Rats were randomly divided into four groups (three test groups and one control group) with 4 rats in each group. Experiment was designed as per OECD guidelines. An initial dose of 50mg/kg b.wt. i.p (as per OECD guidelines) was used. Then dose was serially increased to 100, 150, and 200 mg/kg b.wt. Animals were kept under constant observation for consecutive 14 days and examined regularly for any clinical signs as well as mortality due to toxicity. On 15th day all the animals were sacrificed. The viscera and internal organs were examined for gross lesions and the blood, liver, kidney and brain samples were collected to evaluate hematological, biochemical and antioxidative parameters. Blood samples collected were subjected for ALT, AST, cholesterol, urea, creatinine, glucose, total protein, albumin, globulin and albumin: globulin ratio. Haematological parameters were estimated in blood as per the method given by Jain (1986) and Natt and Herric (1952). Lipid peroxidation (LPO) and reduced glutathione (GSH) were estimated in diluted RBC and tissues as per the method of Rehman (1984), Prins and Loos (1969) and Sedlak and Lindsay (1968), respectively. Data obtained in the present study were statistically compared by analysis of variance (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Acute toxicity of buparvaquone was performed in the wistar rats to test the safety of the formulation. None of the adult male rats receiving buparvaquone @ 50- 150 mg/kg i.p. upto 14 days showed any change in behavioral signs and symptoms. Hematological profile of rats treated with Theilex® (buparvaquone, 5% w/v injection) is shown in Table 1. Results showed that there was significant decrease in the value of Haemoglobin (Hb) , packed cell volume (PCV), total erythrocyte count (TEC) and total leucocyte count (TLC). Haemolysis and shrinkage in RBC might be the reason for decline in cell count (TEC, TLC) and Hb in treated groups at high dose of buparvaquone (Singh *et al*., 2004). Pooling of the circulating blood cells in the spleen or other reservoirs secondary to decreased sympathetic activity also explains the decrease in Hb, PCV and TLC (Mohammed *et al*., 2001).

Effect of buparvaquone on biochemical profile of rats is depicted in Table 2. No significant change in the estimated biochemical parameters (glucose, creatinine and total cholesterol) could be observed in test groups as compared to control. There were significant decrease in

Table 1:
Effect on haematological parameters profile following single i.p. administration of Theilex® (buparvaquone, 5% w/v Injection) in rats

Parameter	Control	Test Group I	Test Group II	Test Group III
Haemoglobin(g/dl)	13.75±0.32	11.63±0.24[a]	13.13±0.66	11.75±0.14[a]
PCV(%)	41.75±0.85	37.25±1.11[a]	40.25±1.65	37.25±0.75[a]
TEC(10^6/ul)	6.93±0.15	6.44±0.21	6.13±0.14[a]	5.88±1.18[a]
TLC(10^3/ul)	8.44±0.21	9.39±0.80	7.13±1.11	5.51±0.64[a]

Table 2:
Effect on serum biochemical profile and serum enzymatic profile following single i.p. administration of Theilex® (buparvaquone, 5% w/v Injection) in rats

Parameter	Control	Test Group I	Test Group II	Test Group III
Total protein (g/dl)	8.49±0.35	9.25±0.41	7.07±0.06[a]	6.59±0.29[a]
Albumin(g/dl)	4.2±0.17	3.64±0.12	3.89±0.28	3.67±0.05
Globulin(g/dl)	4.57±0.21	5.97±0.25[a]	3.62±0.30[a]	3.06±0.36[a]
Glucose (mg/dl)	164.31±5.23	201.22±4.62	161.74±28.88	172.35±14.69
Creatinine (mg/dl)	1.14±0.15	0.98±0.35	0.56±0.08	1.02±0.31
Total cholesterol(mg/dl)	66.27±1.49	55.74±4.51	51.12±10.16	61.36±10.51
Urea (mg/dl)	28.69±1.45	27.13±0.62	20.99±0.74[a]	16.46±1.20[a]
SGOT (U/L)	75.43±0.95	75.84±3.27	86.99±4.71	92.37±5.52
SGPT (U/L)	72.18±0.79	69.61±2.03	41.05±1.33[a]	83.89±17.46

Table 3:
Effect on antioxidative profile following single i.p. administration of Theilex® (buparvaquone, 5% w/v Injection) in rats.

Parameter	Tissue	Control	Test Group I	Test Group II	Test Group III
LPO (nM MDA.ml^{-1})	RBC	34.95±2.38	36.60±3.60	43.90±1.38	34.55±4.09
	Liver	6.35±0.17	8.87±0.31	7.75±0.57	7.72±1.85
	Kidney	43.24±0.92	34.05±1.25[a]	25.07±0.79[a]	35.27±3.40[a]
	Brain	42.66±1.13	50.37±0.88	40.35±6.83	44.87±1.76
GSH (mM.ml^{-1})	RBC	0.16±0.01	0.28±0.03	0.54±0.10[a]	0.95±0.13[a]
	Liver	0.73±0.02	0.59±0.04[a]	0.57±0.02[a]	0.60±0.05[a]
	Kidney	0.55±0.01	0.60±0.04	0.59±0.02	0.66±0.03[a]
	Brain	0.60±0.01	0.59±0.02	0.59±0.02	0.49±0.03[a]

Values in table are Mean ± S.E. (n = 4); a= Significant (P<0.05) as compared to group I with same column.

urea, total protein and globulin levels but no significant change was observed in albumin levels of test groups as compared to control. Also no significant changes were observed in enzymatic profile (SGOT and SGPT) of test groups as compared to control. Reduced serum total protein in rats may be due to decrease in secretion of insulin.

Effect of buparvaquone on RBC and tissue antioxidative profile of rats is depicted in Table 3. No significant changes could be observed in the whole RBC and tissue LPO profile of the animals, but significant reduction was observed in kidney LPO level of rats. Also significant changes in the RBC and tissue GSH profile (liver and brain) were observed in the test groups. Fall in glutathione level might have been due to its utilization for scavenging the reactive oxygen radicals (ROS) produced in excess. Increased level of GSH in kidney and RBC after buparvaquone administration might be attributed to antioxidant activity of buparvaquone. Thus, an elevation in GSH level in present study might have occurred due to enhanced cellular response to overcome the oxidative stress imposed by the buparvaquone administration in rats. There were no gross lesions in the rats of test group I and II in which the drug was administered at the dose rate of 50 and 100 mg.kg^{-1} b.w., respectively. However, in rats of test group III (150 mg.kg^{-1}), liver showed slight necrosis. Marked petechiae and focal areas of necrosis were found in viscera of rats that died at the dose rate of 200 mg.kg^{-1} b.w. Thus, the results of this investigation revealed that theilex can produce significant changes in haematological and biochemical profiles without causing any hepatotoxic effects at higher doses. Buparvaquone did not elevate the oxidative stress in erythrocytes while slight oxidative stress was found to occur in liver at high dose level

REFERENCES

Jain, N.C. (1986). Schalm's Veterinary Haematology. 4th Ed. Philadeiphia, Lea and Febringer.

Kayser, O. and Kiderlen, A. F. (2003). Delivery strategies for antiparasitics. *Expert. Opin. Investig.* Drugs **12(2)**: 197-207.

McHardy, N. (1988). Buparvaquone, the new antitheilerial: a review of its efficacy and safety. *In*: Proceedings of a Workshop on East Coast Fever Immunisation, Lilongwe, Malawi .pp. 158–165.

Mohammed, A., Igbokwe, I.O. and Yidawi, J.P. (2001). Comparision of clinical and hematological changes following i.m administration of xylazine, ketamine or

xylazine-ketamine combination to Sahel goats. *Nig. Vet. J.* **22**:14-21.

Natt, M.P. and Herrick, C.A. (1952)**.** A new blood diluent for counting the erythrocytes and leucocytes of the chicken. *Poult. Sci.* **31**: 735-778.

Prins, H. K. and Loos, J. A. (1969). Glutathione, *In:* Yunis, J.J. ed. Biochemical Methods in Red Cell Genetics. New York, Academic Press. pp. 115-137.

Rehman, S.U. (1984). Lead induced regional lipid peroxidation in brain. *Toxicol. Lett.* **21**: 333-337.

Sedlak, J. and Lindsay, R.H. (1968). Estimation of total protein-bound and nonprotein sulfhydryl groups in tissues with Ellman's reagent. *Anal. Biochem.* **24(25)**: 192-205.

Singh, M., Sandhir, R. and Kiran. (2004). Erythrocyte antioxidant enzyme in toxicological evaluation of commonly used organophosphate pesticides. *Ind. J. Exp. Biol.* **44(7):** 580-583.

Snedecor, G.W. and Cochran, W.G. (1967). Statistical methods. 61, Oxford IBH.

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Research Article

INFLUENCE OF ACETAMINOPHEN ON SEROTONERGIC MEDIATED BEHAVIOURS IN MICE

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ABSTRACT

Male Swiss albino mice were subjected to subacute toxicity study following administration of acetaminophen (ACM) @ 0.1mg/kg and 1mg/kg, s.c.) for 14 days. Chronic tail suspension test (TST) and combination study with 5-HT agonists and antagonists were performed for the assessment of antidepressant activity of ACM. Cortical and hippocampal 5-HT levels were determined by HPLC. The behavioral data indicated that chronic administration of ACM enhanced the antidepressant effect of paroxetine and imipramine. It was found that both paroxetine and imipramine decreased immobility of mice in the TST and ACM (0.1mg/kg) enhanced this effect. ACM (0.1mg/kg) in combination of antagonists, failed to counteract the increased duration of immobility caused by 5-HT depletor, p-chlorophenylalanine (pCPA), and ondansetron, even on the 14th day of the study. Interestingly, pCPA (200mg/kg) reduced antioxidant level and enhanced MDA level in brain whereas ACM neutralized the effect of pCPA.

Keywords: Acetaminophen, cycloxygenase, depression, HPLC, pCPA, serotonin, superoxide dismutase

INTRODUCTION

Major depression and bipolar disorders are the most widespread psychiatric disorders in contemporary society. About 16% and 1% of the population are predictable to be affected by major depression and bipolar disorder one or more times during their lifetime. SSRIs (citalopram, escitalopram, fluoxetine, paroxetine, fluvoxamine and sertraline) are widely used as the prototype drugs for the treatment of depression because of its greater tolerability and easy dosing pattern. However, till now, no antidepressant has proven to be the most effective even in severe depression but certain markers of endogenecity has a good response towards antidepressants which include circadian variation, weight loss, absence of mood reactivity towards the environment, anorexia and psychomotor retardation (Joyce *et al*., 1989). Apart from the delayed onset of action, psychiatrists all over the world identified side effects and predictability of response as the unmet need with existing antidepressants (Dahl *et al*., 1993).There is an extreme need to develop antidepressants that not only augment the onset of therapeutic response but also rapidly enhances sleep so that there is lesser obligation of hypnotics along with antidepressants.

Acetaminophen (ACM) or paracetamol, a commonly used analgesics and antipyretic with low anti-inflammatory effect due to its highly selective inhibitory action on COX-2 (Hinz *et al*., 2008), induced elevation of social behavior in mice due to 5-HT receptor agonism (Gould *et al*., 2012).. A study showed that the antinociceptive effect of ACM was antagonized by 5-HT_3 (Pickering *et al*., 2006), 5-HT_{1A} and 5-HT_4 receptor antagonists (Mallet *et al*., 2008). Furthermore, lesions of bulbospinal 5-HT pathways in mice treated with 5,7-dihydroxytryptamine antagonized the analgesic effects of ACM which suggests that there is a straight interplay between 5-HT and ACM. Since there are no methodical reported data of ACM in lower doses (0.1mg/kg and 1mg/kg) for the treatment of depression, therefore, the objective of the present study was to investigate the possible influence of ACM at low dose range in serotonergic mediated behaviors in mice.

MATERIALS AND METHODS

Experimental animals

Male Swiss Albino mice (20-28g), 3-4 months old were selected for the behavioral study. Mice were divided into 18 groups, consisting of 6 animals per group as per the experimental protocol. They were housed on a 12:12-h light–dark (LD) cycle, in opaque polypropylene cages, (three mice/cage) with corn-husk bedding material and maintained at 25 ± 2 °C with food and water available *ad libitum*. Before start of the experiment, all animals were allowed acclimatize at least 7 days in the environment. Mice were transferred to the testing room followed by a 30min acclimatization period that preceded each test. All experiments were conducted between 9:00 A.M. and 12:00 P.M. All procedures were reviewed and approved by the Institutional Animal Ethics Committee (IAEC). *IAEC approval no. HPI/2013/60/IAEC/PP-0130.*

Subacute toxicity study

The toxicity study was carried out according to the protocol of National Toxicology Program, Technical Report Series (no. 394), Toxicology and Carcinogenesis

Table 1:
Table representing the various treatment groups in a 14-day TST study in mice.

GroupsTreatment (n=6)		Dose	Dose schedule
1	Benzyl alcohol + saline	-	Day 1 to day 14
2	ACM	0.1mg/kg	Day 1 to day 14
3	ACM	1mg/kg	Day 1 to day 14
4	Normal Saline	-	Day 1 to day 14
5	Imipramine	5mg/kg	Day 1 to day 14
6	Imipramine + ACM	5mg/kg, 0.1mg/kg	Day 1 to day 14
7	*Normal saline*	-	Day 1 to day 14
8	*Paroxetine*	34mg/kg	Day 1 to day 14
9	*Paroxetine + ACM*	34mg/kg, 0.1mg/kg	Day 1 to day 14
10	Distilled water	-	Day 1 to day 14
11	Buspirone	10mg/kg	Day 1 to day 14
12	Buspirone + ACM	10mg/kg, 0.1mg/kg	Day 1 to day 14
13	Normal saline	-	Day 1 to day 14
14	Ondansetron	1mg/kg	Day 1 to day 14
15	Ondansetron + ACM	1mg/kg, 0.1mg/kg	Day 1 to day 14
16	Normal saline	-	Day 12 to day 14
17	pCPA	200mg/kg	Day 12 to day 14
18	pCPA+ACM	200mg/kg, 0.1mg/kg	Day 12 to day 14

Studies of ACM vials of strength 150mg/2ml 1% w/v benzyl alcohol was used. In this study, 15 numbers of mice were taken and grouped into 3 consisting of 5 mice each. Group 1 received normal saline + benzyl alcohol, Group 2 received 0.1 mg/kg and Group3 received 1 mg/kg ACM s.c, daily for 14 consecutive days. Water and feed were available *ad libitum*, except nocturnal fasting before the day of sacrifice. Mice were observed twice daily; clinical findings noted during the daily checks were recorded. The animals were weighed before starting the experiment and on days 7 and 14. Feed consumption was measured weekly. On the 12th day of study, three more treatment groups of mice were added which received pCPA (200mg/kg), pCPA (200mg/kg) + ACM (0.1mg/kg) and a control group, respectively. On the 15th day, each animal was sacrificed humanely by xylazine anaesthesia. The levels of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP) for liver function tests and serum creatine kinase (CK) for renal function were estimated using diagnostic kits using a spectrophotometer.

Tail suspension test

In this study, as described above, 18 groups of mice were taken consisting of 6 mice per group. The TSTs were performed according to the conventional method (Steru *et al*., 1985). Mice were hung from the tip of its tail for a duration of 6 minutes and the total duration of immobility was calculated. Doses of different agonists and antagonists were selected in accordance to their LD_{50} values (David *et al*., 2003; Popik *et al*., 2003; Njung'e and Handley, 1991). Animals were administered with the drugs for 14 consecutive days. Behavioral study was carried out on the 1st , 7th and 14th day except for the pCPA treated groups (Group 16, 17 and 18) that received the drugs on the last 3 consecutive days of the study (Raz and Berger, 2010) and the behavioral study for these 3 groups were carried out. The various treatment groups and dose schedule are given in Table 1.

Table3:
Effect of ACM (0.1mg/kg and1mg/kg) administration on mice cortex and hippocampal 5-HT levels.

GROUPS	5-HT level (ng/mg wet tissue) (day 15th)
Control	22.84 ± 2.42
ACM (1mg/kg)	48.25 ± 3.63
ACM (0.1mg/kg)	37.91 ± 2.71
pCPA	10.52 ± 2.86
pCPA ± ACM	19.63 ± 4.09

After completion of the TST study for 14 days, the ACM treated groups along with its control group and the groups for combination study with pCPA were sacrificed under euthanasia and the brains were isolated in cold condition and stored at -22^0 C for proceeding into the *in vivo* antioxidant study.

In vivo antioxidant study

On 15th day of the experiment, after sacrificing the animals of all the groups, their brains were isolated and homogenised at 4^0 C and centrifuged at 10000 x g for 15 minutes with potassium phosphate buffer in the ratio 1:10 (Pitchaimani *et al*., 2012). The homogenate buffer contains a mixture of 5.22 gm of K_2HPO_4 and 4.68 gm of NaH_2PO_4 in 150 ml distilled water (solution A). Then 6.2 gm of NaOH is dissolved in 150 ml distilled water (solution B). Solution A and solution B were mixed to make a final volume of 300 ml homogenate buffer. The supernatant was collected and taken for estimation of protein, reduced glutathione (GSH), lipid peroxidase (LPO), catalase and sodium dismutase (SOD).

Determination of 5-HT concentrations in prefrontal cortex and hippocampus of brain

The prefrontal cortex and hippocampus of the 18 groups of mice were isolated and the level of 5-HT measured using UHPLC (Thermo Scientific Dionex Ultimate 3000, USA and Coulochem III electrochemical detector, Acclaim® 120 C18 Column 4.6x250 mm 5 μm.

Statistical analysis

All the results were expressed as mean ± SEM. Data was statistically evaluated by two-way ANOVA followed by post hoc Bonferroni's test using Graph Pad

Table 2:
The activities of SGOT, SGPT, ALP and creatine kinase in the sera of mice treated with ACM.

GROUPS	SGOT(U/L)	SGPT(U/L)	ALP(U/L)	CK(U/L)
Control	23.2 ± 2.30	11.97 ± 2.10	206.56± 16.80	122.73 ± 9.89
ACM (0.1mg/kg)	23.26 ± 2.04	10.82 ± 1.75	153.79± 12.70	139.4 ± 11.80
ACM (1mg/kg)	18.25 ± 1.98	11.69 ± 1.54	99.85 ± 7.57	139.5 ± 10.80

Prism software (version 5.01). *P < 0.05, **P < 0.01, ***P < 0.001 was considered as statistically significant when normal control group compared with the treated groups.

RESULTS

It was observed in the sub acute study, that mice treated with 0.1mg/kg and 1mg/kg, s.c., survived till the end of the study. There was no significant change in the feed consumption and the final mean body weight of mice in any of the treatment group as compared to the control group. No compound-related lesions were found at the time of dissection.

The level of SGOT, SGPT, ALP and CK in the sera of mice treated with ACM 0.1mg/kg and 1mg/kg compared to control group are given in Table 2. There was no significant alteration of serum biochemical parameters of the ACM treated groups compared to that of control group.

The effect of ACM (0.1mg/kg, 1mg/kg and 10mg/kg) in TST as compared to control and standard (imipramine) is shown in the Fig 1. At the lower dose, ACM 0.1mg/kg have shown a significant decrease in immobility time as compared to the control group. Therefore, this dose was selected for the combination study with different agonists and antagonists.

Among the three agonists (imipramine, paroxetine and buspirone), imipramine and paroxetine showed significant decrease in duration of immobility (Fig 2 and Fig 3) when used in combination with ACM (0.1mg/kg) than that of when used alone. ACM (0.1mg/kg) did not exhibit any response when used in combination with buspirone (Fig 4).

From the results of combination study with 5-HT antagonists, it was determined that ACM (0.1mg/kg) when used in combination, counteracted the increased duration of immobility caused by pCPA on day 1 and day 3 (i.e. 12th and 14th day of the study respectively) and ondansetron. The result is depicted in Fig. 5 & 6.

The concentration of 5-HT was evaluated and presented in Table 3. The results showed that pCPA (200mg/kg) persuaded a depletion of 5-HT concentration in the cortex and hippocampus of the brain of mice on day 15 , whereas, ACM in combination with pCPA neutralized the effect of pCPA by subsequently increasing the brain 5-HT levels. The values are expressed as the mean ± SEM. Values from the 5-HT determination are expressed as ng/mg of wet tissue.

The concentration of MDA, and status of antioxidants (GSH, Catalase and SOD) in the brain of ACM and pCPA treated group following TST study was evaluated and presented in the following graphs (Fig.7A-D). The data illustrated that ACM, at either doses did not affect the brain antioxidant (GSH, SOD and Catalase) level as well as MDA. Again, pCPA in the dose range of 200mg/kg persuaded a reduction in the antioxidants level and enhances MDA level in brain whereas, ACM neutralized the effect of pCPA. Hence, it can be interpreted that ACM

DISCUSSION

In these series of experiments TST was used to assess the antidepressant effect of ACM. TSTs are widely used to screen new antidepressant drugs (Steru *et al*., 1985). This test is rather sensitive and relatively specific to all major classes of antidepressant drugs including TCAs, SSRIs, MAO inhibitors, and atypical antidepressants (Steru *et al*., 1985). ANY Maze video (Stoelting Co, USA) analysis system was used reliably and accurately for automatic recording and scoring of duration of immobility.

Our behavioral data indicated that chronic administration of ACM enhanced the antidepressant like effect of paroxetine and imipramine. It has been demonstrated that both the drugs (paroxetine and imipramine) decreased immobility of mice in the TST and

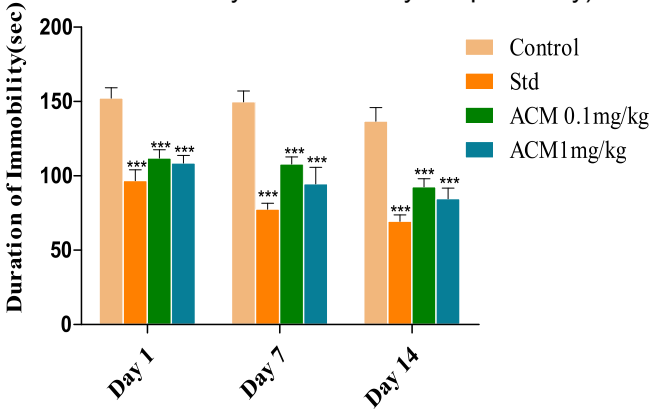


Fig.1
Dose dependent effect of ACM in TST compared to Std and control group. Data are the mean duration of immobility by male mice in a 6 min period. Vertical bars represent SEM (n=6).*P <0.05; **P <0.0l; ***P<0.001.

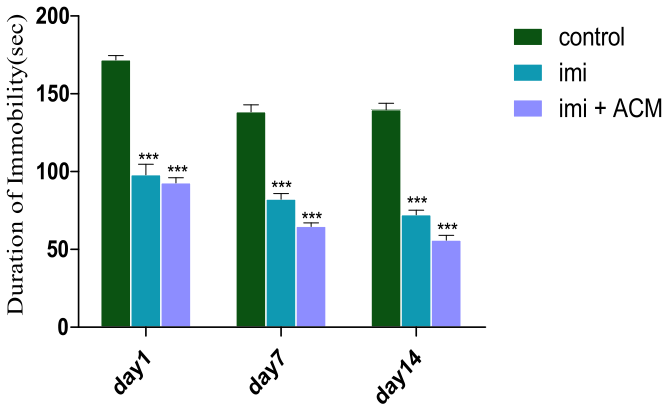


Fig.2:
Effect of combined treatent of ACM with imipramine in TST. Data are the mean duration of immobility by male mice in 6 min period. Vertical bars represent SEM. n=6) *P <0.05; **P <0.0l; ***P<0.001.

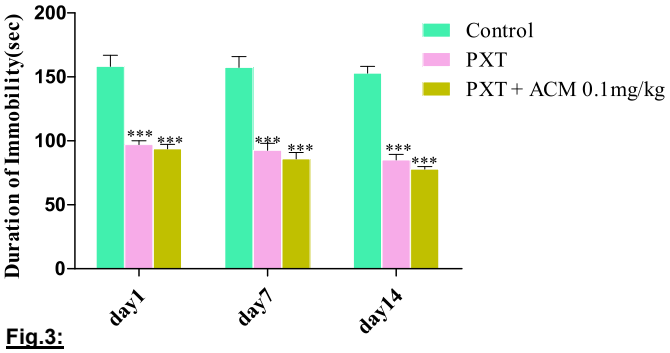


Fig.3:
Effect of combination study of ACM with paroxetine in TST. Data are the mean duration of immobility by male mice in a 6 min period. Vertical bars represent SEM (n=6).*P <0.05; **P <0.0l; ***P<0.001.

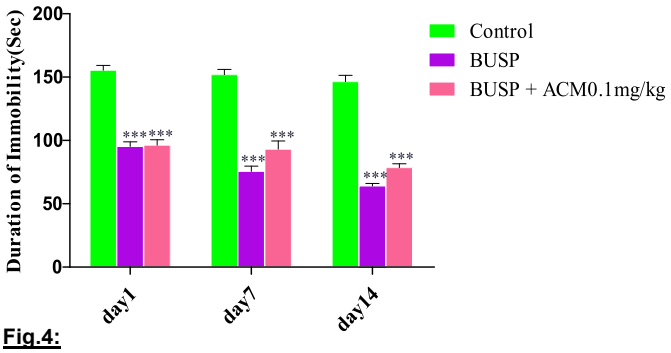
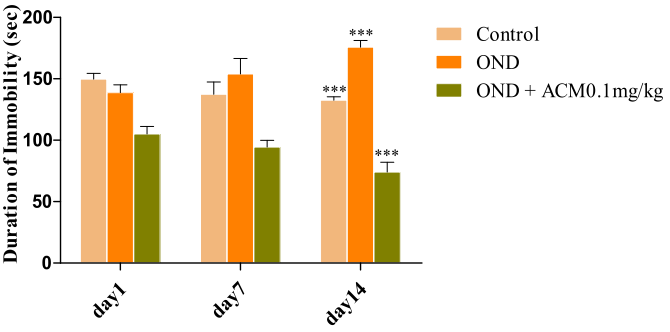


Fig.4:
Effect of combined treatment of ACM with buspirone in TST. Data are the mean duration of immobility by male mice in 6 min period. Vertical bars represent SEM. N=6) *P <0.05; **P <0.0l; ***P<0.001.



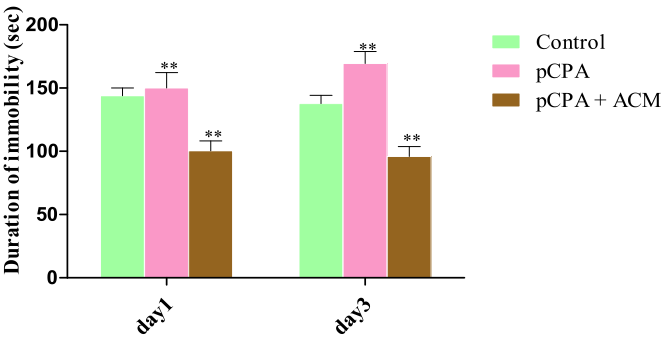


Fig. 5 & 6:
Effect of combined treatment of ACM with Ondansetron and pCPA in TST. Data are the mean duration of immobility by male mice in 6 min period. Vertical bars represent SEM. (n= 6).*P <0.05; **P <0.0l; ***P<0.001.

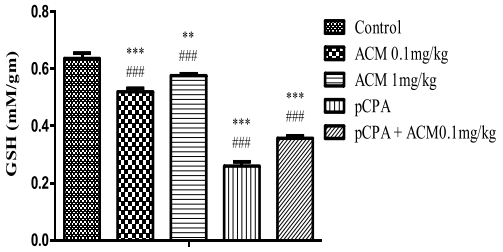


Fig. 7A

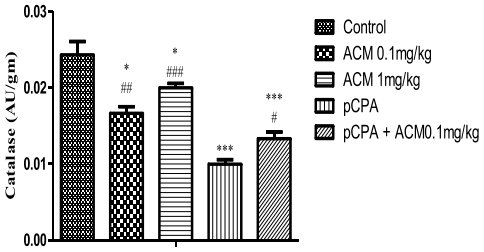


Fig. 7B

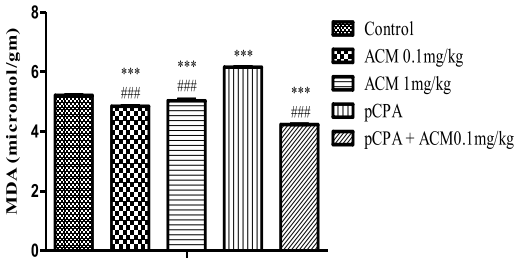


Fig. 7C

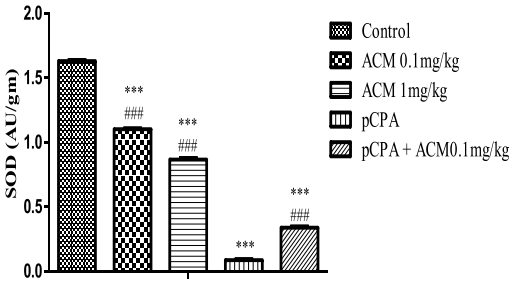


Fig. 7D

(0.1mg/kg and 1mg/kg) act significantly against oxidative stress.

Fig 7:
Regulation of GSH (Fig 7A), Catalase (Fig 7B), MDA (Fig 7C) and SOD (Fig 7D) content in brain of mice treated with ACM alone and combination of ACM and pCPA after undergoing chronic TST. Values are expressed as average ± SEM. **(n= 6).** ***P < 0.01, **P < 0.01 and *P<0.05 vs. control, ###P<0.001, ##P<0.01 and #P<0.05 vs. pCPA

that this effect with ACM (0.1mg/kg) was additive. Buspirone when used alone showed a marked decrease in duration of immobility towards the 14th day of the study. But, ACM (0.1mg/kg) exhibited no effect on duration of immobility when used in combination with buspirone. From the results of combination study with antagonists, it was observed that ACM (0.1mg/kg) when used in combination, failed to counteract the increased duration of immobility caused by pCPA and ondansetron, even on the 14th day of the study.

Our data supports the serotonergic action of ACM which is evident from the HPLC study in the brain of treated mice. There was no scientific data demonstrated that the doses administered in this study are having influence on serotonergic nervous system. In our experimental model, ACM (0.1mg/kg and 1mg/kg) produces contended amount of both 5-HT and NA which is closely related to an essential role in behavioral activity in TST.

Depression is likely to upshot from a combination of innate and environmental factors that each modifies the risk of an individual to become depressed. Stress is one of the most potent environmental factors (Markus, 2003). During stress, activity of the brain 5-HT system and HPA axis rises. 5-HT can modulate the HPA axis, 5-HT overdrive increases CRF and corticosteroid release (Van Praag, 2004). Increased cortisol levels initially cause higher CNS 5-HT turnover by increasing tryptophan availability and stimulation of tryptophan hydroxylase activity (Maccari *et al*., 2003). Given that high cortisol levels initially cause higher CNS 5-HT turnover, hypothetically, during continuous or frequent exposure to stress, availability of brain TRP and 5-HT may reduce and vulnerability to pathology may increase (Markus, 2003).

Since the TST method is based on the observation that if a mouse is placed in a stressful situation, such as suspension by the tail, therefore, it is most likely that chronic involvement of the mice to this method will cause stress induced free radical generation. So, the ACM treated groups used in TST model was considered for carrying out the *in vivo* antioxidant study. Again, the molecule pCPA inhibits cerebral 5-HT synthesis through the inhibition of tryptophan hydroxylase. Therefore, pCPA induced 5-HT reduction in the brain might cause oxidative damage. It was intended to observe if ACM (0.1mg/kg and 1mg/kg) could restore the brain cells from oxidative damage either due to stress or pCPA induced oxidative damage.

ACM (0.1mg/kg and 1mg/kg) was able to normalize brain antioxidant activity. Interestingly, pCPA in the dose range of 200mg/kg persuaded a reduction in the antioxidants level and enhances MDA level in brain whereas, ACM neutralized the effect of pCPA. Most of the currently available antidepressant drugs act through monoamines and in particular 5-HT. It has been demonstrated in the clinics that mood improvement in depressed patients who positively responded to treatment with various classes of antidepressant drugs could be rapidly impaired by 5-HT synthesis inhibition (Delgado *et al*., 1990). Side effects are commonly reported during the chronic treatment, notably insomnia, somnolence, dizziness, akathesia and long term sexual dysfunction. Several strategies are in progress to improve the activity of the conventional antidepressant drugs.

The HPLC results of the present experiment indicate that chronic s.c administration of ACM (0.1 and 1mg/kg) results in elevation of 5-HT levels in cortex and hippocampus in the brain. Comparing the results of HPLC and behavioral study, it could be interpreted that ACM administration mimics the action of 5-HT reuptake inhibitor. This would prevent the reuptake and inactivation of 5-HT thereby potentiating the action of serotonin released by neuronal activity.

Interestingly, recent studies showed that ACM effectively reduced anxiety and increased sociability and induced anxiolytic effects may be mediated by serotonergic system (Gould *et al.*, 2012). In addition, a research report showed that ACM produced changes in brain 5-HT levels due to inhibition of hepatic tryptophan-2,3-dioxygenase activity, which resulted in antidepressant-like effects (Daya and Dukie, 2000).

Thus in view of the fact that ACM shows it's antinociceptive, analgesic and anxiolytic effect by enhancing serotonergic signalling; and 5-HT is implicated in affective disorders like anxiety, depression or compulsive behavior. It could be contemplated that ACM might as well show signs of antidepressant or anticompulsive-like behavior. The recent data showed that chronic treatment of ACM induces subtle changes in 5-HT and NA levels with significant behavioural attention. Earlier research of Daya and Dukie (2000) have linked the rise in 5-HT levels in forebrain with inhibition of hepatic tryptophan 2,3-dioxygenase activity, the enzyme involved in conversion of tryptophan to its metabolite N-formylkynuremine which might be one of the possible mechanism of our study. Thus, as an additional benefit to the potential enhancement of the antidepressant effect, this combination may provide a novel therapy to alleviate symptoms of depression in a better way than existing therapies.

REFERENCES

Dahl, K., Avery, D.H. and Lewy, A.J. (1993). Dim light melatonin onset and circadian temperature during a constant routine in hypersomnic winter depression. *Acta. Psychiatr. Scand.* **88**: 60-66.

David, D.J.P. and Bourin, M., Jego, G., Przybylski, C., Jolliet, P., Gardier, A.M.(2003). Effects of acute treatment with paroxetine, citalopram and venlafaxine in vivo on noradrenaline and serotonin

outflow: a microdialysis study in Swiss mice. *Br. J. Pharmacol.* **140**: 1128–1136.

Daya, S., Kumar, Anoop and Dukie S. (2000). Acetaminophen inhibits liver tryptophan-2,3-dioxygenase activity with a concomitant rise in brain serotonin levels and a reduction in urinary 5-hydroxyindole acetic acid. *Life. Sci.* **67**: 235-240.

Delgado, P.L., Charney, D.S., Price, L.H., Aghajanian, G.K., Landis, H. and Heninger, G.R. (1990). Serotonin function and the mechanism of antidepressant action. Reversal of antidepressant-induced remission by rapid depletion of plasma tryptophan. *Arch. Gen. Psychiatry.* **47:**411–418.

Gould, G.G., Seillier, A., Weiss, G., Giuffrida, A., Burke, T.F., Hensler, J.G., Rock, C., Tristan, A., McMahon, L.R., Salazar, A., O'Connor, J.C., Satsangi, N., Satsangi, R.K., Gu, T., Treat, K., Smolik, C. and Schultz, S.T. (2012). Acetaminophen differentially enhances social behavior and cortical cannabinoid levels in inbred mice. *Neuro-Psychopharmacol. Biol. Psychiatry*. **38**: 260-269.

Hinz, B., Cheremina, O., Brune, K. (2008). Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. *The FASEB Journal: official publication of the Fed. American Soc. Exp. Biol.* **22**: 383–390

Joyce, P.R. and Paykel, E.S. (1989). Predictors of drug response in depression. *Arch. Gen. Psychiatry.* **46**: 89-99.

Maccari, S., Darnaudery, M., Morley-Fletcher, S., Zuena, A.R., Cinque, C. and Van Reeth, O. (2003). Prenatal stress and long-term consequences: implications of glucocorticoid hormones. *Neurosci. Biobehav. Rev.* **27**: 119–127.

Mallet, C., Laurence, D., Bonnefont, J., Ledent, C., Etienne, M., Chapuy, E., Libert, F. and Eschalier, A. (2008). Endocannabinoid and serotonergic systems are needed for acetaminophen-induced analgesia. *Pain*. **139**: 190–200.

Markus, C.R. (2003). Interactions between stress, food and mood. In: Watson DaD F (Ed). Performance Functional Foods. Woodhead Publishing: Cambridge: 5–20.

Njung'e, K. and Handley, S.L. (1991). Effects of 5-HT uptake inhibitors, agonists and antagonists on the burying of harmless objects by mice; a putative test for anxiolytic agents. *Br. J. Pharmacol.* **104**: 105-112.

Pickering, G., Loriot, M., Libert, F., Eschalier, A., Beaune, P. and Dubray, C. (2006). Analgesic effect of acetaminophen in humans. First evidence of a central serotonergic mechanism *Clin Pharmacol Therapeutics*. **79**: 371–378

Pitchaimani, V., Arumugam, S., Thandavarayan, R.A., Thiyagarajan, M.K., Aiyalu, R., Sreedhar, R., Nakamura, T. and Watanabe, K. (2012). Nootropic activity of acetaminophen against colchicine induced cognitive impairment in rats. *J. Clin. Biochem.* **50**: 241-244.

Popik, P., Kozela, E. and Krawczyk, M. (2003). Nicotine and nicotinic receptor antagonists potentiate the antidepressant-like effects of imipramine and citalopram. *Br. J. Pharmacol.* **139**: 1196–1202.

Raz, S. and Berger, B.D. (2010). Effects of ûuoxetine and PCPA on isolation-induced morphine self-administration and startle reactivity. *Pharmacol. Biochem. and Behaviour.* **96**: 59–66.

Steru, L., Chermat, R., Thierry, B. and Simon, P. (1985). The tail suspension test: A new method for screening antidepressants in mice. *Psychopharmacol.* **85**: 367-370.

Tjolan, A., Lund, A. and Hole, K. (1991). Antinociceptive effect of paracetamol in rat is partly dependent on serotonergic system. *Eur. J. Pharmacol.* **193**: 193-201.

Van Praag, H.M. (2004). Can stress cause depression? *Prog. Neuropsychopharmacol. Biol. Psychiatry.* **28**: 891–907.

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Research Article

HYPOURICEMIC EFFECTS OF AQUEOUS AND ALCOHOLIC EXTRACTS OF *CURCUMA LONGA* RHIZOMES IN RATS

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ABSTRACT

The study was planned to evaluate hypouricemic effect of aqueous and alcoholic extracts of *Curcuma longa* rhizomes following its 28 days repeated oral administration in potassium oxonate induced hyperuricemic rats. The study was conducted on forty two (42) male Sprague-Dawely rats divided equally into seven groups. Group I, II and III served as vehicle control group, gout control group and standard treatment control group, respectively. Group IV and V received aqueous extracts of *Curcuma longa* (200 and 400 mg/kg, P.O.) and group VI and VII received alcoholic extracts of *Curcuma longa* (200 and 400 mg/kg, P.O.) for 28 days. At the end of study, blood and serum were analyzed for haematological and biochemical parameters, respectively. Hyperuricemic rats treated with aqueous and alcoholic extracts of *Curcuma longa* at dose rate of 200 and 400 mg/kg b wt and allopurinol @ 5 mg/kg b wt showed reduction in platelets count and serum creatinine, uric acid, blood urea nitrogen and xanthine oxidase enzyme level as compared to rats of gout control group. Aqueous and alcoholic extracts of *Curcuma longa* rhizomes showed effectiveness in dose-dependent manner in potassium oxonate induced hyperuricemic rats.

Key words: *Curcuma longa* rhizomes, Hypouricemic effect**,** Xanthine oxidase, Rats

INTRODUCTION

Hyperuricemia or gout is a metabolic disorder of purine metabolism characterized by increased urate level (urate levels >6.8 mg/dl) and recurring attacks of arthritis and in later stages chronic arthritis, tophi formation and a tendency to renal failure (Golding, 1989). Avoidance of purine-rich foods is important for gout management (Beneke, 2003). The most important approach in the treatment of hyperuricemia is the development of xanthine oxidase (XO) inhibitors, which are effective in reducing plasma and urinary urate levels and reverses the development of tophaceous deposits (Nuki and Simkin, 2006). Food components which inhibit xanthine oxidase activity can reduce the formation of uric acid and alleviate inflammation. This is because xanthine oxidase is a key enzyme playing a role in hyperuricemia, catalyzing the oxidation of hypoxanthine to xanthine and then to uric acid (Unno *et al.,* 2004). Allopurinol is the most common clinically used xanthine oxidase inhibitor prescribed for the treatment of gout. But use of this drug may result in various side-effects including hepatitis, nephropathy and allergic reactions (Hammer *et al.,* 2001). Thus, the development of novel hypouricemic agents of herbal origin with greater efficacy and a broader safety proûle is greatly needed.

Turmeric (*Curcuma longa*) is a medicinal plant extensively used in Ayurveda, Unani and Siddha medicine as home remedy for various diseases (Eigner and Scholz, 1999). Turmeric and its constituents mainly curcumin and essential oils shows a wide spectrum of biological actions. These include its anti-inflammatory, antioxidant, anti-carcinogenic, anticoagulant, antifertility, wound healing activity, anti-diabetic, antibacterial, antifungal, antiprotozoal, anti-fibrotic, antiulcer, hypotensive and hypocholesteremic activities (Kumar *et al.,* 2011). As there is lack of literature for data regarding hypouricemic effect of aqueous and alcoholic extracts of *Curcuma longa* on gout rat model, the present study was planned to explore anti-gout effect of this plant extracts following oral administration at various doses in rats along with hemato-biochemical evaluation.

MATERIALS AND METHODS

Experimental animals

The study was conducted on 42 healthy male Sprague-Dawley rats of 8-12 weeks of age. Rats were procured from Zydus Research Centre (ZRC), Ahmedabad, India. The animals were housed under standard management conditions and fed on pellet diet and deionized water *ad libitum* throughout the experiment. After acclimatization for 5 days, rats were grouped for experiment. Rats were kept under constant observation during entire period of study. The study was conducted after clearance from IAEC.

Drugs and chemicals

Potassium oxonate (Oxonic acid potassium salt), allopurinol and xanthine oxidase activity assay kit were purchased from Sigma-Aldrich (Spruce Street, St. Louis, MO, U.S.A.). Glycerin (Glycerol about 98% Purified) was purchased from Merck Specialities Pvt. Ltd. India. Potassium oxonate used for induction of hyperuricemia and allopurinol used as a positive control were dissolved

in 0.9% saline solution. Reagents used for serum biochemical analysis were purchased from Merck Specialities Pvt. Ltd. India.

Plant materials

Fresh turmeric rhizomes (*Curcuma longa)* were purchased from the Medicinal and Aromatic Plants Unit, Anand Agricultural University, Anand and were duly identified taxonomically.

Preparation of plant extracts

Small pieces of *C. longa* rhizomes were taken and dried under shade, then powdered by mechanical grinder, sieved (sieve no: 10/44) and stored in air tight containers. The dried powder of *C. longa* was separately placed in a continuous extraction apparatus and subjected to successive extraction using water, then alcohol. Exactly 100g of coarse powdered material of *C. longa* was successfully extracted in soxhlet extractor with water and also with alcohol. The extracts obtained were concentrated in rotary evaporator at 50°-60°C under reduced pressure leaving a dark brown residue. Aqueous and alcoholic extracts of *C. longa*, thus obtained was transferred to a petri dish and kept over water bath (50°C) until the solvent gets completely evaporated. It was stored at 4^0C for future use. Aqueous and alcoholic extracts of *C. longa* were dispersed in water using the same amount of glycerin solution. For the healthy control group, the vehicle was prepared through dissolving the same amount of glycerin in water.

Induction of hyperuricemia in rats

Experimentally-induced hyperuricemia in rats (due to inhibition of uricase enzyme with potassium oxonate) was used to study hypouricemic effects of *C. longa* (Yonetani and Iwaki, 1983). After 1 hour fasting, all the animals except vehicle control group were administered potassium oxonate at 250 mg/kg b wt intraperitoneally by dissolving it in 0.9% saline solution, throughout the study period (28 days) and then after 1 hour all those rats were administered test compounds.

Experimental design

Forty two male Sprague Dawley rats were randomly and equally divided into seven groups. Three groups served as control (one healthy control, second gouty control and third positive control), where rats did not receive any extracts. After 1 hour fasting, all the animals except healthy control group were administered 250 mg/kg (I.P.) potassium oxonate, throughout the study period (28 days) and then after 1 hour all those rats were administered test compounds. Allopurinol used as a positive control, administered at 5 mg/kg of b wt (P.O.) to animals of group III. Group IV and V administered aqueous extracts of *C. longa* at dose of 200 and 400 mg/kg (P.O.) and group VI and VII administered alcoholic extracts of *C. longa* at dose of 200 and 400 mg/kg (P.O.), respectively, for 28 days of dosing period. The extracts and allopurinol solutions were administered to rats directly in oesophagus by using rat oral gavage needle with 2 ml BD syringe for 28 days. At the end of study period, on 29^{th} day blood samples were collected from all the animals by retro-orbital plexuses puncture under light ether anesthesia with the help of capillary tube (Sorg and Buckner, 1964). Blood was collected into sterile centrifuge tubes with K_3EDTA as anticoagulant for hematology and plain centrifuge tubes without anticoagulant for serum biochemistry. Blood was allowed to clot at room temperature (26 ± 2 °C) and serum was harvested after centrifugation at 3000 rpm for 10 minutes at 10 °C (Eppendorf 5804 R, Germany) and stored at -35 °C and analyzed within 24 hours.

Estimation of hemato biochemical parameters

Blood samples collected in test tubes with K_3EDTA were subjected to estimation of various hematological parameters by auto hematology analyzer (Mindray BC-2800 Vet, China). On the day of blood collection, red blood cells (RBC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), total leucocytes count (TLC/WBC) and platelets were estimated. Serum biochemical parameters were estimated in clinical serum biochemistry analyzer (NOVA 2021 Biochemistry Analyzer, Analytical Technologies Limited, Gujarat, India) including creatinine, uric acid and blood urea nitrogen (BUN). Serum xanthine oxidase activity was detected by using kits (Coupled enzyme assay), purchased from Sigma-Aldrich (Spruce Street, St. Louis, MO, U.S.A.) by following the manufacturer's protocol. XO activity was determined by spectrophotometric multiwell plate reader (Infinite M200, NanoQuant, TECAN) having 570 nm wavelength. XO activity was reported as milliunit/ml, where one milliunit of XO is defined as the amount of enzyme that catalyzes the oxidation of xanthine, yielding 1.0 µmole of uric acid and hydrogen peroxide per minute at 25ÚC.

Statistical analysis

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

RESULTS

Effect of aqueous and alcoholic extracts of *C. longa* for 28 days in normal and gouty rats on various hematological and serum biochemical parameters are presented in Table-1 and Table-2 respectively. Except, platelet count most of the hematological parameters did not show any significant changes in any experimental group. There was significant ($p<0.05$) increase in platelets count in gout control rats as compared to vehicle control

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

Groups	RBC (10^6/µl)	Hb (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	TLC (10^3/µl)	PLT (10^5/µl)
I	8.27±0.27	17.88±0.42	43.88±0.43	53.78±0.29	20.69±0.48	39.76±0.29	9.25±0.67	9.58±0.85^{a}
II	8.23±0.25	17.28±0.41	42.35±0.28	53.27±0.66	21.35±0.49	40.55±0.84	8.01±0.58	12.92±0.43^{c}
III	8.55±0.30	17.60±0.64	43.45±0.34	53.53±0.40	21.42±0.42	40.49±0.30	8.88±0.38	10.52±0.45^{ab}
IV	9.57±0.42	18.22±0.39	44.19±0.41	52.95±0.30	21.62±0.37	39.32±0.35	9.40±0.25	11.18±0.92^{abc}
V	9.47±0.60	18.73±1.04	44.55±0.67	52.30±0.36	20.97±0.42	40.20±0.35	8.12±0.39	11.84±0.58^{abc}
VI	9.52±0.62	18.83±0.74	43.92±0.74	53.40±0.43	21.82±0.72	40.87±0.61	9.05±0.46	12.55±0.35^{bc}
VII	8.67±0.30	17.50±0.28	43.03±0.40	52.50±0.62	20.35±0.41	39.80±0.29	9.35±0.27	12.32±1.11^{bc}

Mean value with dissimilar superscript in a column vary significantly at p<0.05; Treatment groups: Group II – VII given potassium oxonate (250 mg/kg) for induction of hyperuricemia; I: Vehicle Control; II: Gout Control; III: Standard Treatment Control; IV: Aqueous Extract *C. longa* (200 mg/kg); V: Aqueous Extract *C. longa* (400 mg/kg); VI: Alcoholic Extract *C. longa* (200 mg/kg); VII: Alcoholic Extract *C. longa* (400 mg/kg). RBC: Red Blood Cells; Hb: Hemoglobin; PCV: Packed Cell Volume; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; TLC: Total Leukocyte Count, PLT: Platelets count.

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

Groups	Creatinine (mg/dl)	Uric acid (mg/dl)	BUN (mg/dl)	XO activity (milliunit/ml)	XO Inhibition %
I	0.39±0.03^{a}	8.86±0.14^{a}	18.41±0.31^{a}	1.69±0.17^{a}	-
II	3.01±0.07^{d}	15.50±0.37^{c}	23.83±0.81^{d}	3.33±0.51^{b}	-
III	0.45±0.09^{a}	10.06±0.28^{ab}	19.05±0.44^{ab}	1.70±0.30^{a}	48.94
IV	0.93±0.06^{b}	11.44±0.25^{b}	20.95±0.34^{c}	2.23±0.27^{a}	33.03
V	0.47±0.10^{b}	10.90±0.62^{b}	20.43±0.49^{bc}	2.18±0.29^{a}	34.53
VI	1.50±0.10^{c}	11.41±0.47^{b}	21.05±0.49^{c}	1.95±0.19^{a}	41.44
VII	1.04±0.06^{b}	11.05±0.64^{b}	20.95±0.38^{c}	1.93±0.11^{a}	42.04

Mean value with dissimilar superscript in a column vary significantly at p<0.05; Treatment groups: Group II – VII given potassium oxonate (250 mg/kg) for induction of hyperuricemia; I: Vehicle Control; II: Gout Control; III: Standard Treatment Control; IV: Aqueous Extract *C. longa* (200 mg/kg); V: Aqueous Extract *C. longa* (400 mg/kg); VI: Alcoholic Extract *C. longa* (200 mg/kg); VII: Alcoholic Extract *C. longa* (400 mg/kg). BUN: Blood Urea Nitrogen, XO: Xanthine Oxidase

group. Animals of group III, which was given standard treatment allopurinol showed significant reduction whereas animals of group IV, V, VI and VII, which were given aqueous and alcoholic extracts of *C. longa* revealed reduction in platelets count as compared to gout control group.

There was significant (p<0.05) increase in serum creatinine, uric acid, BUN and xanthine oxidase enzyme level in the animals of gout control group as compared to vehicle control group. There was significant (p<0.05) decrease in level of serum creatinine, uric acid, BUN and xanthine oxidase enzyme level in the allopurinol treated group as compared to gout control rats. Animals of group IV, V, VI and VII, which were given aqueous and alcoholic extracts of *C. longa* also revealed significant (p<0.05) reduction in dose dependent manner as compared to gout control group.

DISCUSSION

Hypouricemic effect of *Curcuma longa* in potassium oxonate induced gout in rats was investigated in the present study. Intra-peritoneal injections of Potassium oxonate at dose rate of 250 mg/kg body weight throughout the study period (28 days) has lead to increased serum concentration of creatinine, uric acid, blood urea nitrogen and xanthine oxidase enzyme in rats of gout control group as compared to rats of vehicle control group. Such changes in serum biochemical parameters established successful gout rat model. These increased values were indicative of kidney damage which was reflected in terms of an increase in serum biochemical parameters. Similar to these findings, plasma samples collected from rats after 3 and 6 hours from oxonate injection at dose of 250 mg/kg body weight have also demonstrated rise in uric acid (Mohamed and Al-Okbi, 2008).

In the present study, there was significant (p<0.05) increase in mean platelets counts in rats of gout control group as compared to vehicle control rats. This might be occured due to inflammatory reactions in rats of gout control group. It is also a symbol of chronic inflammation or inflammation activation (Frenette and Wagner, 1997; Hartwell and Wagner, 1999). Increased number of platelet count is also seen in other inflammatory conditions like rheumatoid arthritis (Gasparyan *et al.,* 2010), inflammatory bowel disease (Voudoukis *et al.,* 2014) and nephritis. Upon activation, platelets secrete a large number of biologically active molecules, which are able to induce or amplify an inflammatory process whereas in hyperuricemic rats treated with aqueous and alcoholic extracts of *C. longa* at doses of 200 and 400 mg/kg b wt reduced mean platelets counts as compared to gout control rats.

Present study indicated that the group of rats given standard reference compound allopurinol at 5 mg/kg b wt

significantly (P<0.01) reduced serum creatinine, uric acid, BUN and xanthine oxidase enzyme level in hyperuricemic rats. Here the extent of reduction in xanthine oxidase enzyme level by allopurinol was much higher than that observed with *C. longa* extracts in hyperuricemic rats. Hyperuricemic rats receiving aqueous and alcoholic extracts of *C. longa* at dose rate of 200 and 400 mg/kg body weight for 28 days also showed significant reduction in serum creatinine, uric acid, blood urea nitrogen and xanthine oxidase enzyme level as compared to rats of gout control group in dose- dependent manner.

A significant (P<0.01) decline in elevated uric acid has also been reported by Mohamed and Al-Okbi (2008) who studied the anti-gout activity of methanol and petroleum ether extracts of celery leaves, celery seeds, rosemary, cinnamon and turmeric in potassium oxonate treated rats (250 mg/kg b wt, I.P.) and results showed that oral administration of different extracts (500 mg/kg body weight) significantly (P<0.01) reduced plasma and urine uric acid levels. The reduction in uric acid levels in gouty rats after oral administration of different extracts may be attributed to the presence of phytochemical constituents such as curcumin, phenolic compounds, plant sterols, long chain fatty acids and to a lesser extent unsaturated fatty acids. Phenolic compounds have also been reported to possess anti-inflammatory activity ((Mohamed and Al-Okbi, 2008). Numerous studies have showed that phenolic compounds found in plants, such as anthrocyanins and quercetin, which are structurally related to xanthine, inhibit xanthine oxidase activity, thus reducing hyperuricemia (Mo *et al.,* 2007). Based on hemato-biochemical evaluation, 28 days repeated oral administration of aqueous and alcoholic extracts of *C. longa* @ 200 and 400 mg/kg body weight have been found to produce hypouricemic and xanthine oxidase inhibitory effects in gouty rats in dose dependent manner.

REFERENCES

Beneke, M. (2003). Avoid purine-rich foods, drink a lot, reduce weight. The most important recipes against hyperuricemia: MMW. *Fortsch. Med*. **145**: 36-45.

Eigner, D. and Scholz, D. (1999). *Ferula asafetida* and *Curcuma longa* in traditional medicinal treatment and diet in Nepal. *J. Ethnopharmacol*. **67**: 1-6.

Frenette, P. S. and Wagner, D. D. (1997). Insights into selectin function from knockout mice. *Thromb. Haemost*. **78**(1): 60–64.

Gasparyan, A. Y.; Stavropoulos-Kalinoglou, A.; Mikhailidis, D. P.; Toms, T. E.; Douglas, K. M. J. and Kitas, G. D. (2010). Platelet Function in Rheumatoid Arthritis: Arthritic and Cardiovascular Implications. *Rheumatol. Int.* **31**(2): 153-164.

Golding, D. N. (1989). A Synopsis of Rheumatic Diseases. 5th ed., Page bros, Ltd, Norwich, England p. 156.

Hammer, B.; Link, A.; Wagner, A. and Bohm, M. (2001). Hypersensitivity syndrome during therapy with allopurinol in asymptomatic hyperuricemia with a fatal outcome. *Dtsch. Med. Wochenschr.* **126**: 1331–1334.

Hartwell, D. W. and Wagner, D. D. (1999). New discoveries with mice mutant in endothelial and platelet selectins. *Thromb. Haemost*. **82**(2): 850–857.

Kumar, A.; Dora, J. and Singh, A. (2011). A review in spice of life *Curcuma longa* (Turmeric). *Int. J. Appl. Biol. Pharm. Tech*. **2**(4): 371-379.

Mo, S. F.; Zhou, F.; Lv, Y. Z.; Hu, Q. H.; Zhang, D. M. and Kong, L. D. (2007). Hypouricemic action of selected flavonoids in mice: structure– activity relationships. *Biol. Pharm. Bull*. **30**(8): 1551-1556.

Mohamed, D. A. and Al-Okbi, S. Y. (2008). Evaluation of anti-gout activity of some plant food extracts. *Pol. J. Food Nutr. Sci*. **58**(3): 389-395.

Nuki, G. and Simkin, P. A. (2006). A concise history of gout and hyperuricemia and their treatment. *Arthritis Res. Ther.* **8** (Suppl. 1): S1-S5.

Sorg, D. A. and Buckner, B. (1964). A simple method of obtaining venous blood from small laboratory animals. *Proc. Soc. Exp. Biol. Med.* **115**: 1131-1132.

Unno, T.; Sugimoto, A. and Kakuda, T. (2004). Xanthine oxidase inhibitors from the leaves of *Lagerstroemia speciosa* (L.) Pers. *J. Ethnopharmacol*. **93**: 391-395.

Voudoukis, E.; Karmiris, K. and Koutroubakis, I. E. (2014). Multipotent role of platelets in inflammatory bowel diseases: A clinical approach. *World J. Gastroenterol.* **20**(12): 3180-3190.

Yonetani, Y. and Iwaki, K. (1983). Effects of uricosuric drugs and diuretics on uric acid excretion in oxonate-treated rats. *Jpn. J. Pharmacol.* **33**(5): 947-954.

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Research Article

VASORELAXATION EFFECT OF SODIUMNITROPRUSSIDE AND 3-MORPHOLINOSYDNONIMINE IN ENDOTHELIUM DENUDED GOAT INTERNAL ILIAC ARTERY

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ABSTRACT

The experiment was undertaken to study the effect of sodiumnitroprusside (SNP) and 3-morpholinosyndonimine (SIN-1) on endothelium denuded goat internal iliac artery (GIIA). SNP and SIN-1 were used to study the tonicity of internal iliac artery in both endothelium intact and denuded vessels. We observed that removal of endothelium did not completely inhibited the relaxation response of both SNP and SIN-1 on NA precontracted internal iliac artery of goat. The mean pD_2 values produced by SNP and SIN-1 in endothelium intact GIIA rings pre-contracted with NA were 6.35 ± 0.2 and 6.83 ± 0.29. With respect to endothelial intact vessels, there was no significant change in pD_2 values of all cases of endothelial denuded vessels. The mean E_{max} values produced by SNP and SIN-1 in endothelium intact GIIA rings pre-contracted with NA were 84.32 ± 4.6 % and 91.03 ± 3.2 %, $p<0.01$) whereas in endothelium denuded vessels, E_{max} of SNP and SIN-1 were decreased with 79.0 ± 2.16 % and 70.9 ± 4.07 %. From this study, it was concluded that endothelium denudation inhibited SIN-1 induced relaxation, but not SNP in goat internal iliac artery.

Keywords: Sodium Nitroprusside (SNP), 3-morpholinosyndonimine (SIN-1), endothelium, goat internal iliac artery (GIIA).

INTRODUCTION

Nitric oxide (NO) is a small, diffusible, lipophilic free radical gas that mediates significant and diverse signaling functions in nearly every organ system in the body. NO, liphophilic in nature, acts in part by stimulating smooth muscle cell soluble guanylate cyclase (sGC) which converts GTP to cGMP. cGMP is an intracellular second messenger that activates cGMP dependent protein kinase, as well as other cGMP –dependent proteins, resulting in diverse effects including relaxation of vascular smooth muscle (Lucas *et al*., 2000). The first demonstration of endothelium dependent relaxation in response to acetylcholine in rabbit aorta by Furchgott and Zawadzki (1980). Later on, NO-donor-mediated endothelium independent relaxation by organic nitrates such as sodiumnitroprusside (SNP), SIN-1 has been reported in arteries from NO deficient animals (Brandes *et al*., 2000). SNP, a potent nitrovasodilator (Gryglewski *et al*., 1992) and SIN-1(Petersson *et al*., 2000) cause vasodilation by producing NO. These organic nitrovasodilator were used in this study to investigate the significance of endothelium in vascular smooth muscle relaxation of goat internal iliac artey (GIIA).

MATERIALS AND METHODS

Goat internal iliac artery collection and tissue preparation

Goat internal iliac artery branches were collected, from local abattoir within 20-30 min of slaughter and kept in cold, aerated Modified Kreb's Henseleit solution (MKHS). MKHS of the following composition (mM/L) NaCl, 118.0 (6.9); KCl, 47 (0.28); $NaHCO_3$, 11.9 (1.0); KH_2PO_4, 1.2 (0.16); Glucose, 11.1 (2.0); $MgSO_4.7H_2O$, 1.2 (0.295) and $CaCl_2.2H_2O$ 2.5 (0.28). Parenthesis indicates g/L of the ingredient. The right GIIA branches were carefully dissected and cleaned of its fat and connective tissue and cut into rings of 2-2.5 mm length and 2-3 mm external diameter. For denudation of endothelium, the lumen of GIIA was gently rolled with horse tail hair.

Drugs and chemicals

3-Morpholino-sydnonimine (SIN-1): M.W. 206.6 g/mol, 10^{-4} M solution was prepared in DMSO. Due to its photosensitive nature, all the experiments of SIN-1(Cayman Chemical, USA) were conducted in dark room. Noradrenaline (NA, 10^{-5}M) (Sigma, USA) stock solution was prepared in 0.1 N HCl containing 0.01 % of ascorbic acid, as an antioxidant. Sodium nitroprusside (SNP, 10^{-4} M) (LOBA Chemie, India) solution was prepared in analytical grade water.

Tissue mounting on organ bath

The goat internal iliac arterial ring was mounted between two L-shaped stainless steel hooks made of 28 gauge stainless steel wires and then the stainless steel hooks were immersed into MKHS solution containing tissue bath in a thermostatically controlled (37.0 ± 0.5 ^{0}C) organ bath of 20ml capacity were continuously aerated with carbogen (95 % O_2 + 5 % CO_2), anchoring into a

tissue holder. The free end of the stainless hook was gently kept on the isometric force transducer. (Model: MLT 0201, Power Lab, Australia) sensitive to 5mg– 25mg positioned on a micromanipulator (Panlab.S.I., Spain). The arterial rings were then kept under 1.2 g tension for a period of 90 minutes calibrating with help of the software chart 6 Pro. During this period, the bathing fluid was changed every 15 min. After equilibrating for 90min, internal iliac artery rings were contracted with NA. At the plateau of NA, ACh (1.0µM) was added to determine endothelial integrity. A relaxant (70 %) response to ACh confirmed the presence of a functional endothelium, while absence of appreciable relaxation (10%) after mechanical denudation confirmed satisfactory endothelium removal.

Experimental technique

NA (10^{-5} M) induced contraction and relaxed by SNP / SIN-1 in GIIA

To investigate the contribution of NO and its endothelial dependence, the internal iliac artery relaxed with SNP / SIN-1 in both endothelium intact and denuded. The arterial ring segments first contracted with NA (10^{-5} M), after 90 min of equilibration, which was relaxed with cumulative doses of SNP / SIN-1 (10^{-9} to 10^{-4} M) in both endothelium intact and denuded at 4min interval with increment of 0.5 log unit. The time to plateau and plateau tension were recorded for each experiment. The pD_2 and E_{max} were recorded for each experiment and compared.

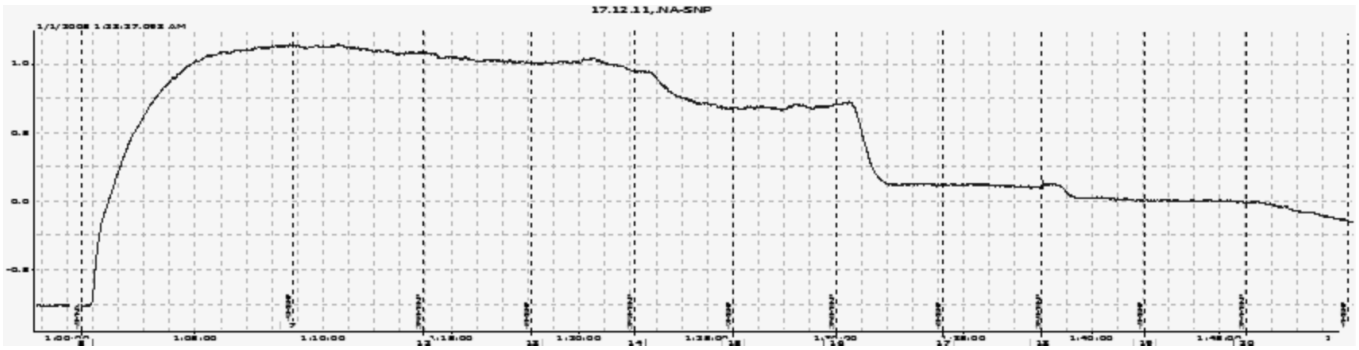
Table 1:
pD_2 and E_{max} of Vasorelaxation of SNP and SIN-1 (1çM-100ìM) on NA (10ìM) induced contraction in presence and absence of endothelium.

Characters	pD_2		E_{max} /E_{Bmax} (%)	
	ENDO (+)	ENDO (-)	ENDO (+)	ENDO (-)
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
SNP	6.35±0.20	5.94±0.05	84.32±4.60	79.0±2.16
SIN-1	6.83±0.29	6.51±0.24	91.03±3.20	70.9±4.07**

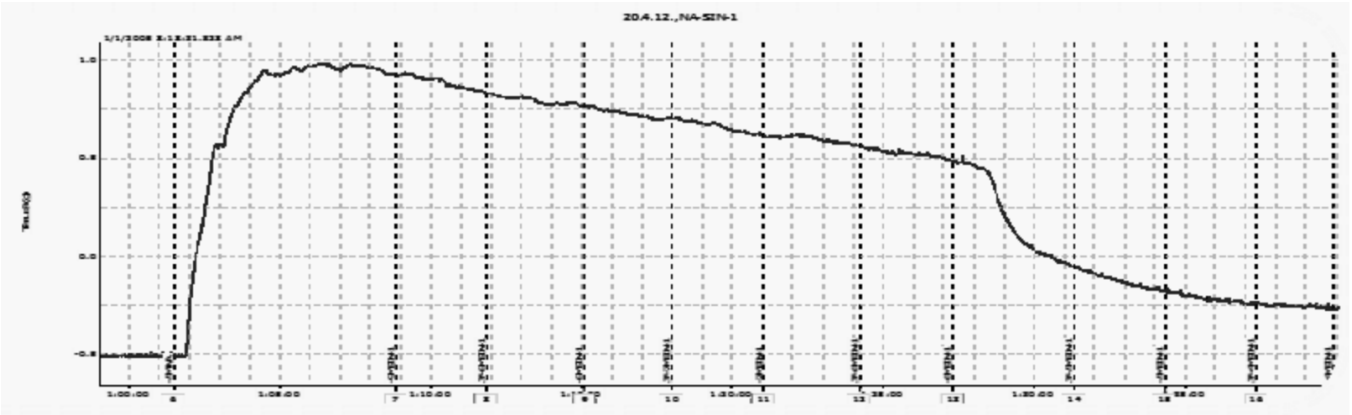
* Values differ significantly wh en compared with control (**P<0.01 and ***P<0.001)

Data analysis

Relaxation responses were expressed as the percentage reversal of NA induced contraction. Emax (the maximal relaxation) was determined by non-linear regression analysis (sigmoidal dose response with variable slope) using Graph Pad Prism version 4.0 (Graph Pad software, Inc, San Diago, CA). Potency was expressed as pD2= -logEC50. Results are expressed as mean ± standard error of the mean, with n being the number of vascular rings. Data were analyzed by 2-way analysis of

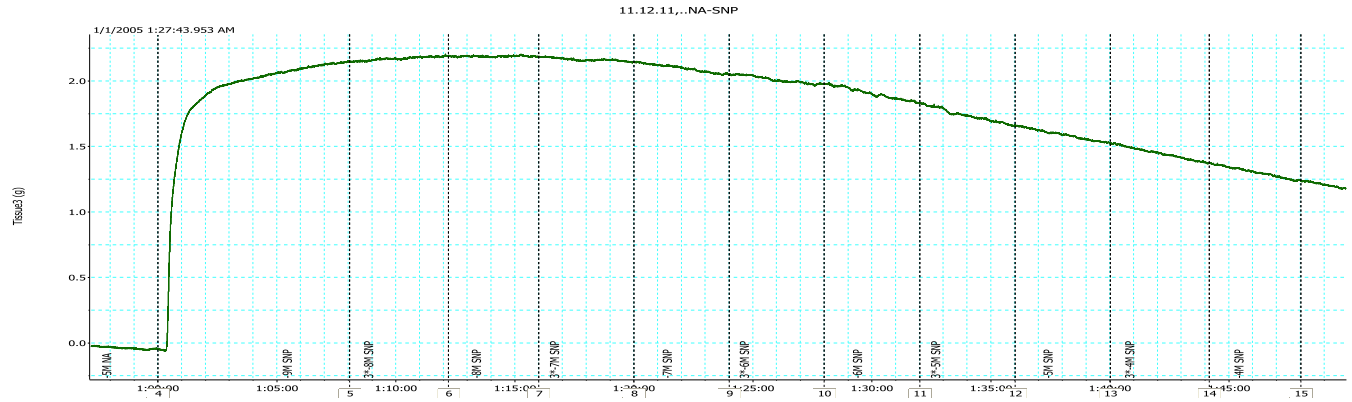


Trace 1a:

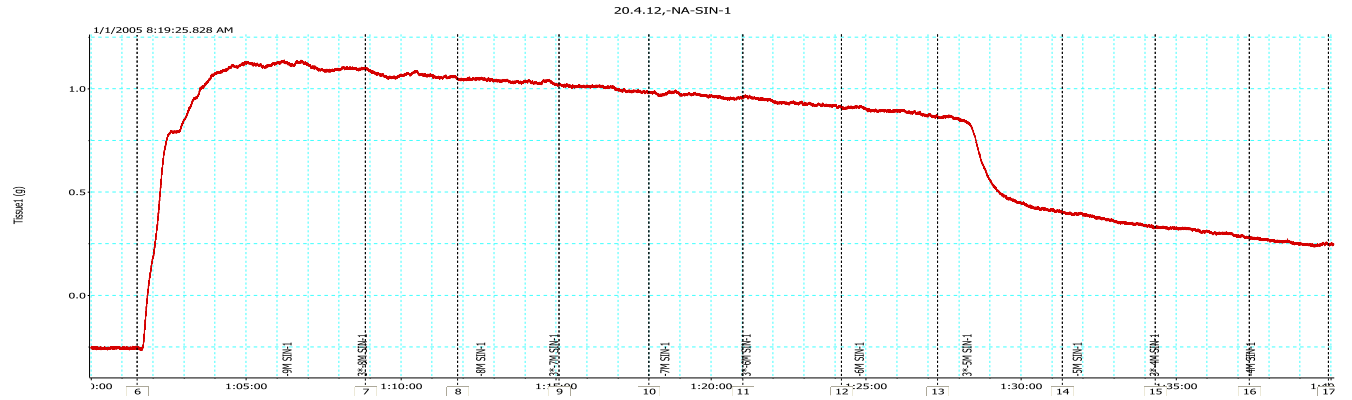


Trace 1b:

Trace: Representative physiograph recording showing concentration dependent relaxation of SNP (trace 1a) and SIN-1 (trace 1b) on NA induced pre-contraction in intact endothelium of GIIA.

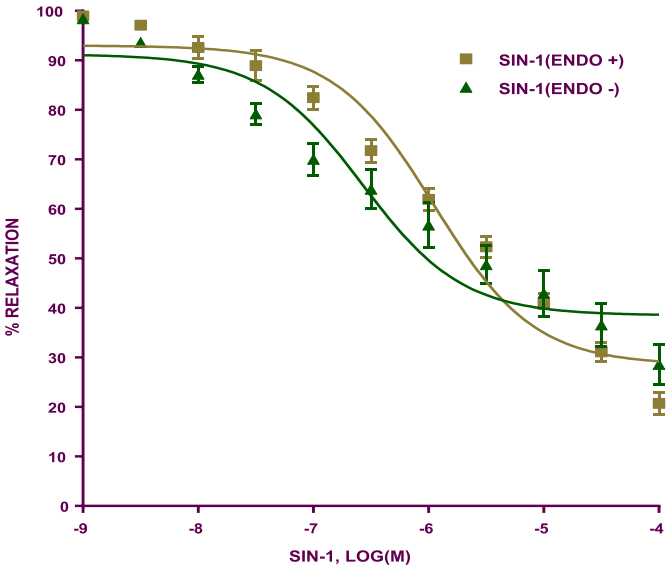


Trace 2a:



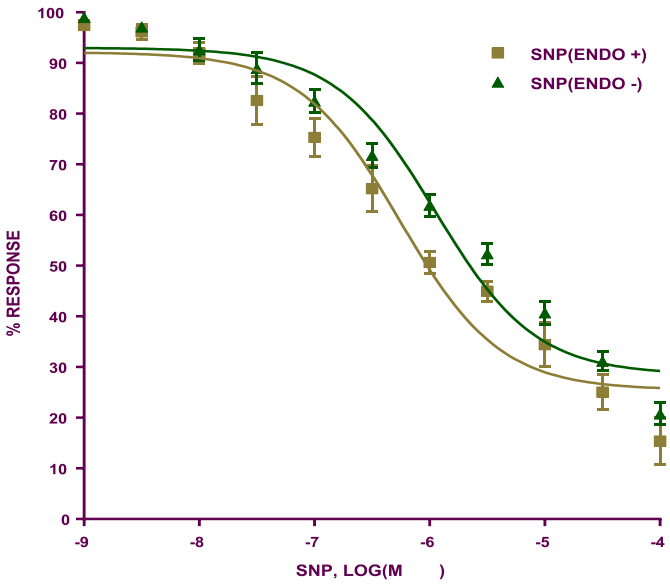
Trace 2b:

Trace: Representative physiograph recording showing concentration dependent relaxation of SNP (trace 2a) and SIN-1 (trace 2b) on NA induced pre-contraction in denuded endothelium of GIIA.



Effect of NA (10^{-5}M) inducedc contraction and relased with SNP (10^{-9} to 10^{-4}M) in endothelium intact and denuded goat internal illac artery

Fig 2



Effect of NA (10^{-5}M) inducedc contraction and relaxed with SIN-1 (10^{-9} to 10^{-4}M) in endothelium intact and denuded goat internal illac artery

Fig 1

variance (ANOVA) for multiple comparisons followed by Bonferroni post hoctest. Student's t-test was employed for comparison between two groups.

RESULTS AND DISCUSSION

On NA (10^{-5} M) induced sustained contraction, cumulative doses (10^{-9} to 10^{-4} M) of SNP (Fig 1) and SIN-

1 (Fig 2) were produced vasorelaxation in both endothelium intact (Trace 1a and 1b) and denuded (Trace 2a and 2b) GIIA. The mean pD_2 of SNP and SIN-1 (10^{-9} to 10^{-4} M) induced a concentration dependent relaxation in endothelium intact GIIA rings pre-contracted with NA were 6.35 ± 0.2 and 6.83 ± 0.29. With respect to endothelial intact vessels, there was no significant change in pD_2 of SNP and SIN-1 values of all cases of endothelial denuded vessels (5.94 ± 0.05 and 6.51 ± 0.24). In endothelium denuded vessels, the vasorelaxation of SNP and SIN-1 were decreased with decrease in the E_{max} (79.0 ± 2.16 % Vs 84.32 ± 4.6 % and 70.9 ± 4.07 % Vs 91.03 ± 3.2 %, $p<0.01$) (Table 1). Thus, endothelium denudation significantly affect SIN-1 vasorelaxation of goat internal iliac artery.

SNP and SIN-1 were produced vasorelaxation in both endothelium intact and denuded goat internal iliac artery. In endothelium denuded GIIA rings, these vasodilators caused a slight decrease in the mean E_{max} of SNP (5 %) but significant decrease in the mean E_{max} of SIN-1 (20 %) induced concentration dependent relaxation pre-contracted with NA, when compared with endothelium intact rings of the same artery. These results indicates that endothelium independent NO release prominent in SNP than SIN-1 vasodilator.

Endothelium denudation significantly affect the vasorelaxation of SIN-1 rather than SNP, which indicates SIN-1 may under the influence of endothelium mediated metabolic conversion to become active or inhibiting some contracting agents released from endothelium, when compare to SNP. The effects of SIN-1 in isolated canine basilar arteries wherein, the inhibition of endothelium-dependent contractions could be due in part to chemical interactions between endothelium-derived contracting factor (superoxide anion) and the NO liberated by SIN-1 (Katusic *et al*. 1989). The inhibitory effect of SIN-1 on Ca^{2+}-induced contraction as observed by Deka *et al*. (2005) was also found to have predominant inhibitory effect of the nitrovasodilator on Ca^{2+} current in frog myocytes (Wahler and Dollinger, 1995). Similarly, SNP was reported to attenuate Ca^{2+} influx in rat aorta (Magliola and Jones, 1990) and to decrease inward Ca^{2+} current in single smooth muscle cells isolated from pulmonary artery.

Eventhough SNP and SIN-1 releases NO, there will be slight change in their vasorelaxation effect, which activates soluble guanylyl cyclase in VSM, causing cGMP accumulation and activation of cGMP-dependent protein kinase, which phosphorylates and activates the K_{ATP} channel, allowing K^+ efflux, resulting in a hyperpolarization. The difference in hyperpolarizations due to SIN-1 and nitroprusside, suggest that NO initiates the hyperpolarization, rather than some other pharmacological effect of the NO-generating agents. The role of cGMP-dependent protein kinases in NO induced hyperpolarizations is likely, but is not directly shown in the present study.

REFERENCES

Brandes, R.P., Kim, D., Schmitz-Winnenthal, F.H., Amidi, M., Godecke, A., Mulsch, A. and Busse, R. (2000). Increased nitrovasodilator sensitivity in endothelial nitric oxide synthase knockout mice:role of soluble guanylyl cyclase. *Hypertension*. **35**:231-236.

Deka, D.K., Raviprakash, V. and Mishra, S.K. (2005). Sodium nitroprusside relaxes goat coronary artery through activation of calcium. *Indian J. Exp. Biol*. **43**:324-329.

Furchgott, R.F. and Zawadzki, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* **288**:373-6.

Gryglewski, R.J., Zembowicz, A., Salvemini, D., Taylor, G.W. and Vane, J.R. (1992). Modulation of the pharmacological actions of nitrovasodilators by methylene blue and pyocyanin, *Br. J. Pharmacol*. **106**:838-845.

Kobzik, L., Reid, M.B., Bredt, D.S. and Stamler, J.S. (1994). Nitric oxide in skeletal muscle. *Nat.* **372**:546-548.

Lucas, K.A., Pitari, G.M., Kazerounian, S., Ruiz-stewart, I., Park, J., Schulz, S., Chepenik, K.P. and Waldman, S.A. (2000). Guanylyl Cyclases and Signaling by cyclic GMP. *Pharmacol. Rev.* **52:**375-413.

Magliola, L. and Jones, A.W. (1990). Sodium nitroprusside alters Ca^{2+} flux components and Ca^{2+} dependent fluxes of K^+ and Clin rat aorta. *J. Physiol.* **421**:411-24.

Petersson, J., Ryman, T.and Hogestatt, E.D. (2000). Vasodilator effects of KRN2391, levcromakalim and 3-morpholino-sydnonimin in human pial and omental arteries. *Naunyn Schmiedebergs Arch. Pharmaco*l. **362(1)**:68-73.

Wahler, G.M. and Dollinger, S.J. (1995). Nitric oxide donar SIN-1 inhibits mammalian cardiac calcium current through cGMP-dependent protein kinase. *Am. J. Physiol.* **268**:C45-C54.

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Research Article

TISSUE RESIDUES OF ENROFLOXACIN AND CIPROFLOXACIN FOLLOWING ORAL ADMINISTRATION OF ENROFLOXACIN IN CHICKENS

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ABSTRACT

This study was designed to determine the tissue residues following oral administration of enrofloxacin in broilers chicken at therapeutic (TD) and extra-label doses (ED). Broiler chickens were divided into three groups (I, II & III) with 8 birds in each group and orally administered enrofloxacin at TD and ED doses. Group II and III received enrofloxacin at TD (10 mg.kg $^{-1}$ x 5 days P.O) and EL (20 mg.kg $^{-1}$ x 5 days P.O) doses, respectively. While Group I was maintained as untreated control and was given placebo (distilled water). After oral dosing for enrofloxacin (Enrogen®)for 5 days, the birdswere slaughteredat different time intervals and residues (enrofloxacin and its metabolite ciprofloxacin) were analyzed in plasma, breast muscle, liver and kidney by using HPLC. Residue profile revealed higher residue levels (>prescribed MRL) upto 36 and 60 hours in the TD and ED groups, respectively. In the therapeutic dose group, ciprofloxacin was not detected in plasma and breast muscle a 96 hours post-withdrawal. However, it was detected in liver and kidney in traces; nevertheless extra labeled dose group showed ciprofloxacin residues at 84 hours post- withdrawal. Tissue residue depletion profile of both enrofloxacin and ciprofloxacin were in the order of liver >kidney>breast muscle in both the treatment groups. Conclusively, it is suggested to adopt a four days withdrawal period following therapeutic or twice the therapeutic (extra-label) administration of enrofloxacin in broiler chickens.

Key words: Enrofloxacin and ciprofloxacin residues in chickens

INTRODUCTION

Enrofloxacin is a synthetic antimicrobial of fluoroquinolone group which is extensively used in the veterinary practice including poultry for the treatment of wide range of infections of respiratory and alimentary tract caused by Gram-negative bacteria (Martinez *et al*., 2005). It inhibits DNA gyrase enzyme of bacteria which affects DNA super coiling. After oral administration, enrofloxacin gets absorbed through the gut, distributed into tissues and excreted through urine and faeces (Prescott *et al*., 2000). Metabolite ciprofloxacin is formed consequent to hepatic metabolism of enrofloxacin which possess bactericidal activity (Myllyniemi *et al*., 1999). Enrofloxacin and its metabolite ciprofloxacin both require to be assayed for assessing its residues profile. Although these residues could be detected by several methods (microbiological, immunological, and chromatographic methods) like other fluoroquinolones, HPLC has been the most widely employed method. Since food safety compliance requires residue monitoring, method used must detect residues at or below the MRL levels (Aerts *et al.,* 1995). The MRL for combined enrofloxacin and ciprofloxacin in the EU is 100and 200 ng/g for chicken meat and liver, respectively (Cinquina *et al.,* 2003). Consequent to the rising concerns about the fluoroquinolones residues in meat, a study was designed to determine residue profile of enrofloxacin and its metabolite ciprofloxacin in broiler chickens upon oral administration at therapeutic and extra-label doses so as fix drug withdrawal period to address public health risk associated with residues.

MATERIALS AND METHODS

Experimental birds

Broiler chicken (N=24) aged three weeks (Vencob®)were procured from a commercial broiler farm and acclimatized for two weeks prior to experiment. Commercial broiler feed and bore-well water was provided without any medication and Institutional Animal Ethics Committee (IAEC) approval was obtained (No. 44/LPM/IAEC/2012, Veterinary College, Hebbal, Bangalore).

Drugs and chemicals

For oral administration, commercial formulation Enrogen®(Indian Immunologicals, Hyderabad, India*a.i.*100mg/mLenrofloxacin) was used. HPLC grade enrofloxacin ($C_{19}H_{22}FN_30_3$) and ciprofloxacin ($C_{17}H_{18}FN_3O_3$) analytical standards(purity e"98 per cent, Cipla Pharmaceutical Pvt. Ltd, Mumbai); and HPLC grade solvents like acetonitrile, methanol, triethylamine, acetic acid and water (Qualigens, Fisher Scientific Ltd, Hyderabad) were used for the study.

Experimental design

Broiler chicken were divided randomly into three groups of 8 each (Group I, II and III,Table 1).Group Iwas kept as control without administration of enrofloxacin treatment and was administered distilled water. Group II and III were kept as experimental groups and administered enrofloxacin through drinking water at therapeutic (10 mg/kg, P.O. daily) and extra-label dose (20 mg/kg body, P.O. daily) for 5 consecutive days (*i.e*. from day 36thto 40thof bird age). From 40th day onwards birds from each group were slaughtered at 12, 24, 36, 48, 60, 72, 84 and 96 hours interval.

Sample collection

Liver, kidney and breast muscle samples were collected from the slaughtered chicken individually in separate polythene bags. Blood samples were collected in polypropylene micro centrifuge tubes and plasma was separated. Tissue and plasma samples were stored in deep freezer (-20°C)until further analysis. The residue analysis comprised of residue extraction, detection and quantification protocols as per Graci*aet al*., (2001).

Extraction of residues

Liquid-liquid partition was used to extract enrofloxacin and ciprofloxacin residues from the tissue samples. Briefly, 2 g of tissue was homogenized with 4 mL of phosphate buffer and centrifuged for 5,000 rpm for 8 minutes. To the 300 µl supernatant fluid was collected in a fresh tubeand 450 µl of acetonitrile was added (1:1.5) and vortexed for 30 seconds. The mixture was centrifuged at 5,000 rpm for 12 minutes and resultant clear supernatant was transferred into a 2 mL micro centrifuge tube, filtered through 0.2µm membrane filter and 20 µl was injected into the HPLC system.

HPLC assay

Enrofloxacin and ciprofloxacin residues in plasma and tissue samples were determined by high performance liquid chromatography (HPLC; Shimadzu Prominence, Japan). The recovery percentage, repeatability and assay precision were calculated using standard methods.

Analytical HPLC system comprised of double pump (LC-20AT), rheodyne manual injector (20 µl loop), dual wavelength UV detector (SPD-20A) and LC Solution®data analysis software. A reverse phase C_{18} column (250 x 4.5 mm, particle size 5±0.3µm, pore diameter 100±10 A°, Merck®, USA) was used as a stationary phase; and mixture of water, methanol and acetonitrile (80:5:15,v/v, pH 3 adjusted using 0.4% of triethylamine) containing 0.4 per cent of orthophosphoric acids as the mobile phase and was run at 1mL per minute.The UV detector (SPD-20A) at excitation wavelength of 278 nm and emission wavelength of 455 nm was used; and 20µl volume was injected in to the system. Calibration curve was constructed for the standard antibiotics by spiking the drug free plasma and meat tissues. Standard curve obtained by plotting concentration versus peak area (Fig. 1) showed linearity in the range of 0.5 to 100 µg.g^{-1} (r^2 0.998). The limit of detection (LOD) of 0.075 and 0.0375 was recorded for enrofloxacin and ciprofloxacin, respectively. Residue levels in the samples were determined using calibration curve of spiked samples. Linear regression equation of calibration curve was used to calculate the concentration of enrofloxacin and ciprofloxacin in plasma and tissue samples. Recovery of enrofloxacin and ciprofloxacin in the spiked samples were 82.75, 77.73, 83.39, 82.80 and 82.75, 82.73, 84.90, 86.28 per cent, respectively for plasma, breast muscle, and liver and kidney tissues. The intra- and inter-day assay coefficient of variation were <10% for each analyte.

Statistical analysis

The experimental data was expressed as mean±SD and using MS-Excel program.

RESULTS AND DISCUSSION

Concentrations of enrofloxacin and its metabolite ciprofloxacin in plasma or tissue samples (*viz.,* liver, kidney and breast muscle) determined at different time intervals is shown in Table 1. Concentrations of both analytes (enrofloxacin and ciprofloxacin) were found higher in plasma followed by liver, kidney and breast muscle in broilers that received both therapeutic and extra-label doses of enrofloxacin. Ciprofloxacin was detected in tissue samples from 12 hours of post-slaughter up to 72 hours and its levels were higher than that of enrofloxacin. Plasma or tissue samples of control group (group I) analyzed simultaneously did not show any residues.

Plasma enrofloxacin reached peak concentration of 564.5 and 446.7 µg.ml^{-1} at 12 and 24 hours post-administration; and its concentration showed declining trend from 36 to 84 hours (120.2 to 0.68 µg.ml^{-1}) (ppb) when given at therapeutic doses (group II). Similar trend was noticed in the tissues *i.e.*, higher concentrations of 128.8 & 130.4, 483.1 & 458.6, 343.4 & 344.2 µg.g^{-1} in breast muscle, liver and kidney samples, respectively at 12 and 24 hours. From 36 hour onwards concentrations of enrofloxacin further reduced in the tissues (Table 1).

Similar trend was observed with respect to ciprofloxacin in plasma samples at 12, 24, 36 and 48 hours post- withdrawal (967.4, 656.9, 233.3 and 112.1 µg.ml^{-1}) in the TD group (II) and was significantly reduced thereafter (Table 1). However, in extra labeled dosing (ED, group III) plasma showed ~1.5 times higher concentration than the TD (group II).

In the therapeutic dose group, ciprofloxacin was not detected in both plasma and breast muscle samples at 96 hours post-withdrawal. However, ciprofloxacin was detected in liver and kidney samples in traces.In extra label dosed group ciprofloxacin residues up to 96 hours post- withdrawal was detected.The Depletion pattern of

Table 1:
Concentrations of enrofloxacin and ciprofloxacin residues in plasma (µg.ml^{-1}) and tissues (µg.g^{-1}) following oral administration of enrofloxacin in broiler chicken after therapeutic or extra-label dose.

Enrofloxacin								
Post- withdrawal period (Hours)	Group II Therapeutic dose (TD) 10 mg.kg^{-1}b.wt.; P.O× 5 days				Group IIIExtra labeled dose (ED) 20 mg.kg^{-1}b.wt; P.O× 5 days			
	Plasma	B. muscle	Liver	Kidney	Plasma	B. muscle	Liver	Kidney
12	564.5	128.8	483.1	343.4	732.2	178.8	517.3	634.1
24	446.7	130.4	458.6	344.2	719.8	170.4	612.2	568.3
36	120.2	81.0	222.4	257.5	465.1	139.2	489.3	456.4
48	92.1	33.8	88.2	176.3	324.5	45.1	346.5	298.6
60	26.2	12.3	44.6	59.8	128.7	34.3	142.4	119.0
72	8.4	2.1	10.0	15.0	48.4	4.5	59.3	32.9
84	0.68	ND	1.5	2.4	5.4	0.96	11.2	8.4
96	ND	ND	ND	ND	ND	ND	3.8	1.2
				Ciprofloxacin				
12	967.4	106.5	817.8	753.7	1254.6	112.2	1132.1	823.3
24	656.9	74.2	554.3	443.2	1098.2	96.4	864.9	389.6
36	233.3	54.9	321.1	210.8	679.0	83.6	578.3	291.1
48	112.1	34.8	168.4	196.3	431.5	68.3	538.7	255.5
60	22.5	10.3	56.9	33.6	190.7	59.5	154.4	184.2
72	6.2	0.86	23.5	4.5	67.4	17.5	160.4	98.7
84	0.23	0.12	7.4	0.96	21.5	1.8	55.2	34.4
96	ND	ND	0.4	0.2	4.6	0.6	19.8	5.4

ND= Not detected

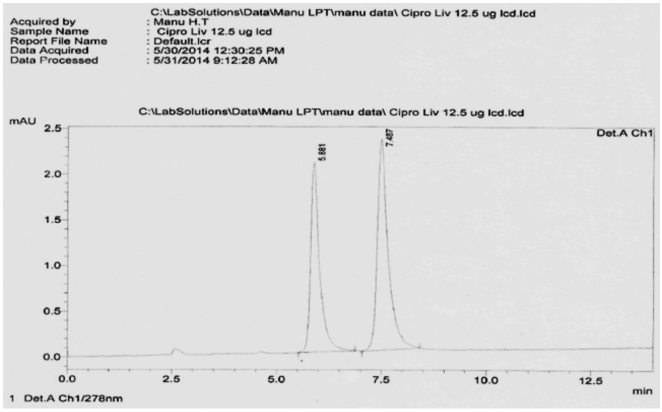

Fig.1.
Standard chromatogram of enrofloxacin (7.487) and ciprofloxacin (5.881)/

enrofloxacin and ciprofloxacin residues in broiler chicken plasma or tissues (*viz.*, breast muscle, liver and kidney) following withdrawal of enrofloxacin after therapeutic (10mg. kg^{-1} x 5 days P.O) or extra label(20mg.kg^{-1} x 5 days P.O) administration of enrofloxacin (Enrogen®) depicted in Figure 2.

Pharmacokinetic studies revealed that peak seru mconcentration of enrofloxacin occur within 72 hours, and undergoes wide distribution in to tissues on account of lipid solubility (Prescott, *et al*.,2000. Further, enrofloxacin undergoes extensive biotransformation in to ciprofloxacin in chickens (Anadon *et al.,*1995; Okerman *et al.,*2004) apart from minor metabolites, *viz:*oxociprofloxacin, dioxociprofloxacin.In the present study enrofloxacin residues levels were higher than MRL (EEC, 2003) levels in TD or ED after its withdrawal.Similarly, residues of ciprofloxacin in kidney tissues were also above the MRL levels at 12 and 24 hours post-withdrawal.

The residue depletion profile of enrofloxacin and ciprofloxacin in tissues were in the order of liver Ãkidney>breast muscles in both the groups (TD & ED).Our results with respect to depletion pattern of enrofloxacin in the liver, kidney and breast muscles of chickens were analogues to observations of Schneider and Donoghue (2006) in chickens, where in enrofloxacin and ciprofloxacin concentrations were 4 - 4.7 times higher in the liver and kidney during 6 and 12 hr post withdrawal and correlation decreased to 2.5 times at 24 hour after the last dose residues of enrofloxacin were 3.8 times higher than the MRL in liver as compared to muscle tissues in chickens after five days of therapeutic (10mg.kg^{-1}, P.O) dose with enrofloxacin in chickens (Petrovic*et al.,* 2006).The residue levels of enrofloxacin and ciprofloxacin were below the MRL levels, respectively at 36 and 84hr post withdrawal of drug in TD group. However, the residues of enrofloxacin and ciprofloxacin were persisted up 60 hr and 48 hours (Fig.2), respectively after the withdrawal of enrofloxacin (Enrogen®) in extra-label dose (20 mg.kg^{-1}).Our observation is in conformity with Jelena *et al*. (2006) and Schneider and Donoghue (2006).

Knoll *et al.*(1999) observed higher levels (2-10 times) of ciprofloxacin residues in the meat, liver and kidney after enrofloxacin administration in chicken. Similar to our observations the residue levels of ciprofloxacin were relatively higher (567.3 µg.gm^{-1}) in liver when compared to muscle tissue (328.8 µg.gm^{-1}). Elkholy *et al*. (2009) concluded that withdrawal time of 5 days was required in layers to deplete residues of enrofloxacin in eggs or tissues

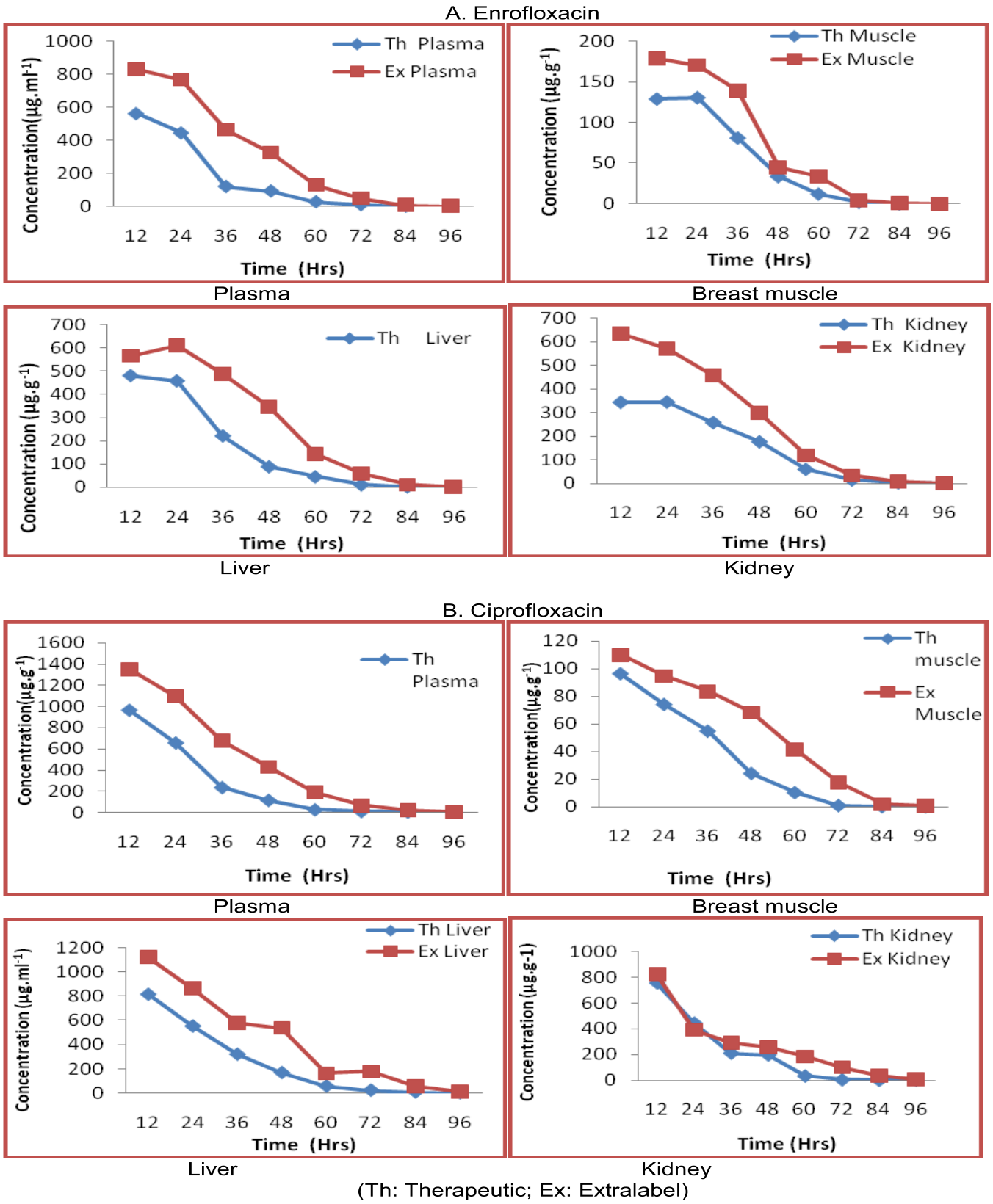

Fig.2:
Depletion pattern of enrofloxacin and ciprofloxacin residues in broiler chicken plasma and tissues following withdrawal of enrofloxacin after therapeutic (10mg kg $^{-1}$ x 5 days P.O.) or extralabel administration (20mg kg $^{-1}$ x 5 days P.O.).

after five days of per oral treatment with two formulations (Enrotryl® and Baytril®). Anadon *et al.* (1995) observed relatively higher concentrations of enrofloxacin(990ng.g^{-1}) and ciprofloxacin (960ng.g^{-1}) residues in liver tissue than in muscle tissues (respectively, 540 and 650 ng.g^{-1}) one day after the withdrawal of enrofloxacin treatment in chickens (10mg.kg^{-1} x5days, PO). The present study revealed higher residual concentrations of ciprofloxacin in breast muscle (112 µg.gm^{-1}) and liver (1132.1µg.gm^{-1}) when compared to enrofloxacin (128.8 µg.gm^{-1} in muscle and 567.3 µg.gm^{-1} in liver tissue) after TD or ED of enrofloxacin (Enrogen®) in broiler chickens. Therefore, conclusively a four days withdrawal period for enrofloxacin (Enrogen®, 10%w/v) is suggested in broiler chickens receiving either therapeutic (10mg.kg^{-1}) or extra-label dose (20mg.kg^{-1}) for five consecutive days.

Based on the depletion profile of enrofloxacin and ciprofloxacin in chicken tissues in the present study it can be concluded that twice the therapeutic dose of enrofloxacin in broilerchickens would lead to accumulation of enrofloxacin and ciprofloxacin residues in tissues above the MRL levels and pose public health hazards. The present study suggests that four days withdrawal period following the last dosing of enrofloxacin formulations (10%w/v) allowed the drug concentration to decrease to an acceptable level prior to slaughter (below the prescribed EU MRL for enrofloxacin and ciprofloxacin). Further, the present study stresses on the adaptation of stricter norms for use of antimicrobials in the poultry industry as well as need to monitor residues in meat prior to marketing by concerned regulatory authorities in India.

REFERENCES

Aerts, M.L., Hogenboom, A.C. and Brinkman, U.A. (1995).Analytical strategies for the screening of veterinary drugs and their residues in edible products.*J.Chromatogr. B: Biomed. Sci. Appl.* **667** (1): 1-40.

Anadón, A., Martínez-Larrañaga, M.R., Díaz, M.J., Bringas, P., Martínez, M.A., Fernàndez-Cruz, M.L., Fernández, M.C. and Fernández, R. (1995).Pharmacokinetics and residues of enrofloxacin in chickens.*Am. J. Vet. Res*. **56 (**4): 501-506.

Cinquina, A.L., Roberti, P., Gianetti, L., Longo, F., Draisci, R., and Brizioli, N.R. (2003). Determination of enrofloxacin and its metabolite ciprofloxacin in goat milk by high-performance liquid chromatograph with diode-array detection: optimization and validation. *J. Chromatography A.* **987:** 221-226.

EEC, 2003.European Union Council Regulation (EEC).*Off. J. Eur. Com.* pp.2377-2390.

Elkholy, H.M., Elkholy, A.A., Awidat, S.K and Elmajdoub, A.A. (2009).Tissue and egg residues and adverse effect of two oral enrofloxacin preparations: Baytril® and Enrotryl®.*Global Veterinaria.***3**: 363-368.

Gracia, M.A., Solans, C., Hernandez, E., Puig, M. and Bregante, M.A. (2001).Simultaneous determination of enrofloxacin and its primary metabolite ciprofloxacin in chicken tissues.*Chromatographia.***54:** 3-4.

Jelena, P., Balti, M., Stefanov, S. and Dragica, S. (2006).Residues of enrofloxacin and its main metabolite ciprofloxacin in broiler chickens.*ActaVeterinaria (Beograd)*.**56:** 497-506.

Knoll, U., Glunder, G., Kietzmann, M. (1999).Comparative study of the plasma pharmacokinetics andtissue concentrations of danofloxacin and enrofloxacin in broiler chickens.*J. Vet.Pharmacol.Therap.*22: 239-46.

Martinez, M., Mcdermott, P. and Walker, R. (2005). Pharmacology of the fluoroquinolones: a perspective for the use in domestic animals. *The Vet.Journal*.**18**: 24-76.

Myllyniemi, A.L., Rintala, R., Backman, C. and Niemi, A. (1999).Microbiological and chemical identification of antimi-crobial drugs in kidney and muscle.*Food Addit.Contam.***16:** 339-351.

Okerman, L., De Wasch, K., Van Hoof, J. and Smedts, W. (2004).Simultaneous determination of different antibiotic residues in bovine and in porcine kidneys by solid-phase fluorescence immunoassay.*J. AOAC Int.* **86:** 236-240.

Petrovic, J., Baltic, M., Jupic, V., Stefanovic, S.I and StogjanovicDragica.(2006). Residues of enrofloxacin and its main metabolite ciprofloxacin in broilers.*ActaVeterinaria*.**56**: 497-506.

Prescott, J.F., Baggot J.D. and Walker, R.D. (2000).Fluoroquinolones for antimicrobial therapy in veterinary medicine, 3rd ed. Ames: Iowa State University Press.pp.315-339.

Schneider, M.J. and Donoghue, D.J. (2006). Comparison of bioassay and liquid chromatography-fluorescence-mass spectrometry method for the detection of incurred enrofloxacin residues in chicken tissue. *Poult. Sci.* **83** (5)**:** 830-834.

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Research Article

PHARMACOKINETICS AND DOSAGE REGIMEN OF CEFOPERAZONE FOLLOWING SINGLE DOSE INTRAVENOUS ADMINISTRATION IN SURTI GOATS

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ABSTRACT

The pharmacokinetics and appropriate dosage regimen of cefoperazone was studied following its single intravenous (IV) administration at the dose of 20 mg/kg body weight to surti goats (n=6). Cefoperazone concentrations in goat plasma were assayed by high performance liquid chromatography (HPLC). The elimination half-life ($t_{1/2\beta}$) was 2.96 ± 0.54 h. The mean values of apparent volume of distribution (Vd_{area}) and volume of distribution of drug at steady-state (V_{dss}) were calculated to be 0.45 ± 0.12 and 0.36 ± 0.05 L/kg, respectively. The mean value of total body clearance (Cl_B) was 3.23 ± 0.14 ml/min/kg. The average values for area under plasma drug concentration-time curve (AUC) and area under first moment of curve (AUMC) were 104.08 ± 4.48 μg.h/ml and 199.28 ± 36.10 μg.h^2/ml. The average value of mean residence time (MRT) was 1.88 ± 0.30 h. Based on the pharmacokinetic parameters, an appropriate dosage regimen for intravenous administration to maintain a minimum plasma drug concentration of ≥ 1.0 μg/ml would be of 11.0 mg/kg of body weight repeated at 12 h intervals in surti goats.

Key words: Cefoperazone, pharmacokinetics, dosage regimen, Surti goats

INTRODUCTION

Cefoperazone, is a semi-synthetic third generation, piperazin β-lactam antibiotic that possesses broad spectrum activity against a wide range of aerobic and anaerobic gram-positive and gram-negative bacteria. Cefoperazone is suitable for the treatment of bone and joint infections of horses (Soraci *et al*., 1998), calf diseases such as diarrhea and pneumonia associated with gram-negative bacteria resistant to commonly used antibiotics (Soback *et al*., 1986). The minimum inhibitory concentration (MIC) for a majority of Cefoperazone sensitive bacteria is in the range of 0.05 to 4 μg/ml (Jones *et al.,* 1983; Neu, 1984). The pharmacokinetics of cefoperazone has been determined in dogs (Montesissa *et al*., 1981), unweaned calves (Soback and Ziv, 1989), weaned calves (Gupta *et al*., 2007), buffalo calves (Goyal *et al*., 2003), sheep (Soni *et al.,* 2012) and horses (Soraci *et al*., 1996). Owing to its high efficacy, broad spectrum of activity, rapid tissue penetration, high safety and very low development of bacterial resistance, cefoperazone is gaining popularity among practitioners. Considering its great potential for its use in treatment of bacterial infections of goats, the present study was planned to determine the pharmacokinetics and appropriate dosage regimen of cefoperazone in surti goats following its single intravenous (IV) administration at the dose of 20 mg/kg body weight.

MATERIALS AND METHODS

The study was conducted on six Surti goats of 2-3 years of age weighing between 22.0 to 32.0 kg. Intravenous injection of the drug was given through a jugular vein using 20G x 25mm needle. Cefoperazone sodium (Megnamycin, Pfizer International Ltd., Mumbai, India) equivalent to cefoperazone 1 gram for injection was procured from the local market. Potassium dihydrogen phosphate (KH_2PO_4, AR grade), perchloric acid, acetonitrile, methanol and water (HPLC grade) were purchased from Hi Media Laboratories Pvt. Ltd. and Merck India Ltd., Mumbai, India. Blood samples (2 ml each) were collected in heparinized centrifuge tube using IV catheter fixed contra-laterally to the jugular vein at 0 minutes (before drug administration), 2, 5, 10, 15, 30, 45 minutes and at 1, 2, 4, 8, 12, 18, 24 and 36 h after intravenous administration of drug. Plasma was separated soon after collection by centrifugation at 3000 rpm for 10 minutes at 10°C (Eppendorf 5804 R, Germany). Separated plasma samples were transferred to labeled cryovials and stored at -40 °C until assayed.

Plasma cefoperazone concentration was determined by adopting procedure as reported by Haghgoo *et al*. (1995) with minor modifications using high performance liquid chromatography (HPLC) apparatus of Laballiance (USA). Chromatographic separation was performed by using reverse phase C_{18} column (Whatman®, PARTISIL ODS-3 RAC-II; 4.60 X 100 mm ID) at room temperature. The data integration was performed using software clarity (Version 2.4.0.190). The mobile phase was a mixture of 30 mM KH_2PO_4 buffer: methanol (70:30) at a pH of 5.0. The 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 266 nm wavelength. Standard curve of cefoperazone was prepared using drug-free goat plasma. The sensitivity of assay method for cefoperazone was 1 µg/ml. The assay was sensitive, reproducible and linearity was observed between 1 to 200 µg/ml. The mean correlation coefficient (r^2) was 0.9995. The various pharmacokinetic parameters were calculated from plasma concentration of cefoperazone using software PK solution (version 2.0, Summit Research Services, USA). Based on the pharmacokinetic data, the dosage regimen of cefoperazone were also determined (Baggot, 1977).

RESULTS AND DISCUSSION

All animals remained in good health throughout the acclimatization and study periods. The plasma levels of cefoperazone after its single IV administration (20 mg/kg) in healthy goats are depicted in Figure 1. Following intravenous administration of the drug, plasma cefoperazone levels of 196.73 ± 3.39 and 1.19 ± 0.26 µg/ml of drug were measured at 0.033 and 8 h, respectively. Similar therapeutic concentration in plasma up to 8 h (155.31 ± 13.29 µg/ml and 2.29 ± 0.46 µg/ml) have been reported in sheep (Soni *et al.,* 2012). Various pharmacokinetic parameters calculated from plasma concentration – time profile after single dose IV administration of cefoperazone (20 mg/kg) in healthy goats are depicted in Table 1.

The elimination half life of 2.96 ± 0.54 h of cefoperazone following intravenous administration was observed in goats. Similarly half life of 3.80 ± 0.60 h has been reported in sheep following intravenous administration of cefoperazone (Soni *et al.,* 2012). Nevertheless, value of elimination half-life of cefoperazone observed in present study is shorter than the half-life of 5.65 h reported in buffalo calves (Goyal *et al*., 2003). The total body clearance of cefoperazone in the present study was 3.23 ± 0.14 ml/min/kg. The total body clearance in the present study is faster than 1.96 ± 0.05 and 0.72 ± 0.18 ml/min/kg reported in dogs (Montesissa *et al*., 1981) and horses (Soraci *et al*., 1996), respectively. However, the total body clearance in the present study is slower than body clearance of 11.5 ± 0.33 and 8.16 ± 1.60 ml/min/kg as reported in cross-bred cow calves (Gupta *et al*., 2007) and unweaned cow calves (Soback and Ziv, 1989), respectively. Shorter elimination half life and faster total body clearance suggests rapid elimination of cefoperazone in goats.

The mean apparent volume of distribution (Vd_{area}) observed following intravenous administration was 0.45 ± 0.12 L/kg. However, mean value of Vd_{area} have been reported 0.23 L/kg in dogs (Montesissa *et al*., 1981) and 0.31 ± 0.09 L/kg in horses (Soraci *et al*., 1996). However, higher values of Vd_{area} of 1.06 ± 0.13 L/kg in sheep (Soni *et al.,* 2012), 1.69 L/kg in unweaned cow calves (Soback and Ziv, 1989) and 1.3 L/kg in buffalo calves (Goyal *et al*., 2003) have been reported. The area under plasma drug concentration time curve (AUC) following IV administration was observed as 104.08 ± 4.48 µg h/ml which is higher than the value of 86.43 ± 5.15 µg h/ml in sheep (Soni *et al.,* 2012), 37.57 ± 5.55 µg h/ml in horse (Soraci *et al*., 1996) and 29.0 ± 1.03 µg h/ml in crossbred calves (Gupta *et al*., 2007).For intravenous dosage regimens the priming dose (D) and maintenance dose (D') of cefoperazone based

Table 1:
Pharmacokinetic parameters (mean ± SE) of cefoperazone after single dose IV administration (20 mg/kg) in healthy goats (n=6)

Pharmacokinetic parameters	Unit	Intravenous
Cp^0	µg/mL	136.55 ± 6.67
$t_{½\alpha}$	h	0.46 ± 0.02
$t_{½\beta}$	h	2.96 ± 0.54
$AUC_{(0-\infty)}$	µg.h/mL	104.08 ± 4.48
AUMC	µg.h^2/mL	199.28 ± 36.1
$Vd_{(area)}$	L/kg	0.45 ± 0.12
$Vd_{(ss)}$	L/kg	0.36 ± 0.05
$Cl_{(B)}$	mL/min/kg	3.23 ± 0.14
MRT	h	1.88 ± 0.30

Cp^0: Concentration at time 0; $t_{1/2\alpha}$: Distribution half-life; $t_{½\beta}$:Elimination half-life; $AUC_{0-\infty}$: Area under plasma drug concentration-time curve; AUMC: area under first moment curve; Vd_{area}: Apparent volume of distribution; Vd_{ss}: Volume of distribution at steady-state; Cl_B: Total body clearance; MRT: Mean residence time.

Table 2:
Calculated intravenous dosage regimens of Cefoperazone, required to maintain specific plasma cefoperazone concentration in surti goats

MIC (µg/ml)	Dose (mg/kg) and Dosing intervals (h)					
	6		8		12	
	D	D'	D	D'	D	D'
0.05	0.1	0.08	0.17	0.15	0.52	0.5
0.10	0.19	0.16	0.34	0.3	1.04	1
0.50	0.97	0.79	1.69	1.51	5.18	5
1.00	1.93	1.57	3.38	3.02	10.36	10
2.00	3.86	3.14	6.76	6.04	20.72	20
5.00	9.66	7.86	16.9	15.1	51.8	50

D = Priming dose. D' = Maintenance dose.

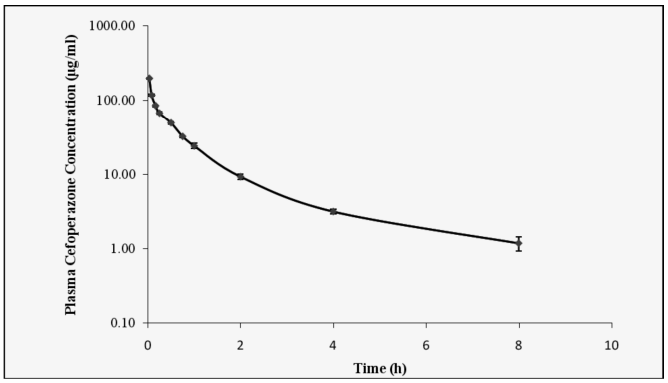

Fig. 1:
Semilogarithmic plot of cefoperazone concentration in plasma versus time following IV administration in goats (Each point represents mean ± SE of six animals)

on the desirable minimum plasma concentration was calculated by using equations; $D = C_P^{\alpha}(min).V_d(e^{\beta\tau})$ and $D' = C_P^{\alpha}(min).V_d(e^{\beta\tau} - 1)$. Where, C_P^{α} is the minimum inhibitory concentration, V_d is the volume of distribution at steady state, e represents the base of natural logarithm, β is overall elimination rate constant and τ (Tau) is the dosage interval (Baggot, 1977).

Computed intravenous dosage regimens of cefoperazone for goats are presented in Table 2**.** Therapeutic dosage regimens have been calculated for the maintenance of minimum concentrations of 0.05, 0.10, 0.5, 1, 2 and 5 μg of cefoperazone per ml of plasma and for administration at intervals of 6, 8 and 12 h. Table 2 shows the priming (D) and maintenance doses (D') of cefoperazone to be administered at various time intervals for clinical use in goats. To maintain MIC ≥ 1.0 μg/ml, satisfactory intravenous dosage regimen would be 10.36 mg/kg body weight as priming dose followed by maintenance doses of 10 mg/kg body weight per 12 h. It is concluded from this investigation the most suitable dosage schedule of cefoperazone for MIC of ≥ 1.0 μg/ml would be 10.36 mg/kg followed by 10 mg/kg repeated at 12 h intervals in surti goats.

REFERENCES

Baggot, J.D. (1977). Principles of drug disposition in domestic animals. The basis of veterinary clinical pharmacology. Ist edn., W.B. Saunders Co., Philadelphia, U.S.A, pp. 144-189.

Goyal, S., Chaudhary, R. K. and Srivastava, A. K. (2003). Pharmacokinetics of cefoperazone in buffalo calves following its intravenous administration. *J. Vet. Pharmacol. Toxicol.* **3**:104-108.

Gupta, J. K., Chaudhary, R. K and Dumka, V. K. (2007). Cefoperazone pharmacokinetics following single intravenous administration in cross bred calves. *Isrl. J. Vet. Med*. **62:**87-90.

Haghgoo, S., Hasegawa, T., Nadai, M., Wang, L., Nabeshima, T. and Kato, N. (1995). Effect of a bacterial lipopolysaccharide on biliary excretion of a -lactam antibiotic, cefoperazone, in rats. *Antimicrob. Agents Chemother.* **39**(10):2258–2261.

Jones, R.N., Barry, A.L. and Packer, R.R. (1983). BMY-28142, cefbuperazone and sch-34343; antimicrobial activity against 94 anaerobes compared to seven other antimicrobial agents. *Diag. Microbial. Infect. Dis*. **3**(3): 263-268.

Montesissa, C., Villa, R., Anfossic, P., Zanonic, R. and Carlib, S. (1981). Pharmacodynamics and pharmacokinetics of cefoperazone and cefamandole in dogs following single dose intravenous and intramuscular administration. *Vet. J.* **166**:170-176.

Neu, H. C. (1984). The pharmacokinetics of new cephalosporins: significance in clinical practice. *Bull. N.Y. Acad. Med.* **60**(4):327-339.

Soback, S. and Ziv, G. (1989). Pharmacokinetics of single doses of cefoperazone given by the intravenous and intramuscular routes to unweaned calves. *Res. Vet. Sci*. **47**:158-163.

Soback, S., Bor, A., Paz, R. and Ziv, G. (1986). Clinical pharmacology of mecillinam in calves. *J. Vet. Pharmacol. Ther*. **9**: 385-393.

Soni, H. H., Patel, R. J., Sadariya, K. A., Devada, S. S., Bhavsar, S. K. and Taker, A. M. (2012). Single dose pharmacokinetics of cefoperazone following intravenous and intramuscular administration in sheep. *Isr.I J. Vet. Med***. 67**(4): 220-224.

Soraci, A. L., Mestorino N. and Errecalde, J. (1998). Concentration of cefoperazone in synovial fluid and in bone after intramuscular administration. *Revist. Med. Vet. Buen. Aire.***79:**148-150.

Soraci, A. L., Mestorino, N. and Errecalde, J. (1996). Pharmacokinetics of cefoperazone in horses. *J. Vet. Pharmacol. Ther*. **19:** 39-43.

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Research Article

EFFECT ON HAEMATOLOGY AND SERUM ELECTROLYTES OF CHRONIC TOXICITY OF PESTICIDES INCOCKERELS

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ABSTRACT

Eight weeks old, white leghorn male chicks were randomly divided into seven groups comprising of six birds each and were fed on medicated ration with alphamethrin @100 and 200mg/kg in group II and III; butachlor @200 and 400 mg/kg in group IV and V and monocrotophos @ 2.5 and 5 mg/kg in group VI and VII, respectively, group I served as control, to evaluate the effect on haematological parameters and serum electrolyte on 10 and 20 weeks post administration. A significant ($P<0.01$) reduction in TEC, TLC and Hb on haematological examination and heterophil and lymphocyte count on DLC and serum levels of Na^+, Cl^-, and Ca^{++} ions was observed 10 weeks post administration of alphamethrin and monocrotophos on high dose levels, however, potassium significantly ($P<0.01$) increased in all the treated groups. This study indicated that prolonged subclinical exposure of pesticides, altered serum electrolyte levels with haematological change in birds.

Key words: Alphamethrin, butachlor, monocrotophos, haematology, electrolytes, poultry

INTRODUCTION

Pesticides translocated in the body tissues after their consumption either directly or indirectly following short or long term exposure and likely to cause toxic effects on hepato-renal, and other vital organs. Insectides alphamethrin, a synthetic pyrethroid (WHO, 1992) and monocrotophos, an organophosphate (Rao, 2004) and herbicide butachlor (Wilson *et al.*, 2000), are used extensively in various agriculture and animal husbandry practices. However, meager information is available on their impact on haematological and major blood electrolyte following chronic toxicity in birds. The present study was, therefore, designed to evaluate the effect of chronic oral dose on haematological and blood electrolytes in WLH cockerels.

MATERIALS AND METHODS

Experimental animals and design

Eight weeks old, white leghorn male chicks procured from PRC, were randomly divided into seven groups of six birds each. The feed and water was given ad libitum during the study. After acclimatization for two weeks, forty two chicks were divided randomly and equally into seven groups and were treated with alphamethrin @100 and 200 mg/kg b.wt.in group II and III; butachlor @ 200 and 400 mg/kg b.wt. in group III and IV and monocrotophos @ 2.5 and 5.0 mg/kg in group VI and VII; group I served as control. Chicks were kept under constant observation oral administration recording behaviour, general response and other clinical manifestations.Haematological and blood electrolyte analysis was done at 10 and 20 wks interval during the 20 weeks study.

Haemamtological examination

Blood samples were collected from wing vein at 10 and 20 wks interval in two test tubes, 2 ml blood in the heparinised test, tube and 5 ml in other test tube without anticoagulant for serum separation for analysis of electrolytes. TEC, TLC (Natt and Harric, 1952) Hb (jain, 1986) and DLC (Lucas and Jambroz, 1961) were estimated from blood without anticoagulant.

Serum electrolytes

Sodium and potassium levels of the serum were determined by flame photometric method after diluting the specimen 100 times with triple distilled water. Stock solutions of sodium containing 200 mmo l/L and potassium 10 mM/L were appropriately diluted to prepare standard curve (Oser, 1971). Level of chloride (mM/L) in serum was estimated according to the method as mentioned by Oser (1971) and serum calcium level (mM/L) as described by Wotton (1974).

RESULTS

As shown in Table 1, a significant ($P<0.01$) diminution in TEC, TLC, PCV and Hb was observed in alphamethrin, butachlor and monocrotophos treated cockerels at 10 weeks and 20 weeks interval. Cockerels given monocrotophos showed significantly ($p<0.01$) lower values when compared with alphamethrin and butachlor on 20 weeks post administration at higher doses.

Table 2 depicts DLC of heterophils, lymphocytes, monocytes, eosinophils and basophils. The count of heterophils was significantly ($p<0.01$) higher in alphamethrin treated group at 10 and 20 weeks post administration. No significant change in the relative population of heterophils was observed in butachlor treated groups but significantly ($p<0.01$) decreased in

monocrotophos treated group in comparison to control at both 10 and 20 weeks intervals. A significant ($p<0.05$) declines in differential count of lymphocytes was recorded in all the treated groups at low and high dose group at 10 and 20 weeks interval. The count of eosinophils, basophil and monocytes were increased significantly ($p<0.01$) at 10 and 20 weeks interval of post administration of the insecticide.

Serum electrolyte levels have been depicted in Table 3. Serum sodium level (mM/L) decreased significantly ($p<0.01$) in all the treated cockerels at 10 and 20 week interval. A significant ($p<0.01$) increase in the serum potassium level (mM/L) was recorded in all the treated cockerels at 10 and 20 weeks interval. Cockerels of high dose groups of alphametherin revealed significantly ($p<0.01$) higher serum chloride levels at 20 weeks intervals and significantly ($p<0.01$) decreased in all treated groups at low and high doses of pesticides group in comparison to control. In comparison to control, serum calcium level (mM/L) declined significantly ($p<0,01$) in both dose groups of all treated cockerels at 10 weeks post intoxication. Interestingly at 20 weeks, group of alphamethrin treated revealed a significant ($p < 0.01$) increase in the level of calcium (Table 3).

DISCUSSION

A significant decrease was noted in the TEC and TLC in cockerels fed alphamethrin and monocrotophos in this study. Reduction in leucocyte and differential lymphocyte count could be considered as a depressant action of alphamethrin on bone marrow and spleen. Haemolytic effects of synthetic pyrethroids in chicken lymphocyte culture have been also observed in cypermethrin and deltamethrin intoxicated chicks, dogs and goats (Mohmed and Adam, 1990). Butachlor proved to be less haematotoxic than other anilide herbicides such as propachlor which lowered PCV and Hb values in rats (Panshina, 1976). Similar haematological findings in toxicity studies of other organophosphorus insecticides also have been reported by Witherup *et al.* (1971).

Depletion of sodium in extracelluar fluid in chronic toxicity may result from increased influx of sodium in the axoplasm, loss of sodium ions in feces and urine and less absorption from GIT. The decreased levels

Table 1:
Effect pesticides on haematological parameters of cockerels (n=6).

Treatment	Dose (mg/kg)	TEC (Mean ± SEM) Interval (Weeks)		TLC (Mean ± SEM) Interval (Weeks)		PCV (Mean ± SEM) Interval (Weeks)		Hb (Mean ± SEM) Interval (Weeks)	
		10	20	10	20	10	20	10	20
Control	0	3.237 ± 0.033^{ap}	3.212 ± 0.039^{ap}	13.61 ± 0.40	13.75 ± 0.36^{abcde}	$39.25\pm0.53a$	39.83 ± 0.33^{abcpq}	$128.50\pm1.49ap$	132.67 ± 1.33^{abcd}
Alphamethrin	100	3.127 ± 0.058	3.172 ± 0.058^{b}	13.01 ± 0.19	11.45 ± 0.30^{ap}	37.25 ± 0.56	35.92 ± 0.47^{dep}	$130.67\pm1.91bc$	114.50 ± 2.77^{aef}
	200	2.925 ± 0.047^{ab}	2.925 ± 0.047^{q}	12.23 ± 0.51	10.16 ± 0.21^{bfq}	$35.17\pm0.56abp$	30.42 ± 0.55^{adfghi}	$108.67\pm1.76abdq$	97.33 ± 2.56^{begh}
Butachlor	200	3.293 ± 0.032^{bc}	3.160 ± 0.042^{c}	13.75 ± 0.44	13.21 ± 0.15^{fgpr}	39.50 ± 0.18	39.17 ± 0.33^{fj}	130.00 ± 1.37^{dr}	122.33 ± 1.89^{gip}
	400	3.110 ± 0.057	2.987 ± 0.052^{d}	14.36 ± 0.39^{a}	11.71 ± 0.38^{eq}	38.58 ± 0.57	34.58 ± 0.90^{bgk}	124.67 ± 2.76^{q}	124.00 ± 2.96^{hjk}
Monocrotophos	2.5	3.110 ± 0.046	2.883 ± 0.058^{p}	12.56 ± 0.31	11.56 ± 0.30^{dr}	38.75 ± 0.60^{p}	36.33 ± 0.95^{hlq}	$117.67\pm4.25e$	109.33 ± 1.68^{gp}
	5	2.993 ± 0.034^{cp}	2.554 ± 0.121^{abcdq}	11.88 ± 0.42^{a}	10.12 ± 0.23^{eg}	36.42 ± 0.82	29.00 ± 0.65^{eijkl}	112.83 ± 3.78^{gpr}	97.60 ± 2.13^{dfik}

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

Tables 2:
Effect of pesticides on differential leucocyte count (DLC) of cockerels (n=6).

Treatment	Dose (mg/kg)	Heterophil (Mean ± SEM) Interval (Weeks)		Lymphocyte (Mean ± SEM) Interval (days)		Monocyte(Mean±SEM) Interval (Weeks)		Eosinophil(Mean±SEM) Interval (days)		Basophil (Mean±SEM) Interval (days)	
		10	20	10	20	10	20	10	20	10	20
Control	0	45.3±0.67ab	46.5±1.20	45.2 ± 0.068^{aq}	44.7 ± 0.33^{abcd}	4.5±0.43	3.2 ± 0.30^{abcd}	4.3 ± 0.56^{pq}	4.5±0.56abc	1.3±0.42p	1.3 ± 0.40^{abc}
Alphamethrin	100	46.2±0.87	47.7±0.65	43.3 ± 0.91^{q}	38.5 ± 1.23^{g}	4.7±0.49	5.3 ± 0.49^{p}	5.0 ± 0.63^{r}	6.7 ± 0.55^{de}	1.5±0.22	2.0±0.63
	200	44.8±0.87	46.0±1.15	39.7 ± 1.25^{p}	33.5 ± 1.00^{bef}	5.7±0.49	7.0 ± 0.36^{ade}	8.2 ± 0.79^{p}	10.3 ± 0.42^{afgj}	1.7±0.21p	3.2 ± 0.16^{q}
Butachlor	200	44.8±0.08	45.5±0.99	43.0 ± 0.68^{r}	43.8 ± 0.47^{egf}	5.3±0.61	4.3 ± 0.42^{ddr}	5.3±0.51	5.0 ± 0.45^{fg}	1.7±0.49	2.0±0.25
	400	45.3±0.79	44.8±0.79	42.7 ± 1.25^{s}	43.5 ± 0.56^{fij}	$5.0\pm0.45b$	4.0 ± 0.25^{fq}	6.0±0.97	4.7 ± 0.42^{aik}	1.9±0.47	1.5±0.34
Monocrotophos	2.5	47.0±1.33	45.2±1.07	37.3 ± 0.80^{aqrs}	34.3 ± 1.11^{agj}	4.8±0.40	6.8 ± 0.47^{bfg}	6.7±0.49	10.8 ± 0.83^{dhj}	1.5±0.34	2.9 ± 0.47^{a}
	5	44.5±0.76	44.0±0.84	40.0±1.00	34.4 ± 0.74^{dhj}	6.2±0.48	7.0 ± 0.45^{gfr}	9.4 ± 1.02^{qr}	11.2 ± 0.73^{celk}	2.5±0.43	3.0 ± 0.29^{b}

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

Table 3:
Effect of pesticides on serum electrolyte levels of cockerels (n=6).

Group	Dose (mg/kg)	Chloride (Mean ± SEM) Interval (Weeks)		Calcium (Mean ± SEM) Interval (Weeks)		Sodium (Mean ± SEM) Interval (Weeks)		Potassium (Mean ± SEM) Interval (Weeks)	
		10	20	10	20	10	20	10	20
Control	0	117.14±1.070^{pq}	116.07±0.245^{ab}	2.417±0.097	2.296±0.032^{ab}	158.00±1.31a	156.83±0.40^{a}	5.042±0.150a	4.917±0.140^{abp}
Alphamethrin	100	112.70±0.984^{p}	111.89±1.800	2.421±0.023^{a}	2.348±0.039^{cde}	159.50±0.48	156.83±0.70^{b}	5.283±0.209	5.667±0.124^{cdpq}
	200	115.33±0.352	117.47±0.507^{cd}	2.370±0.024^{b}	2.409±0.079^{bh}	158.33±0.33	154.17±0.48^{cd}	6.075±0.149^{abcdp}	6.517±0.130^{acefg}
Butachlor	200	115.42±0.239	114.92±1.075	2.126±0.059	2.066±0.026^{cfijk}	160.50±0.43	163.00±0.82^{abcdefq}	4.625±0.107^{b}	4.583±0.083^{dehr}
	400	114.82±0.649	111.80±0.594	2.353±0.036^{c}	2.594±0.041^{im}	161.00±0.37^{b}	159.67±0.42^{dgp}	5.00)±0.145^{c}	5.333±0.189^{grs}
Monocrotophos	2.5	114.25±0.751	108.25±1.039	2.137±0.073	1.739±0.039^{adgjl}	162.50±0.43^{ac}	157.17±0.79^{e}	4.46)±0.078^{d}	4.917±0.083^{iq}
	5	108.26±1.789^{q}	113.10±0.447^{bd}	1.955±0.071^{abc}	1.666±0.068^{behklm}	155.83±0.87^{bc}	154.00±0.55^{fg}	5.167±0.0735^{p}	6.150±0.128^{bgls}

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

of sodium and potassium observed during acute toxicity study could be due to inhibitory effect of alphamethrin insecticides on Na^+- K^+ ATPase activity in the renal tubular epithelium resulting in enhanced excretion of electrolyte (Shalaby *et al*., 1991).

Monocrotophos decreased the level of serum Ca^{++} and Cl^- ions as against an elevation in the level of Na^+ and K^+. This disturbed regulatory mechanism could explain electrolyte profile. Organophosphates affect thyroid function which may lead to alterations in serum electrolytes (Hammond *et al.,*1982). Alteration in Na^+, K^+ and Ca^{++} has also been observed in monocrotophos intoxicated buffalo calves (Sandhu and Jindal, 1990) and also repored in cockerels by Singh (2011). It is concluded from this study that exposure of alphamethrin, butachlor and monocrotophos at sublethal dose levels produced mild to moderate haemotoxic effects and altered serum electrolyte levels 10 weeks post intoxication in WLH cockerels.

REFERENCES

Hammond, P.S., Brunstein, H., Kenedy, J.M., Bardawy, S.M.A. and Fukut, T.R. (1982). Mode of action of delayed toxicity of O, O, S methyl phosphorothioate in rats. Pestic. *Bicochem. Physiol*. **18**: 77-89.

Mohmad, O.S.A. and Adam, S.E.I. (1990). Toxicity of sumicidium (Fenvalerate) to Nubian goats. *J. Comp. Pathol.* **102**: 1-6.

Natt, M.P. and Harric, C.A. (1952). A new diluent for counting erythrocyto and leneocyte of the chicken. *Poult. Sc.* **31**: 735-738.

Oser, B.L. (1971). Hawk's physiological chemistry. 14th ed. Tata Mc Graw Hill Pub. Comp. Ltd., New Delhi.

Panshina, T.N. (1985). IRPTC scientific reviews of Soviet Literature on toxicity and hazards of chemicals No. 83: Propachlor (Ramrod). Moscow, Centre of International project GKNT, 19 pp. (cited in WHO, 1993).

Paulov, S. (1991). Effect of the pyrethroid insecticide on erythrocytes and aminotransferase. *Biol. Bratislava*. **46:** 597-601.

Roa, J.V. (2004). Effects of monocrotophos and its analogs in acetylcholinesterase activity's inhibition and its pattern of recovery on euryhaline fish, Oreochromis mossambicus. *Ecotox Environ. Safe,* **59**: 217-222.

Sandhu, H.S. and Jindal, R. (1990). Effect of acute toxicity of monocrotophos on blood macroelements in Bubalus bubal is. *Indian Vet. J*. **67 (4):** 315-317.

Shalaby, S.I.A., Abdel Razic, A.M.A., Nawito, M.F., Noseir, M.B., Razic, A.M.A. Abdel, (1991). Effect of baythroid insecticide (cyfluthrin) on the level some minerals in the blood of pregnant Barki ewes. *Egypt J. of Comp. Pathol. Clin. Pathol*. **4(2):** 279-290.

Singh, S. P. (2011). Effect of acute toxicity of pesticides on haematological and serum electrolytes cockerels. *Indan J. Vet. Pharmacol & Toxicol, **10(1&2):**45-48.*

W.H.O. (1992). Alphacypermethrin. *Environmental Health Criteria. Geneva*. **142:** 1-112.

Witherup, S., Jolley, W.J., Summer, K. and Pitzer, E.A. (1971). Chronic toxicity studies C 2, 3- dichlorovinyl dimethyle PO4 (DAVP) in dogs and rats. Toxicol. Appl. Pharmacol., **19**: 377.

Wilson, A.G.E. and Takei, A.S. (2000). Summary of Toxicology studies with butachlor. *J. Pesticide Sci.* **25**: 75-83.

Wotton, I.V.P. 1974. Microanalysis in medical biochemistry, 5th ed. Churchill Livingstone, U.K. p. 68.

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Research Article

MOLECULAR CHARACTERIZATION OF RARE SEROVARS OF *SALMONELLA* IN GANGETIC WATER BY REP- PCR.

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ABSTRACT

Salmonella is one of the cardinal pathogenic bacteria present in contaminated water. Repetitive Extragenic Palindromic(REP)PCR has been reported to be polymorphic at serovars level in *Salmonella. Salmonella* isolates (rare serovars) collected from 10 different stations of Ganga River were studied for REP-PCR. Twenty isolates belonging to seven different serovars (*S*. Abuja, *S*. Lagos, *S*. Chinkual, *S*. Zwickau, *S*. Pontypridd, *S*. Goldenberg and *S*. Oritamerin) were studied for the REP-PCR. Out of 20 isolates 19 different profiles were observed and one sample has not shown REP sequence. There was no host specific, type specific, and region specific profile pattern but REP-PCR was found rapid and reproducible and can be used for differentiation of different *Salmonella* isolates.

Keywords: Repetitive Extragenic Palindromic(REP), *Salmonella,* Rare Serovars, Discriminative Index, Dendrogram.

INTRODUCTION

Ganga the national River is one of the biggest rivers in Indian sub-continent and is considered to be sacred and has been declared as National River by Indian Government in Year 2008. The fertile soil of the river basin measuring around one million kilometers square is a key to agricultural economy of the country and supports one of the highest human density populations. The large quantity of pollutant such as untreated sewage material in large volume (approximately one billion liters per day) (Hamner *et al*., 2007) are disposed off in river Ganges that leads to accumulation of hazardous pathogens like *Shistosoma, Escherichia* coli 0157:H7, *Shigella* and *Salmonella* (Hamner *et al*., 2007, Tikko *et al*., 2001) and expose the surrounding population to these diseases.

Salmonella is considered a dreaded pathogen causing several diseases such as endocarditis (Moreno *et al*., 2000) Typhoid and Paratyphoid fever (Hasan *et al*., 2005), Pneumonia (Gezen *et al*., 2008) and meningitis (Swe *et al*., 2008). Identification and strain differentiation are two cardinal requisites for epidemiological analysis and controlling salmonellosis. The conventional approaches used for strain differentiation and identification in epidemiological studies are time consuming but with development of molecular biology techniques like PCR genome has been targeted for this purpose. In the genome of the bacteria REP-PCR has been extensively used for taxonomic purpose (Gonzalez *et al*., 2003). The *Salmonella* serovars REP sequence has been studied and using REP-PCR reported to be serovars specific (Jenson *et al*., 1993). The basis of differentiation of serovars was based on variation in number and type of REP sequences found within the profiles (Brosius, *et al*., 1981). The present study was undertaken to differentiate seven serovars of *Salmonella* isolated from Ganges river water by targeting REP sequence.

MATERIALS AND METHODS

Twenty isolates of *Salmonella* were collected form ten different locations (stations) of Ganga viz, Gangotri, Uttarkashi, Rishikesh, Haridwar, Hastinapur, Garh Mukteshwar, Narora, Kanpur, Allahabad, and Varanasi. These isolates were serotyped at National *Salmonella* Research Centre, IVRI Izatnagar U.P. India, and were characterized as *S*. Abuja, *S*. Lagos, *S*. Chinkual, *S*. Zwickau, *S*. Pontypridd, *S*. Goldenberg and *S*. Oritamerin (Table 1). Genomic DNA was isolated from all isolates by C-TAB method (Wilson *et al*., 1987). After that *Salmonella* specific PCR was carried out(fig.1).

REP-PCR

Five µl (40ng) of genomic DNA was used for50 µl of PCR reaction mixture, containing 5pmol of each primers (Primer1- 5' CGG NCT ACN GCN NNN G 3') and (Primer

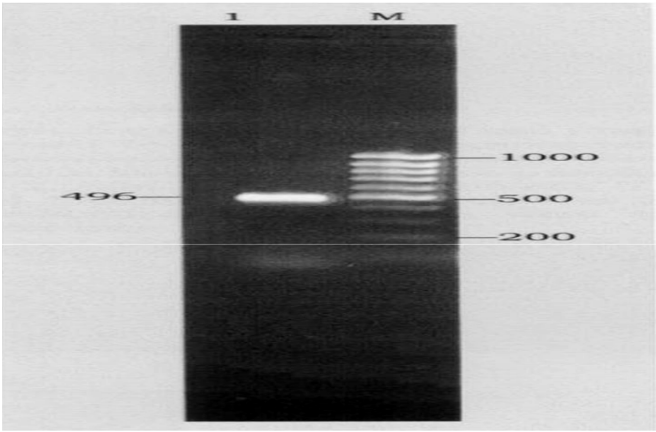

Fig 1. Amplicon of hisA gene of salmonella specific PCR

Table-1: Description of *Salmonella* isolates (serovars, place of isolation, band profile and molecular type) isolated from various locations from river Ganga.

Sl. No.	Isolate No.	Serovar	Place of isolation	Band size (bp)	Profile
1.	G-2	S. *Abuja*	Hastinapur	2130, 1796, 1610, 1375, 1119, 946, 932, 806, 588, 267, 235, 200, 168, 138, 122	R1
2.	G-4	S. *Abuja*	Hastinapur	2130, 1610, 1119, 932, 806, 588, 267, 235, 200, 168, 138, 122	R2
3.	G-6	S. *Abuja*	Hastinapur	2130, 1796, 1610, 1119, 932, 806, 588, 267, 235, 200, 122	R3
4.	G-7	S.*Pontypridd*	Kanpur	3227, 2130, 1796, 1610, 1119, 932, 806, 588, 267, 235, 200, 168, 138, 122	R4
5.	G-9	S *Abuja*	Hastinapur	3227, 2130, 1796, 1610, 1571, 1119, 932, 806, 588, 267, 235, 200, 168, 122	R5
6.	G-12	S.*Lagos*	Haridwar	2130, 1796, 1610, 1571, 932, 806, 588, 168	R6
7.	G-13	S.*Lagos*	Haridwar	No band	R
8.	G-15	S.*Lagos*	Haridwar	1796, 1610, 1571, 1119, 806, 588, 508, 200, 168, 138	R7
9.	G-17	S.*Chinkual*	Varanasi	**3587**, 3227, 2130, 1796, 1610, 1119, 932, 806, 588, 508, 267, 190, 138, 122	R8
10.	G-19	S.*Abuja*	Hastinapur	3227, 2130, 1796, 1610, 1571, 1375, 1119, 932, 806, 588, 508, 267, 200, 190, 168, 138, 122	R9
11.	G-22	S.*Abuja*	Hastinapur	3227, 2130, 1796, 1610, 1571, 1119, 806, 588, 508, 267, 190, 138, 122	R10
12.	G-24	S.*Chinkual*	Varanasi	3227, 2130, 1796, 1610, 1571, 1375, 1119, 932, 806, 588, 508, 267, 190, 138,122	R11
13.	G-25	S.*Zwickau*	Narora	3227, 2130, 1796, 1375, 1119, 806, 588, 508, 200, 190, 168, 138, 122	R12
14.	G-26	S.*Goldenberg*	Narora	3227, 2130, 1796, 1571, 1375, 1119, 932, 806, 588, 508, 267, 138, 122	R13
15.	G-27	S.*Lagos*	Haridwar	3227, **2408**, 2130, 1796, 1571, 1119, 932, 806, 588, 508, 267, 138, 122	R14
16.	G-30	S.*Pontypridd*	Narora	1692, 818, 643, 424, 324, 235, 200, 168	R15
17.	G-31	S.*Abuja*	Hastinapur	1692, **825**, 818, 643, 424, 324, 200, 168, 112	R16
18.	G-32	S.*Abuja*	Hastinapur	1692, 987, 818, 643, 424, 324, 245, 200, 168, 112	R17
19.	G-39	S.*Oritamerin*	Narora	2130, 1692, 987, 818, 643, 424, 324, 245, 200, 168, 112	R18
20.	G-43	S.*Oritamerin*	Narora	1692, 818, 643, 324, 245, 200, 168, 112	R19

Table 2: Biochemical Characterization of Samples

S. N.	Strain No.	Serovar	M.R. Test	V.P. Test	Urease Test	Citrte Test	Lysin Test	H2s Test	ONPG Test	Lactose Test	Arabinose Test	Maltos Test	Sorbitol Test	Dulcitol Test
1	G - 2		+	-	-	+	+	+	-	+	+	-	-	-
2	G - 4	S. Abuja	+	-	-	+	+	+	-	-	-	+	-	-
3	G - 6	S. Abuja	+	-	-	+	+	+	-	-	+	-	-	-
4	G - 7	S. Abuja	+	-	-	+	+	+	-	-	-	+	+	-
5	G - 9	S.Pontypridd	+	-	-	+	+	+	-	+	+	+	-	-
6	G - 12	S. Abuja	+	-	-	+	+	+	-	-	-	+	-	-
7	G - 13	S. Lagos	+	-	-	+	+	+	-	+	-	+	+	-
8	G - 15	S. Lagos	+	-	-	+	+	+	-	+	+	+	-	+
9	G - 17	S. Lagos	+	-	-	+	+	+	-	+	-	+	-	-
10	G - 19	S.Chinkual	+	-	-	+	+	+	-	-	+	-	-	-
11	G - 22	S. Abuja	+	-	-	+	+	+	-	-	+	+	+	+
12	G - 24	S. Abuja	+	-	-	+	+	+	-	-	+	+	-	-
13	G - 25	S. Chinkual	+	-	-	+	+	+	-	+	+	-	-	-
14	G - 26	S. Zwickau	+	-	-	+	+	+	-	+	+	+	-	-
15	G - 27	S. Goldenberg	+	-	-	+	+	+	-	+	+	-	+	+
16	G - 30	S. Lagos	+	-	-	+	+	+	-	-	-	+	-	-
17	G - 31	S. Pontypridd	+	-	-	+	+	+	-	-	+	-	-	-
18	G - 32	S. Abuja	+	-	-	+	+	+	-	-	-	+	-	+
19	G - 39	S. Abuja	+	-	-	+	+	+	-	+	+	-	+	-
20	G - 43	S. Oritamerin	+	-	-	+	+	+	-	+	+	-	-	-
		S. Oritamerin												

2- 5' NCG NCT TAT CNG GCC TAC 3'), 100µM of dNTPs, 1.5 Unit of Taq DNA polymerase and 1.5Mm final concentration of MgCl2.All the chemicals used in PCR were procured from MBI- fermentas. PCR Programme was as follows-initial denaturation (94ºC for 5 min.) followed by 30 cycles composed of denaturation (94ºC for 1 min.), annealing (55ºC for 2 min.) and polymerization (72ºC - 5 min.) Final extension was carried out at 72ºC for 10 minutes. Five µl of amplified products was loaded on 2% agarose gel containing ethidium bromide and electrophoresis was conducted in 1X TAE at 5V/cm for 1.5 hours. Each sample was amplified thrice with negative control by electrophoresis to avoid possibilities of artifacts. Molecualar weight of amplicon was measured by comparing 100-300 bp DNA low range rular (Banglore Genei). The NTSYSpc (version-2.11V) software was used for construction of phylogenetic tree and analysis of bands.

Samples showing positive results in '*his*' gene PCR (Fig.1) was subjected to a panel of biochemical tests in *Salmonella* identification kit. Which included methyl red test, vogues proskaur's test, urease test, H_2S production, citrate utilization, lysine utilization, lactose fermentation, arabinose utilization, maltose utilization, sorbitol utilization and dulcitol utilization (Table2).

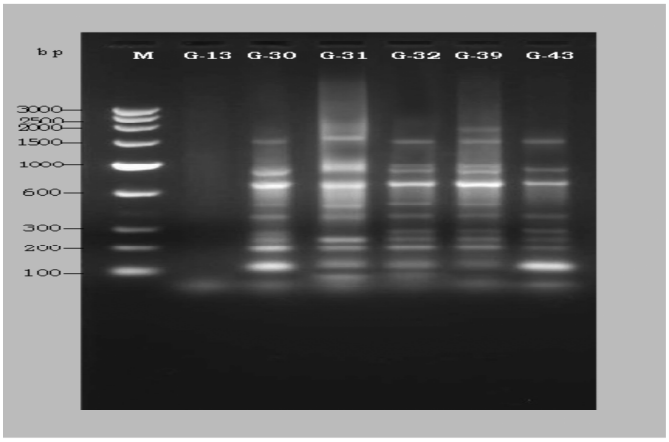

Fig 5. The Band profile of *Salmonella* Rare Isolates in REP-PCR
Lane M : DNA Maker (100-3000 bp)
(DNA low rage DNA rular, Banglore Genei)
Lane 2-14 : *Salmonella* isolates G-2, G-4, G-6, G-7, G-9, G-12, G-15, G-17, G-22, G-24, G-26, G-27

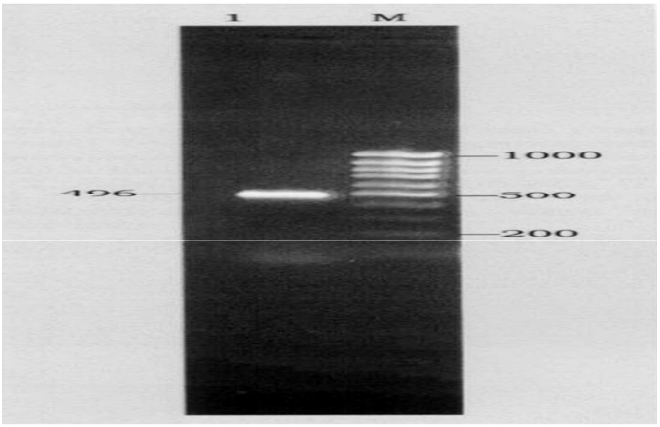

Fig 6. The Band profile of *Salmonella* Rare Isolates in REP-PCR
Lane M : DNA Maker (100-3000 bp)
(DNA low rage DNA rular, Banglore Genei)
Lane 2-6 : *Salmonella* isolates G-13, G-30, G-31, G-32, G-39, G-13

RESULT AND DISCUSSION

In all serovars, Repetitive Extragenic Palindromic sequence was amplified. After separation of PCR product on 2% agarose gel only intense bands were scored. The relative molecular weights of amplicons were compared to the bands with Standard molecular weitghts marker 100-300 bp DNA low range rular (Banglore Genei). Size of bands ranged from from 122 bp to 3587 bp. All the isolates could be grouped into 19 profiles (Table 1). All the profiles are unique because there was no repetition in profiles. No serovars specific profile could be observed in the study. There were some common bands in many of the profiles as 806bp, 588bp, 2130bp, 1119bp, 122bp, and 1610bp. The 806bp and 588bp bands were present in 14 profiles while 2130bp, and 1119bp specific bands were present in 13 profiles, 122bp band occurred in 12 isolates and 1610bp specific band was present in 11 profiles (Fig. 5 & 6). Bands of 3587bp, 2408bp, and 825bp were unique as each of these was present in only one isolate namely G-17(*S*. Chinkual) from Varanasi, G-27(*S*. Lagos) isolated from Haridwar and G-31(*S*. Abuja) respectively. One isolate G-13 (*S*. Lagos) isolated from Haridwar showed no amplicon which may indicate that it lacks of REP sequence but present only in 19 isolates out of 20 and only one sample G-13 (*S*. Lagos). The efficiency of differentiation was calculated by Simpson"s Index of Diversity (D) and it was 0.99.

In this study twenty serovars of *Salmonella* were isolated by molecular methods from Ganges river water at ten different locations (stations). *Salmonella* Oritamerin which is common in rivers of other countries (Ronold *et al*., 1992) but not so frequently reported in India, was present in Ganges water possibly due to the congregation of vast human population from across the diverse geographical boundaries during festival such as Kumbh. On amplification of REP sequence the product size varied from112 to 3587 bp, which is very similar to the results of (Bennasar *et.a.,* 2000). The variation of REP sequence of *Salmonella* genome could be attributed as possible reasons for high genetic variation shown by the isolates. We did not observe any serovar specific profile in the present study. Dandrogram was constructed on the basis of combined molecular typing using the bands obtained in REP PCR at 7 scales, Dandrogram(Fig.2) showed two bands profiles, and at scale 3 three bands profiles. Some other bands profiles also obtained at 3, 4, 6, 7,11,14,17 and 23 scales. With these findings we suggest that Repetitive Extragenic Palindromic sequence is not serovars specific but has a high genetic variation and can be used for the molecular typing of field isolates of *Salmonella*

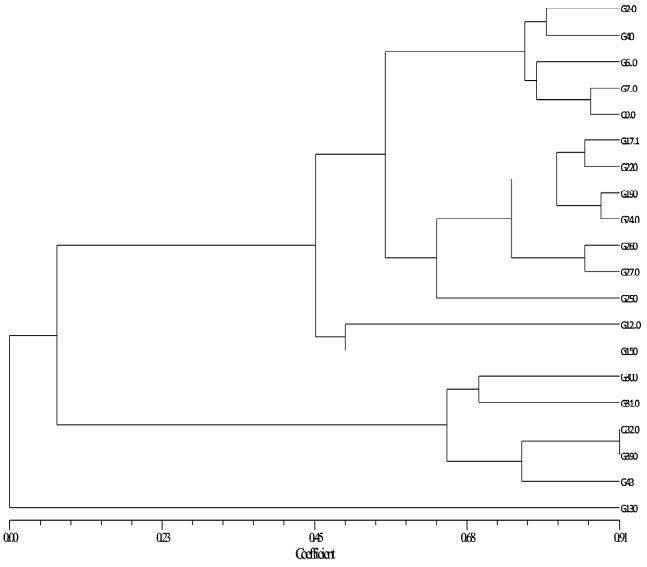

Fig 2. Phylogenatic tree of *Salmonell*a isolates (rare serovars) based on REP-PCR profiles

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REFERENCES

Balwant., Tyagi, A., Singh S.P., Saxena, M.K., and Mishra, D.P. (2014). "Biochemical Characterization of rare serovars of *Salmonella* isolated from Gangetic water". *J. Vety. Pharmacol. And Toxicol.***13:** (1): 79-80.

Balwant., Tyagi, A., Singh S.P., Saxena, M.K., and Mishra, D.P. (2014). "Molecular Characterization OF *Salmonella* (rare serovars) isolated from Gangetic water based on ERIC-PCR".*J. Vety. Pharmacol. And Toxicol.***13:** (2): 152-155.

Bennasar, A., De Luna, G., Cabrer, B., and Lalucat, J., (2000). "Rapid Identification of *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Virchow isolated by PCR Based Fingerprinting Methods." *Int. J. Microbiol.* **16:** (3): 804-808.

Brosius, *et al*., (1981)."Gene Organization of Primary Structure of a Ribosomal RNA Operon from *E. Coli." J. Mol. Bio.* **148:** (2): 107-127.

Fadl, A. A., Nguyen ,A.V. and Khan M.I.(1995) "Analysis of *Salmonella enteritidis* Isolates by Arbitrarily Primed PCR" *J. Clin.Microbiol.* **33**: (4): 987-989.

Gezen J.R., Towle,D.M. and Kravetz J.D., (2008). *Salmonell*a Typhimuriu Pulmonary Infection in an Immunocompitent Patient, *Connect. Med.* **72:** 139-142.

Gonzalez, N., Romero, J. and Espejo, R.T. (2003) Comprehensive Detection of Bacterial Population by PCR Amplification of the 16S-23S RNA Spacer Region, *J.Microbiol. Method*. **55:** (1): 91-97.

Hamner, S., Broadway ,S. C., Mishra V. B., Tripathi. A., Mishra,R. K., Pulcini, E., Pyle,B. H. and Timothy, E.F. (2007)Isolation of Potentially Pathogenic *E. Coli* O157:H7 from Ganges River. *App. Env. Microbiol.* **73:** (7): 2369-2372.

Hasan ,R., Cooke,F.J., Nair S., Harish ,B.N. and Wain, J. (2005). Typhoid and Paratyphoid Fever, *The Lancet*. **366** (9497):1603-1604.

Jenson ,M. A., Webster, J. A., and Straus, N.(1993). Rapid Identification of Bacteria on the Basis of Polymerase Chain Reaction Amplified Ribosomal DNA Spacer Polymorphism, *App. Env. Microbiol.* **59**: (4): 945-957.

Moreno, J.G., Moar, C., Roman F., Perez-Maestu, R. and Lopez de Letona, J.M. (2000). "*Salmonell*a Endocarditis Presenting a Cerebral Hemorrhage,"*Eu. J. Int. Med.* **11:** (2): 96-97.

Ronold , L., Anderson,Y., Biritta J. and Norberg, P. (2000). A Summary of Reported Food Borne Diseases Incidence in Sweden, 1992 to 1997, *J. Food Protec.* **63:** (10): 1315-1320.

Swe, K., Nage, G., Van M. and Hoosen, A.(2008). *Salmonell*a Typhimurium Meningitis in an Adult Patient with AIDS. *J. Clin. Patho*. **61**:(1): 138-139.

Tikko,A., Tripathi ,A.K., Verma,S.C., Agrawal, N., and Nath, G. (2001). Application of PCR Fingerprinting Techniques for Identification and Discrimination of *Salmonell*a Isolates. *Cur. Sci.* **80:** (8): 1049-1052.

Wilson, K. (1987). Preparation of Genomic DNA from Bacteria, *Cur. Proto. in Mol.Biol.* Unit **2.4.1**, Wiley, New York,.

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Research Article

STUDY OF EFFECT OF EXTRACT OF *JATROPHA CURCAS* LEAVES ON IBD VIRUS USING $TCID_{50}$

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ABSTRACT

The present study was carried out to investigate the effect of methanolic extract of *Jatropha curcas* leaf methanolic extract for its cytotoxicity and potential to inhibit replication of infectious bursal disease (IBD) virus in chicken embryo fibroblast (CEF) cell culture. The phytochemical analysis of methanolic extract of *J. curcas* leaves revealed the presence of tannins, alkaloids, flavonoids and steroids. A CEF adopted IBDV isolate UA-Bz-2 strain was propagated in CEF culture at 10^{th} passage. There was marked rounding, aggregation of refractile cells and cytoplasmic vacuolation by 24 hours post infection (hpi). A significant number of rounded dead cells by 48 hpi and marked granulation of cell cytoplasm particularly around nucleus, degeneration of cells showing detachment, clumping and micro-plaques in the cell sheet with a few long cytoplasmic strands were evident by 72 hpi. The uninfected control did not show any changes up to 72 hpi. Harvested virus showed a $TCID_{50}$ of $3.9x\ 10^5$, $3.16x\ 10^6$ and $1.7x\ 10^7$ per ml at 24, 48 and 72 hours post infection, respectively. But after *Jatropha curcas* leaf methanolic extract treatment, the titre was significantly ($p< 0.05$) reduced to 3.16×10^3, 2.37×10^4 and 2.15×10^6 at 24, 48 and 72 hpi, respectively. Treatment of the infected CEF with the extract at concentrations of 50 μg/mL reduced the viral load by 99.18%, 99.25% and 87.35% after 24, 48 and 72 h, respectively The findings of the present study revealed the potential of the bioactive compounds of *Jatropha curcas* methanolic leaf extract to inhibit the replication of IBDV in CEF cell culture .

Key words: IBD, *Jatropha curcas* , chicken embryo fibroblast, cytopathy, $TCID_{5,}$

INTRODUCTION

Jatropha curcas L., a soft wood perennial plant belongs to family *Euphorbiaceae,* commonly known as Ratanjot or Purgative nut. In recent years this plant has received extensive attention of many scientists in view of its great economic importance, medicinal significance and for its seed oil as commercial source of fuel (Datta & Pandy, 1993). Antibiotic activity of *Jatropha* has been observed against organisms including *S. aureus* and *E. coli* (Matsuse *et al..,* 1988). Crude stem bark extracts of *J. curcas* Linn. was reported to inhibit the growth of pathogenic bacteria and fungi (Igbinosa *et al .,* 2009). In addition, crude extract of *J. Curcas* Linn. has been found to inhibit HIV induced cytopathic effects with low cytotoxicity (Sriprang *et al .,* 2010). Based on the above facts, the present study was carried out to investigate the effect of methanolic extract of *J. curcas* leaves for its cytotoxicity and potential to inhibit replication of infectious bursal disease virus in chicken embryo fibroblast cell culture.

MATERIALS AND METHODS

Plant material

Leaves of *Jatopha curcas* were procured through Medicinal Plants Research Drug Centre (M.R.D.C.), G.B. Pant University of Agriculture and Technology, Pantnagar.

Embryonated chicken eggs (9 to 11 days-old) were procured from Instructional Poultry Farm (IPF), GBPUA&T, Pantnagar, and utilized for production of primary embryo fibroblast (CEF) cell culture. Confirmed CEF adapted IBD virus of strain UA-Bz-2 (10^{th} passage) available in the department was used in this study.

Prepartion of extract

Jatropha curcas leaves were dried under hot circulating air at 40ºC for 3-4 days, and ground into a fine powder. The powder was allowed to soak in 50% methanol (v/v) for 48 hours under continuous agitation at 40ºC in a shaking incubator. The mixture was first filtered through muslin cloth, then through filter paper. Filtrate was kept in rotatry evaporator (65ºC, for 3 days) then the extract was obtained by drying the filtrate under hot circulating air at 40ºC followed by lyophilization.

Phytochemical Analysis of the extract

A small portion of the dry extract was subjected to the phyto- chemical test using Trease and Evans (1983); Harbourne (1983) methods to test for alkaloids, tannins, flavonoids, steroids and saponins.

MTT assay of the extract for cytotoxicity

Primary CEF cultures were obtained as per the method described by Cunningham (1973). Ability of the extract of *Jatropha curcas* leaves to induce cytotoxicity was assessed by the MTT [3-(4,5-dimethylthiazol-2-y1)-2,5- diphenyltetrazolium bromide] dye reduction assay, which is based on the ability of a mitochondrial dehydrogenase from viable cells to oxidize the tetrazolium rings of the pale yellow MTT and to form a dark blue formazan crystals. These formazan crystals are largely impermeable to cell membranes and, therefore, accumulate within healthy cells. The concentration of the formazan product formed is a direct representative of the

number of surviving cells.

CEF cells were grown in 96-well plate for 24 hours; 200ìL medium with dilution of the filter sterilized extract was added per well, quadruplicates for each concentration. The extract was used in concentrations ranging from 5μg to 5mg/mL (*i.e.* 5ìg/mL, 10μg/mL, 25μg/mL, 50μg/mL, 75μg/mL, 100μg/mL, 250μg/mL, 500μg/mL, 1mg/mL, and 5mg/mL,) and incubated for 24 hours at 37°C in a CO_2 incubator. Subsequently, 50ìL of aqueous solution of MTT (50ìg/mL) was added to each of the wells, and further incubated for 4 hours. After incubation, dark blue formazan crystals were solubilized with 200μL of DMSO and 25μL of glycine buffer was added, then absorbance was measured at 570 nm using computerized Micro Scan ELISA Reader.

The rate of growth inhibition was calculated as per the following formula:

Per cent growth rate = [OD extract treated/ OD of Control] X 100%

The values of per cent cytotoxicity are expressed with respect to the control cells (untreated cells) *i.e.* Per cent cytotoxicity = (100 – Per cent growth rate) %

Propagation of virus in CEF culture

The culture flasks with 80-90% confluency were taken and the spent media was decanted and then inoculated with 1.0 ml of 100 $TCID_{50}$ of the virus inoculum prepared as per standard procedure per 25cm^2 flasks. These flasks were then incubated for 1 h at 37°C for adsorption of the virus then the inocula were decanted and fresh maintenance medium (MEM with 2% FBS) was added to the flasks and kept in incubator at 37°C and 5% CO_2 for development of cytopathic effects. The inoculated monolayers were observed under inverted microscope daily for cytopathic effect (CPE) which is characterized by small round refractile cells.

May-Grunwald and Giemsa staining

The cytopathic effect produced by IBDV in CEF culture were observed without staining daily at interval of 12 hours post infection (hpi) for appearance of CPE, or else cell cultures were prepared in cover slips and inoculated. The inoculated cultures were stained using May-grunwald and Giemsa stains at different hpi as per the technique described by Merchant *et al*., (1960) with slight medications. The medium from test tubes in which the cover slips were kept was discarded and the monolayers were washed with PBS (pH 7.2). The monolayers were then fixed with absolute methanol for 30 minutes at room temperature. The fixed monolayers were stained with filtered May-Grunwald stain for 30 minutes and then filtered Giemsa stain (1:5 dilution of stock solution) for 30 minutes. The monolayers were washed with distilled water decanting the stain and dehydrated in two changes of acetone and acetone:xylene (1:1) Finally, the coverslips were passed two changes of xylene and mounted on a clean microscopic slide with a drop of DPX mountant. The stained cover slips were examined for CPE under light microscope and photographed.

Calculation of virus titre using $TCID_{50}$

To study the growth pattern of IBDV, virus titration was performed as described by Kibenge *et al* (1988) with slight modifications. For, this culture supernatant was collected at different time intervals of about 0, 24, 48 and 72 hpi of at 10^{th} passage level from 25 cm^2 and 175 cm^2 culture flasks by repeated freezing and thawing culture supernatant collected at different time interval was titrated in CEF cells on microtitre plate. Culture supernatants were serially diluted (10 fold) from 10^{-1} to 10^{-8} dilutions in ependroff tubes. CEF cells were prepared in 96 well microtitre plate. When 80% of confluent monolayer was formed 100 µl of diluted virus was serially added from well no.2 to 9, each dilution in 8 rows. column nos. 10 and 11 were kept for virus and cell controls, respectively. Plates were properly sealed and kept at 37°C. Development of cytopathic effects was observed in each well after every 24 h interval for three days. The reading of CPE was recorded as soon as the CPE was complete in virus control. The titre was calculated as per the method of Reed and Muench (1938).

RESULTS

Phytochemical analysis of *Jatropha curcas* leaf extract

The phytochemical analysis conducted on methanolic extract of *Jatropha curcas* leaves revealed the presence of tannins, alkaloids, flavonoids and steroids. The ûndings may offer an explanation for the traditional use of this plant against viral infection treatments. These compounds are known to support biological activities in medicinal plants and thus supported the antiviral activity of the plant extract used in this study.

Propagation of the IBDV in CEF culture

A CEF adopted IBDV isolate UA-Bz-2 strain was propagated in CEF culture at 10^{th} passage. It started showing the cytopathic effects by 24 hours post infection (hpi). There was marked rounding, aggregation of refractile cells and cytoplasmic vacuolation. By 48 hpi, significant number of

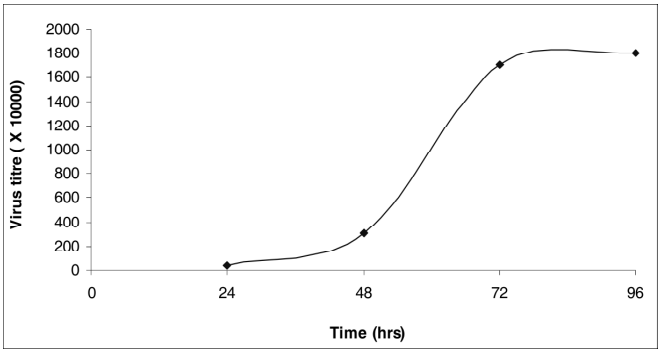

Fig 1:
Virus titre and growth curve of IBDV in CEF

dead cells, which had become rounded, were seen scattered in the monolayer. There was marked granulation of cell cytoplasm particularly around nucleus. By 72 hpi, most of the degenerated cells had detached from the substratum, leaving micro-plaques in the cell sheet. The cells were clumped into patches, leaving behind large spaces and a few long cytoplasmic strands. The uninfected control did not show any changes up to 72 hpi.

Virus titre and growth curve analysis

Virus titre was calculated at 24, 48 and 72 h intervals by using the formula used by Reed & Muench (1938).

The calculated titres were 3.9×10^5, 3.1×10^6 and 1.7×10^7 $TCID_{50}$ per ml at 24, 48 and 72 h intervals, respectively. There was significant increase in titre from 24 to 72 h but after 72 h, marked change in titre was not observed (Fig- 1.1). The virus harvested at 72 hpi and diluted to 100 $TCID_{50}$ was used for future experimental studies.

MTT dye reduction test for cytotoxicity

To eliminate the direct cytotoxic effect *Jatropha curcas* leaf extract, cell viability of chicken embryo fibroblast cells (CEF cell cultures) was assessed by using MTT assay based on the activity of mitochondrial dehydrogenases of living cells. In this assay, the chromogenic substrate 3-(4, 5-dimethylthiazol-2-y1)-2, 5-diphenyltetrazolium bromide (Sigma, USA) is converted into purple formazan and quantified at 570 nm using a spectrophotometer. The graph plotted between % of survival cells and different concentrations of the extract depicted, 50, 75 and 100 ìg/ml of the extract resulted in 100, 97 and 74% survival, respectively (Fig- 1.2). Therefore, the highest concentration without cytotoxicity 50ìg/ml of *Jatropha curcas* leaf extract was tested for their antiviral activity.

3.5 Determination of viral kinetics with and without extract of *Jatropha cucas* leaves treatment

To study the effect of the methanolic extract (50µg/ml) of *Jatropha curcas* on IBDV replication in CEF, the load of virus with and without the extract treatment were determined by $TCID_{50}$. Virus titres at 24, 48 and 72 h were 3.9×10^5, 3.1×10^6 and 1.7×10^7 $TCID_{50}$ per ml, respectively. the titre of virus significantly ($p<0.05$) reduced to 3.16×10^3, 2.37×10^4 and 2.15×10^6 $TCID_{50}$/ml at 24, 48 and 72 h, respectively **(Table- 1)** with the treatment of methanolic extract (50µg/ml) of *Jatropha cucas* leaves**.**

Table 1:
Virus titre with and without the methanolic extract of *Jatropha curcas* leaves (50µg/ml) at different time intervals

Time Interval (hrs)	Extract untreated CEF	Extract treated CEF	% Inhibition of replication of virus
	$TCID_{50}$ / ml	$TCID_{50}$ / ml	
24	3.9×10^5 *	3.16×10^3 *	99.18
48	3.1×10^6 *	2.37×10^4 *	99.25
72	1.7×10^7 *	2.15×10^6 *	87.35

*: between extract treated and untreated virus titre**.**

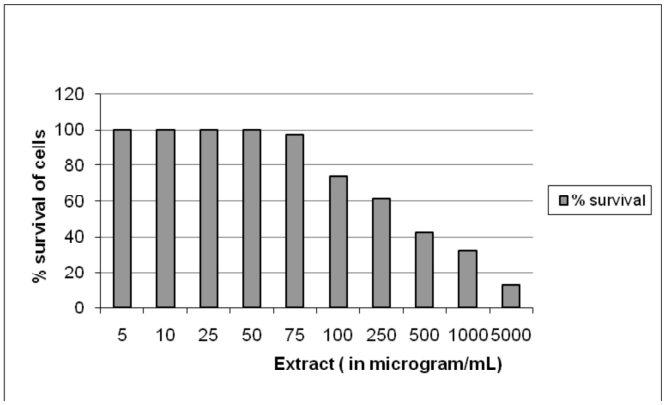

Fig 2:
MTT assay of methanolic extract of *Jatropha curcas* leaves for cytotoxicity

DISCUSSION

IBDV replicates in CEF cells and produces cytopathic effects which include cell rounding, granulation and finally detachment of cells from the culture surface (Yamaguchi *et al.*, 1996). The multiplication cycle in CEF and CEK cell cultures is 10-16 h (1974; Nick *et al.*, 1976).

The field isolate of IBDV (UA-Bz-2) was propagated in CEF. Since it has very well adapted to the CEF culture and started showing CPE from 24 hpi. The virus showed characteristic CPE 36-72 hpi. There was marked rounding, aggregation of refractile cells and cytoplasmic vacuolation which intensified with further passages which were in agreement with the observations made by Cho and Edgar (1969).

In order to determine the dose of inoculam and also to standreized $TCID_{50,}$ virus titration was done in infected CEF after 24, 48 and 72 hpi. In this experiment it was observed that virus titre increased from 24- 72 hpi, and after that there was no significant increase in the titre beyond 72h. Khatri and Sharma (2007) reported titres of cell-associated and cell-free IBD viruses increased between 24h and 72h following infection. Although CPE can be appreciated in unstained CEF culture, by May Grunwald-Giemsa staining, granulation of cell cytoplasm and clumping of cells could be better appreciated.

Absorbance values obtained during MTT assay, performed to assess the direct cytotoxic effect of the methanolic extract of *J. curcas* in CEF cells were used to calculate the percent toxicity (Fig- 2). The findings indicate a dose-dependent nature of the cytotoxicity induced by the extract in CEF cells; higher the concentration of *J. curcas* extract, higher was the magnitude of cytotoxicity appearing in the CEF. *J. curcas* extract at a concentration of 5 mg/mL induced 87% cytotoxicity, the per cent cytotoxicity soared to 68% at the dose of 1 mg/mL.

Intermediate concentrations of 500 ìg/mL and 250 ìg/mL showed 58% and 49% cytotoxicity, respectively. Since the extract was found only to induce cytotoxicity 3% at a concentration of 75 μg/mL, a concentration below this i.e. 50 ìg/mL, which showed 100% cell survival was selected for treating the CEF culture for antiviral studies. However, Inhibitory concentration for cytotoxicity of methanolic extract of *J. curcas* leaves Faria *et al.*(2004) calculated $IC_{50,}$ at a concentration of 54 ìg/mL in Human cell line HL60.

The titre of virus at 10^{th} passage was found to be 3.9×10^5, 3.16×10^6 and 1.7×10^7 $TCID_{50}$/ml after 24, 48 and 72 hpi, respectively as per the method described previously. But when *Jatropha curcas* leaf extract treated, the titre was significantly ($p< 0.05$) reduced to 3.16×10^3, 2.37×10^4 and 2.15×10^6 at 24, 48 and 72 hpi, respectively, (Table 1). Treatment of the infected CEF with the extract at concentrations of 50 μg/mL found to reduce the viral load by 99.18%, 99.25% and 87.35% after 24, 48 and 72 h, respectively. El-Ebiary *et al*. (1997) reported CPE of IBDV grown in CEF became pronounced after nine passages at 2 days of post infection with peak titre of 10^5 $TCID_{50}$ / 0.1 ml at 54 and 60 h, respectively.

The antiviral activities of extracts derived from Jatropha species against inûuenza virus were also conûrmed at the level of nucleic acid by PCR experiments. Reduction of nucleic acid synthesis in comparison with the control was observed. On the basis of findings of researchers regarding inhibitory effect of the extract on influenza and HIV virus, it can be assumed that IBDV being RNA virus, the extract may also have inhibitory effect on replication of the virus possibly through viral RNA synthesis. However, these could be possibly that other mechanism such as protein synthesis, virus assembly etc. might also involve in the inhibition of overall viral replication rate.

An isolate of IBDV (UA-Bz-2) was propagated in chicken embryo fibroblast (CEF) cells. *In vitro* studies revealed characteristic cytopathic effects (CPE) like rounding, aggregation of refractile cells and cytoplasmic vacuolation this intensified with different time intervals. Harvested virus showed a $TCID_{50}$ of $3.9x\ 10^5$, $3.16x\ 10^6$ and $1.7x\ 10^7$ per ml at 24, 48 and 72 hours post infection, respectively. After *Jatropha curcas* leaf extract treatment the virus titre was calculated at different time intervals. The inhibition of IBDV growth by significantly reducing the titre to $3.16x\ 10^3$, $2.37x\ 10^4$ and $2.15x\ 10^6$ per ml at 24, 48 and 72 hours post infection, respectively. The findings of the present study provide an insight into the major role played by the bioactive compounds responsible for the inhibition of replication of IBDV. The present study provides an opening for the development of *Jatropha curcas* based herbal therapeutic agent against Infectious Busral Disease.

REFERENCES

Cho, Y. and Edgar, S.A. (1969). Characterisation of the infectious bursal agent. *Poultry Sci*. **48**: 2102-2109.

Cunningham, C.H. (1973). A Laboratory Guide in Virology, 6th Edn. Burgess Publishing Company, Minnesota.

Datta, S.K. and Pandey, R.K. (1993). *Applied Botany* Abstracts, **1(2)**: pp.108-118.

EL-Ebiary, E. A., Madhy, S. S. and Khodier, M. H. 1997. Trials of using cell cultures for evaluation of IBDV vaccines in Egypt. *Assiut Veterinary Medical Journal*. **36:**105-123.

Faria, M. H. G. (2004). Atividade citotóxica de 12 plantas medicinais do Ceará, Brasil. *Rev. Bras. Farm.,* 83: 9-10.

Harborne,, J. B.(1998). *Phytochemical methods*. 3rd. ed., Chapman and Hall, London, 1–302.

Igbinosa, O.O., Igbinosa, E.O., Aiyegoro, O.A. (2009).Antimicrobial activity and phytochemical screening of stem bark extracts from *Jatropha curcas* (Linn.) *Afr J Pharmacol.* **3**: 58–62.

Khatri, M. and Sharma, J. M. (2007). Replication of infectious bursal disease virus in macrophages and altered tropism of progeny virus. *Vety Immonol Pathol*. **117:** 106-115.

Kibenge, F.S.B., Dhillon, A. S. and Russell, R.G.(1988). Growth of serotype I and II and variant strains of infectious bursal disease virus in Vero cells. *Avian Diseases.* **32(2)**: 298-303.

Merchant, D.J., Khan, R.H. and Murphy, W. Jr. (1960). Handbook of cell and organ culture. Burgess and Publishers.

Matsuse I.T., Lim Y.A., Hattori M., Correa M., Gupta M. P. (1998).A search for anti-viral properties in Panamanian medicinal plants: the effects on HIV and its essential enzymes. *J Ethnopharmacol*. **64**: 15–22.

Nick, H., Cursiefen, D. and Becht, H. 1976. Structural and growth characteristics of infectious bursal disease virus. *J Virol*. **18**: 227-34.

Sriprang, S., Sriprang, N., Sumpradit, T. And Shimbhu,D. (2010).Antibacterial activities of crude extracts from physic nut (*Jatropha curcas*) seed residues. *Sci Asia*. **36**: 346–348.

Trease, G.E. and Evans, W.C. (1983). Pharmacognosy. 14th Edt, Publ. Brown Publications.

Yamaguchi, T., Ogawa, M., Inoshima, Y., Miyoshi, M., Fukushi, H. and Hirai, K.(1996). Identification of sequence changes responsible for the attenuation of highly virulent infectious bursal disease virus. *Virol.* 223: 219–223.

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Research Article

ACUTE TOXICITY STUDY OF OXYTETRACYCLINE IN RATS

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ABSTRACT

The present study was carried out in the forty eight rats randomly and equally divided into six groups. Group I served as control. Groups II and III were injected oxytetracycline (OTC) @ 0, 300 and 1000 mg kg^{-1} b. wt., i.p., in three test groups. Haemato-biochemical and antioxidant parameters were recorded. There was a significant ($P<0.05$) increase in TEC, TLC, AST, total cholesterol and creatinine and decrease in Hb, BUN, urea and creatinine in rats of group III and significantly ($P<0.05$) increases the value of PCV, TEC and TLC in group II. There was a significant ($P<0.05$) reduction in LPO in kidney, liver, brain and erythrocytes in OTC treated rats in comparison to control. There was a significant ($P<0.05$) decline in value of GSH in erythrocytes but significantly elevated in liver, kidney and brain in rats of treated groups as compared to untreated control. It is concluded that oxytetracycline at doses as high as 2000 mg/kg, i.p. caused moderate toxic effects on haemato-biochemical parameters but did not induce oxidative stress in rats.

Key words : Oxyteracycline, acute toxicity, haemato-biochemical profile, rat

INTRODUCTION

Oxytetracycline was first isolated from soil containing the bacteria *Streptomyces rimosus* in 1948 and was the second of the tetracycline antibiotics to be discovered (Hlavka *et al.,* 1992). Oxytetracycline (OTC) is a broad spectrum antibiotics with bacteriostatic activity against gram-positive and gram-negative bacteria, including some anaerobes in livestock, poultry and aquatic animals. OTC is one of the commonly used antibiotics in livestock to treat both common bacterial infections of respiratory tract, sinuses, middle ear and urinary tract and rare infections like anthrax, plague, cholera, Legionnaire's disease etc (Dale and Mandelstam, 2005). It is also active against *Chlamydia, Mycoplasma* and some protozoa and several rickettsial infections including Anaplasma. Because of it's broad spectrum of activity, low toxicity and capacity for diffusion into most body fluids and tissues,it is also an important drug for use at public and private fish hatcheries (USGS, 2003). To produce its antibacterial activity, it inhibits several processes essential for the survival and growth of bacterial cells, mostly synthesis of bacterial proteins (Ophardt, 2003). In view of its wide use in livestock, poultry and aquatic animals, this study was planned to evaluate its effect on haemato-biochemical and antioxidant parameters following its single high intraperitoneal (i.p.) doses in rats.

Chemical and reagents

Oxytetracycline injectable solution (50 mg mL^{-1}) used in this study was provided by Indian Immunologicals. Reagents for this study were procured from Sisco Research Laboratories Pvt. Ltd. The parameters for biochemical analysis were determined by spectrophotometric methods using diagnostic kits

Experimental design

Twenty four albino rats (50 % each of male and female) of 3 to 4 months of age weighing between 150 to 200 g were procured from Laboratory Animal Resource Centre, India Veterinary Research Institute, Barielly and kept in plastic cages under standard managemental conditions. Standard rat feed and water were provided *ad libitum* throughout the study. The study was undertaken after approval of IAEC.

After acclimatization for two weeks, rats were randomly and equally divided into three groups (8 rats in each group). Group I was considered as control and given normal saline, i.p. groups II and III were given single i.p. oxytetracycline injection @ rate of 0, 300 and 1000 mg kg^{-1} b wt. The rats were kept under constant observation for 14 days. On 15th day all the rats were sacrificed for collection of tissues.

Haemato-biochemical parameters

Blood was collected in heparinized tubes for estimation of haematological parameters such as total erythrocyte count, total leucocyte count, packed cell volume and haemoglobin (Jain, 1986) and in non heparinized tubes for the estimation of blood analytes and enzymes in plasma such as aspartate amino transferase (AST) and alanine amino transferase (ALT), alkaline phosphatase, BUN, creatinine, plasma proteins and albumin were determined by spectrophotometric methods using diagnostic kits (ACE Diagnostics & Biotech Ltd., Gurgaon).

Antioxidant parameters

Estimation of different antioxidative parameters such as lipid peroxidation product malondialdehyde (LPO) (Rehman, 1984) and reduced glutathione (GSH) (Sedlak and Lindsay, 1968) was done in liver, kidney and brain and reduced glutathione in erythrocytes (Prins and Loos, 1969).

Statistical analysis

Statistical analysis of data was done by using

ANOVA technique according to the method described by Snedecor and Cochran (1967). Comparison among treated group were made with help of student't' test at 5% level of significance.

RESULTS

No mortality was observed following post administration of single i.p. dose of oxytetracycline (50 mg mL^{-1}) in groups I, II and III.

Haemato-biochemical profile of rats treated with oxytetracycline is shown in Table 1. There was a significant (P<0.05) increase in TEC and TLC and decrease in Hb in rats of group III and of PCV, TEC and TLC were significantly (P<0.05) increased in group II as compared with control. The values of AST, total cholesterol and creatinine in plasma were significantly (P<0.05) increased whereas total urea and BUN were significantly (P<0.05) declined in rats of group II as compared to group I. There was significant (P<0.05) increase in ALT, AST, total cholesterol and urea, BUN and creatinine significantly (P<0.05) decreased in rats of treated group III as compared to rats of control group I.

The lipid peroxidation (LPO) and reduced glutathione (GSH) in brain, kidney, liver and erythrocytes rats treated with oxytetracycline is depicted in Table 2. There was a significant (P<0.05) reduction in LPO in kidney, liver and brain, kidney and erythrocytes in rats of treated groups II and III in comparison to group I.

There was significantly (P<0.05) decline in value of GSH in erythrocytes and significantly whereas elevated in liver, kidney and brain in rats of treated group II and III significantly (P<0.05) as compared to untreated control.

Table 1:
Effect on hemato-biochemical parameters following single i.p. dose of oxytetracycline in rats (Mean+_SE; n=8).

Groups	I	II	III
Dose (mg.kg^{-1})	0	300	1000
Hb (%)	10.90 ± 0.80	10.24 ± 0.66	6.67 ± 0.49*
PCV (%)	27.37 ±2.35	41.37 ±9.29*	34.62 ±3.21*
TEC ($10^6\mu L^{-1}$)	6.89 ±0.44	9.29 ±0.28*	11.14 ±1.64*
TLC ($10^3\mu L^{-1}$)	17.12 ±0.60	23.66 ±1.85*	24.06 ±1.20*
ALT(U L^{-1})	24.65±0.82	27.75±2.61	42.62±2.10*
AST(U L^{-1})	30.0±1.20	48.96±2.60*	81.00±5.30*
Cholesterol(mg.dL^{-1})	94.99±1.35	155.65±4.56*	230.33±7.09*
Urea(mg.dL^{-1})	31.34±1.03	12.83±1.04*	6.54±0.52*
BUN (mg.dL^{-1})	21.22±0.93	15.29±0.45*	12.16±0.74*
Creatinine(mg.dL^{-1})	3.77±0.25	5.49±0.30*	6.82±0.72*

Values in table are Mean ± S.E. P< 0.05 vs. control group

DISCUSSION

A significant difference in values of Hb, TEC and TLC was observed in group III as compared to control. TEC and TLC were significantly increased whereas Hb was decreased in group II and III in comparison to control. Haemolysis and shrinkage in RBC might be the reason for decline in cell count in treated rats of group II and III. An increase in activities of ALT and AST is known to be the indicator of hepatic damage (Cornelius, 1989). A significant increase in the activity of AST and ALT in treated groups might be due to hepatic damage caused by oxytetracycline in this study (Ahmad and Khater, 2001).

A significant increase in cholesterol level was recorded in both the treated groups. Liver is the major site of cholesterol, bile acid and phospholipids synthesis and metabolism. Hepatic cholesterol homeostasis is maintained by equilibrium between the activities of hydroxyl methyl glutaryl CoA (HMG-CoA) reductase and that of acyl CoA, cholesterol acyl transferase (Hochgraf *et al.*, 2000). Thus, alteration in cholesterol level might have been due to effect on metabolism of cholesterol in this study.

The concentrations of urea and BUN were found to be significantly lower in group II and III than control whereas the creatinine was significantly higher in both the treated group as compare to control. Higher level creatinine is suggesting of nephrotoxic effect, however, BUN and urea levels do not favors the effect (Cornelius, 1989).

There was a significant reduction in LPO in low dose group in comparison to control suggested that oxytetracycline did not produce oxidative stress in oxytetracycline treated rats @ 1000mg/kg b wt. The present study showed that the drug administration in higher dose resulted in reduction in LPO in RBCs as evidenced

Table 2:
Effect on LPO and GSH in tissues and erythrocytes following single dose intraperitonial administration of oxytetracycline in rats (Mean+SE).

Groups		I	II	III
Dose (mg.kg^{-1})		0	300	1000
LPO(nM MDA/g)	Liver	26.73±2.10	21.40±0.52*	21.40±0.52*
	kidney	12.71±0.65	8.95±0.32*	5.50±0.51*
	Brain	34.05±1.17	27.04±0.67*	23.62±0.22*
	Erythrocyte	3.66 ± 0.16	3.21± 0.37	0.95± 0.08*
GHS(nM/g)	Liver	0.32±0.01	0.36±0.009*	0.87±0.01*
	kidney	0.28±0.004	0.59±0.085*	0.85±0.005*
	Brain	0.75±0.01	1.44±0.01*	1.58±0.13 *
	Erythrocyte	1.31± 0.12	0.06± 0.009*	0.04± 0.001*

Values in table are Mean ± S.E. P< 0.05 vs. control group

by decline in production of malondialdehyde (MDA). The reported value of GSH in RBCs of rats was significantly declined in RBCs and an increase in other tissues of treated groups in comparison to control which suggested that oxytetracycline did not promote the synthesis of oxidative radicals in tissues of liver, kidney and brain of treted groups.

A significant reduction in LPO in liver, kidney and brain was observed in treated groups II and III. A fall in LPO suggested the reduction in production of MDA. The GSH was found to be higher in brain, kidney and liver of both the treated group II and III than control.

GSH plays an important role against oxidative damage caused by ROS in the cells. In addition, GSH also react non enzymaticaly with ROS. Oxidation reduction coupling of GSH is central to the cellular response to oxidative stress. The balance between oxidation of GSH to glutathione disulphide (GSSG) and a rapid reduction of GSH by glutathione reductase contributes to the maintenance of the cellular homeostatic GSH: GSSG ratio of about 300:1 (Alpert and Gilbert, 1985).

The elevation of GSH level in organs like liver, brain and kidney of treated rats might be due to in response to administration of oxytetracycline. It is concluded from the toxicity study of oxytetracycline in rats that it produced moderate haemotoxic and hepatotoxic effect but did not produce oxidative stress after single dose as high as 2000 $mg.kg^{-1}$.b. wt., i.p. in rats rather it enhanced level of GSH in vital organs indicated its anti-oxidant potential in rats.

REFERENCES

Ahmed, M. B. and Khater, M. R. (2000). The evaluation of the protective potential of Ambrosia maritima extract on acetaminophen-induced liver damage. *J. Ethanopharmacol.***75**: 169-174.

Alpert A. J. and Gilbert H. F. (1985). Detection of oxidized and reduced glutathione with a recycling post column reaction. *Anal Biochem*. **144** : 553-562.

Cornelius C E, 1989. Liver function. In: Kaneko, J.J.*ed*. Clinical biochemistry of domestic animals. Academic press Sandiego, New York. pp. 386.

Dale, M. M. Mandelstam, J. (2005). Antiobiotics. Microsoft Encarta Online Encyclopedia.

Hlavka, J. J., Ellestad G. A. and Chopra I. (1992). Antibiotics (Tetracyclines). Kirth-Othmer Encyclopedia of Chemical Technology, Volume 3 (Fourth Edition). New York: John Wiley & Sons.

Hochgraf, E.; Corgan, V. and Soshama, M. (2000). Dietray oxidized linoleic acid enhances liver cholesterol biosynthesis and secretion in rats. *J.Natr. Biochem.* **11:** 176-180.

Jain, N. C. (1986). Schalm's Veterinary Haematology. 4th Edn. Lea and Febringer, Philadeiphia.

Ophardt, C. E. (2003). Introduction to Drug Action: Other Antibiotics.

Prins, H. K. and Loos, J. A. (1969). Glutathione. *In:* Yunis, J.J. ed. Biochemical Methods in Red Cell Genetics. New York, Academic Press. pp. 115-137.

Rehman, S. U. (1984). Lead induced regional lipid peroxidation in brain. *Toxicol. Lett.* **21** : 333-337.

Sedlak, J. and Lindsay, R.H. (1968). Estimation of total protein-bound and nonprotein sulfhydryl groups in tissues with Ellman's reagent. *Anal. Biochem.* **24(25)**: 192-205.

Snedecor, G. W. and Cochran, W. G. (1967). Statistical methods. 61, oxford IBH.HSDB (Hazardous Substances Data Bank). 2003a. Oxytetracycline.

USGS (U. S. Geological Survey). 2003. Expansion of Oxytetracycline in Immersion and Feed Additive for Control of Bacterial Diseases and Otolith Marking on Cultured Freshwater Fish. USGS Upper Midwest Environmental Sciences Center.

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Short Communication

HEAMATO-BIOCHEMICAL EVALUATION OF *CISSUS QUADRANULARIS* IN LONG BONE FRACTURE HEALING IN DOGS

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ABSTRACT

The present study was carried on 12 dogs weighing 20-30 kg. All the animals were divided into two groups comprising 6 dogs in each group. In animals of Group I, immobilization of fractured bone was done by intra-medullary pinning (control) and in animals of group II, immobilization of fractured bone was done by intra-medullary pinning + inj. of ethanolic extract of *Cissus quadrangularis* @ 50 mg/kg body weight s.c. on alternate days for 20 days postoperatively (treatment). There were non-significant changes in haematological (total erythrocyte count, total leucocytes count, haemoglobin, lymphocyte, neutrophil, monocyte, eosinophil, basophil) and biochemical (serum alkaline phosphatise, creatinine kinase, serum calcium, serum phosphorus) parameters. Study guggests that *C. quadranularis* @50 mg/kg t.w. for 20 days is safe for fracture healing.

Key word- *Cissus quadrangularis,* Long bone fracture, Intra-medullary pinning, Dogs

Cissus quadrangularis Linn (Syn: *Vitis quadrangularis* Wall: family : Vitaceae) is an, indigenous medicinal plant of India. The plant is also known as "Harsankar" in Hindi and Asthisanghar in Sanskrit and in English "Edible Stemmed Vine". The calcium ions, phosphorous and phytoestrogens present in this plant may be of use in the process of ossification and fracture healing (Rao *et al*., 2007). Therefore, the present study was planned to investigate the effect of ethanolic extract of *Cissus quadranularis* on heamato-biochemical parameters in long bone fracture healing in dogs..

The study was conducted on 12 dogs divided into two groups, 6 dogs in each groups I and II. In group, immobilization of fractured bone was done by intra-medullary pinning (control) and in group II with immobilization of fractured bone by intra-medullary pinning + inj. of ethanolic extract of *Cissus quadrangularis* @ 50 mg/kg body weight s.c. was of given on alternate days for 20 days postoperatively. Five ml blood was collected in sterilized glass vials containing EDTA (Acid, 2 mg/ml blood) on 0, 7th 14th, and 28thday for haematological parameters viz. total erythrocyte count (10^{6}/µl), total leucocytes count (thousands/ µl), haemoglobin (gram/dL), lymphocytes (%), neutrophils (%), monocytes (%), eosinophils (%), basophils (%) and biochemical parameters viz. serum alkaline phosphatase (IU/L), creatinine kinase (IU/L), serum calcium (mg/dl), serum phosphorus (mg/dl). The data obtained during the study were analyzed employing the one way ANOVA as per method described by Snedecor and Cochran (1994).

The results of the present study showed that the total erythrocyte count did not vary significantly (p>0.05) in both the groups. There was constant and continuous inclining of total erythrocyte count in group II due to anabolic effect of ethanolic extract of *Cissus quadrangularis* which has stimulated and coapted the reticuloendothelial system.

Mallika *et al.* (2006) reported that the *Cissus quadrangularis* had been proved to be highly effective in relieving pain, reduction of swelling and promoting in the process of wound as well as fracture healing.

The non-significant (p>0.05) variation in the values of heamoglobin was observed in both groups of animal. Similar observations were reported by and Ratnakar (2012) who observed the variation in haemoglobin values. It could be due to the surgical stress and anabolic effect of ethanolic extract of *Cissus quadrangularis.*

In case of differential leucocyte count, there were non-significant changes in (lymphocytes, neutrophils, monocytes, eosinophils and basophils). The observation was accordance with Gahlod (2007) and Ratnakar (2012) who reported that the changes in lymphocyte count, transient increase of neutrophils, eosinophils, basophils, variation in monocyte count due to trauma, inflammation and surgical stress during the treatment period. The values of serum alkaline phosphatase level were showing non-significant (p>0.05) change in both the groups.

Statistically non-significant (p>0.05) decrease was observed in creatinine kinase level in both the groups. Creatine kinase is mostly present in the skeletal muscle, myocardium, brain and intestine.

The mean values of serum calcium was non-significant (p>0.05) in both the treatment group. Deka *et al.* (1994) recorded low level of serum calcium in *Cissus quadrangularis* treated animals during early fracture healing and postulated that parafollicular or "C" cells of thyroid gland release more calcitonin and thereby a decrease in serum calcium level, since calcitonin increase the osteoblastic activity. The change in values of serum phosphorus were non-significant (p>0.05) in both the

Table 1 : Effect of ethanolic extract of *Cissus quadranularis* on heamato-biochemical parameters in long bone fracture healing in dogs.

S.No.	Parameters	Group	0 Day	7th Day	14th Day	28th Day
1	Total erythrocyte count (million/µl)	G1	6.15±0.15	5.23±0.52	5.45±0.32	5.73±0.44
		G2	5.23±0.52	6.05±0.22	6.18±0.82	6.75±0.25
2	Total leucocytes count (thousand/µl)	G1	18.81±4.33	19.78±2.12	19.15±3.35	17.56±3.25
		G2	16.23±1.24	17.16±1.10	15.9±2.31	16.70±2.38
3	Haemoglobin (gm %)	G1	13.33±1.10	13.16±0.22	12.41±1.35	11.25±1.11
		G2	12.58±1.26	12.41±1.35	12.01±1.24	11.83±2.32
4	Lymphocyte (%)	G1	27.33±2.87	25.66±4.37	29.50±6.62	24.66±2.52
		G2	32.66±4.37	28.01±1.82	24.33±3.29	31.37±3.26
5	Neutrophil (%)	G1	62.51±3.32	65.16±6.16	63.13±5.12	63.02±5.29
		G2	59.33±4.47	62.16±1.32	61.53±2.34	56.83±8.72
6	Monocyte (%)	G1	3.83±0.28	3.33±0.31	3.66±0.82	3.16±0.35
		G2	3.16±0.35	3.00±0.67	3.16±0.35	3.16±0.46
7	Eosinophil (%)	G1	3.16±0.61	3.66±0.58	2.83±0.28	3.00±0.51
		G2	3.00±0.13	3.83±0.62	3.33±0.21	3.00±0.13
8	Basophil (%)	G1	0.50±0.16	0.50±0.27	0.33±0.22	0.00
		G2	0.33±0.53	0.33±0.38	0.50±0.18	0.33±0.10
9	Serum alkaline phosphatase(IU/L)	G1	213.05±37.78	197.88±35.83	170.54±77.37	117.37±19.28
		G2	264.04±51.67	210.64±47.64	141.87±23.10	121.24±19.36
10	Creatinine kinase(IU/)	G1	233.06±55.42	186.60±37.27	138.13±23.57	98.73±10.96
		G2	192.16±33.85	172.60±30.79	119.81±19.55	97.32±14.39
11	Serum calcium(mg/dl)	G1	7.73±0.58	7.96±0.45	8.21±0.54	9.49±0.45
		G2	7.74±0.59	8.28±0.56	8.68±0.50	9.89±0.52
12	Serum phosphorus(mg/dl)	G1	2.35±0.42	2.47±0.38	2.72±0.36	3.05±0.59
		G2	2.44±0.31	2.68±0.42	2.90±0.51	3.56±0.59

groups. Saravanan *et al.* (2002) reported similar variation in serum phosphorus after oral and local application of *Cissus quadrangularis* and *Terminalia arjuna* in radial and femoral fracture in dogs respectively. Thus, it is concluded that *Cissus quadrangularis* @ 50 mg/kg body weight s.c. on alternate days for 20 days.

REFERENCES

Deka, D.K., Lahon, L.C., Saikia, J. and Mukit, A. (1994). Effect of *Cissus quadrangularis* in accelerating healing process of experimentally fracture radius- ulna of dog. *Indian Journal of Pharmaceutical Science*, **26**:44-45.

Gahlod, B.M. (2007). Studied on healing of fractures of femur in canine with special reference to use of different implants and anabolic hormone- A clinical study. PhD Thesis, Nagpur Veterinary College, Nagpur India.

Mallika, J. and Shyamala Devi C.S. (2006). Gastroprotective action of *Cissus quadrangularis* extract against NSAID induced gastric ulcer: role of roinflammatory cytokines and oxidative damage. *Biological Interaction,* **161**: 262-270.

Rao, M.S., Bhagath, K.P., Narayana Swamy, V.B. and Gopalan, K.N. (2007). *Cissus quadrangularis* plant extract enhances the development of cortical bone and trabeculae in the fetal femur. *Pharmacology Online*, **3**: 190-202.

Ratnakar, T.R. (2012). Clinical studies on healing of long bone fracture with reference Radiology), Nagpur Veterinary College, Nagpur.

Saravanan, B., Maiti, S.K., Singh, G.R., Hoque, M. and Kumar, N. (2002). Evaluation of the herb, Cissus quadrangularis in accelerating healing process of femur in dog. *Journal of Veterinary Medicine,* Series A (In press).

Snedecor, G.W. and Cochran, W.G. (1994). Statistical Method, 8th Edn., The Iowa state University Press Publishing Co., U.S.A., pp 312-317.

Tembhurne, R.D., Gahlod, B.M., Dhakate, M.S., Akhare, S.B., Upadhye, S.V. and Bawaskar, S.S. (2010). Management of femoral fracture with the use of horn peg in canine. *Veterinary World,* **3**(1): 37-41.

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Short Communication

SAFETY ASSESSMENT FOLLOWING INTRAMUSCULAR ADMINISTRATION OF MOXIFLOXACIN WITH KETOPROFEN IN SHEEP

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ABSTRACT

Moxifloxacin is a novel fourth generation fluoroquinolone with broad spectrum of antibacterial activity against a wide range of aerobic and anaerobic gram-positive and gram negative bacteria. Ketoprofen is non-steroidal anti-inflammatory drug commonly used in veterinary medicine. The study was undertaken to determine blood biochemical profile following daily intramuscular administration of moxifloxacin (5 mg/kg) in combination with intramuscular administration of ketoprofen (3 mg/kg) for five days in sheep. Blood samples were collected daily for 5 days before and after the initiation of drugs administration and proceeded for determination of various haematological (Hb, PCV, TEC, TLC and DLC) and biochemical parameters (AST, ALT, AKP, LDH, BUN, serum creatinine and total bilirubin). No significant alterations ($p < 0.05$) were found in the mean value of haematological and biochemical parameters during treatment period. Repeated intramuscular administration of moxifloxacin (5 mg/kg) in combination with ketoprofen (3 mg/kg) for 5 days in sheep was found safe. Thus, moxifloxacin may be useful in combination of ketoprofen to treat bacterial diseases accompanied by fever, pain and other inflammatory condition in sheep.

Key words: Moxifloxacin, ketoprofen, safety assessment, fluoroquinolone, sheep

Antibacterials are frequently recommended with non-steroidal anti-inûammatory drugs (NSAIDs) for the treatment of various bacterial infections accompanied by other inflammatory conditions in animals. Moxifloxacin is a novel fourth generation fluoroquinolone with broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria, anaerobes and a typical organisms such as *Mycoplasma* and *Chlamydia* spp (Brown, 1996). The drug thus seems to be extremely useful in a variety of infections including those of urinary tract, respiratory tract, soft tissues, bones and joints of animals. Ketoprofen is an aryl propionic acid derivative, non-selective cyclooxygenase inhibitor having anti-inflammatory, analgesic and antipyretic properties. This new combination has the immense potential for clinical use. The data on safety profile of co-administration of moxifloxacin with ketoprofen in sheep are limited. Hence, the present study was undertaken to assess safety following daily intramuscular (IM) administration of moxifloxacin (5 mg/kg) in combination with ketoprofen (3 mg/kg) for five days in sheep.

The study was conducted on six Patanwadi sheep of 2-3 years weighing between 25-30 kg. Each sheep was housed in a separate pen and provided with a standard ration and *ad libitum* water. The sheep were kept under constant observation for two weeks before the commencement of the experiment and subjected to clinical examination to exclude the possibility of disease. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC No. 2010/VPT/80). Moxifloxacin and ketoprofen were administered by intramuscular injection at deep gluteal muscle using 20G x 25mm needle. All animals were observed daily for any side effects or clinical abnormalities throughout the period of experiment. Safety assessment was carried out by monitoring haematological and serum biochemical parameters in sheep.

Blood samples were collected at day 0 (before administration of drugs), 1st, 2nd, 3rd, 4th and 5th from jugular vein into sterile tubes with tri-potassium ethylene diamine tetra-acetic acid for haematological and in plain sterile tube for serum biochemical analysis. Haematological and serum biochemical parameters were estimated using automated haematology analyzer (Abacus Junior Vet 5, Austria) and clinical serum biochemistry analyzer (Junior Selectra, Vital Scientific NV, Netherlands). The statistical analysis was performed using student's t-test using SPSS software (Version 12.0.1). Statistical differences were considered at pd"0.05 and pd"0.01.

The mean values of haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC) and differential leukocyte count (neutrophil, lymphocyte, basophil, eosinophil and monocyte) are presented in Table 1. Whereas, the mean values of serum alkaline phosphatase (AKP), serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum lactate dehydrogenase (LDH), total bilirubin (TB), serum creatinine, blood urea nitrogen (BUN), total protein (TP), albumin and globulin are presented in Table 1. During the experimental period, none of the animal of study group revealed any clinical symptoms and mortality attributable to the 5 days intramuscular administration of moxifloxacin in combination with ketoprofen.

The mean values of any of hematological and serum biochemical parameters after treatment for 1-5 days

Table 1:
Effect of daily intramuscular administration of moxifloxacin (5 mg/kg) and ketoprofen (3 mg/kg) for 5 days on haematobiochemical parameters in sheep (Mean ± SE; n=6).

Parameters	Day					
	0	1st	2nd	3rd	4th	5th
Haematological parameters						
Hb (g/dl)	11.62 ± 0.27	10.98 ± 0.33	10.59 ± 0.45	11.48 ± 0.66	12.11 ± 0.64	11.12 ± 0.60
PCV (%)	32.50 ± 0.43	32.67 ± 0.42	32.00 ± 0.45	32.50 ± 0.43	31.50 ± 0.43	32.50 ± 0.43
TEC (x 10^6/¼l)	10.61 ± 0.37	10.72 ± 0.43	11.07 ± 0.40	10.94 ± 0.56	11.01 ± 0.32	10.78 ± 0.48
TLC (x 10^3/cmm)	8.86 ± 0.13	9.13 ± 0.34	8.95 ± 0.28	9.10 ± 0.65	9.57 ± 0.24	9.63 ± 0.28
Neutrophil (%)	38.17 ± 0.54	39.67 ± 0.92	39.33 ± 1.15	39.83 ± 0.65	38.00 ± 0.77	40.00 ± 0.45
Lymphocyte (%)	57.00 ± 0.73	55.33 ± 1.05	56.00 ± 1.63	55.00 ± 0.77	57.00 ± 1.15	54.67 ± 0.42
Monocyte (%)	1.67 ± 0.21	2.17 ± 0.31	1.83 ± 0.48	2.17 ± 0.31	2.17 ± 0.31	2.00 ± 0.26
Eosinophil (%)	2.50 ± 0.22	2.33 ± 0.42	2.33 ± 0.33	2.50 ± 0.34	2.17 ± 0.40	2.67 ± 0.33
Basophil (%)	0.67 ± 0.21	0.50 ± 0.22	0.50 ± 0.34	0.50 ± 0.22	0.67 ± 0.21	0.50 ± 0.22
Serum biochemical parameters						
AKP (IU/L)	115.56 ± 1.38	115.07 ± 1.30	117.58 ± 2.25	113.29 ± 1.39	113.06 ± 0.95	113.85 ± 1.53
AST (IU/L)	87.23 ± 0.41	84.47 ± 3.63	85.20 ± 1.35	88.59 ± 1.79	88.27 ± 1.17	88.93 ± 1.78
ALT (IU/L)	27.39 ±1.33	28.92 ± 1.99	29.64 ± 2.59	28.68 ± 1.42	28.65 ± 0.79	29.80 ± 1.55
LDH (IU/L)	329.49 ± 1.64	333.65 ± 2.34	333.83 ± 2.50	333.09 ± 2.44	332.42 ± 1.85	330.04 ± 1.63
TB (mg/dl)	0.20 ± 0.03	0.22 ± 0.02	0.16 ± 0.05	0.17 ± 0.02	0.16 ± 0.01	0.21 ± 0.03
Creatinine (mg/dl)	1.27 ± 0.12	1.35 ± 0.03	1.39 ± 0.13	1.42 ± 0.10	1.35 ± 0.09	1.41 ± 0.11
BUN (mg/dl)	16.91 ± 0.47	16.62 ± 1.22	16.41 ± 0.96	15.55 ± 0.54	16.34 ± 0.47	15.84 ± 0.47
TP (g/dl)	6.91 ± 0.13	7.06 ± 0.03	6.86 ± 0.06	6.99 ± 0.05	7.13 ± 0.07	6.72 ± 0.07
Albumin (g/dl)	3.10 ± 0.06	3.14 ± 0.04	3.15 ± 0.07	3.18 ± 0.06	3.02 ± 0.04	3.11 ± 0.05
Globulin (g/dl)	3.81 ± 0.12	3.93 ± 0.03	3.71 ± 0.03	3.81 ± 0.06	4.21 ± 0.15	3.60 ± 0.11

did not differ significantly ($P < 0.05$) in comparison to the corresponding control values (0 day). The findings of the study could be correlated to lack of interaction between moxifloxacin and ketoprofen. Kamali (1994) also reported lack of interaction between ciprofloxacin and fenbufen.

Similar findings of non-significant change in hematological and biochemical parameters following repeated administration of ofloxacin in combination with ornidazole in mice (Chaudhary *et al.,* 2009), ciprofloxacin with metronidazole in human (Werk and Schneider, 1988), ciprofloxacin and ofloxacin in combination with clindamycin in human (Boeckh *et al.,* 1990) have been reported. Additionally, Ciprofloxacin in calves (Bhavsar *et al.,* 2004), enrofloxacin in yak (Khargharia *et al.,* 2007) and levofloxacin in layer birds (Patel *et al.,* 2009) have also been reported to cause non-significant change in hemato-biochemical parameters.

On the basis of observation of various hematological and serum biochemical parameters, multiple intramuscular administration of moxifloxacin (5 mg/kg) along with ketoprofen (3 mg/kg) did not affect hepatic as well as renal functioning. This suggests that intramuscular administration of moxifloxacin at least for 5 days along with ketoprofen is safe in Patanwadi sheep.

REFERENCES

Bhavsar, S.K., Verma, M.P. and Thaker, A.M. (2004). Pharmacokinetics, tissue concentration, and safety of multiple dose intravenous administration of ciprofloxacin in cow calves. *J. Vet. Pharmacol. Toxicol*. **3**(1): 27-34.

Boeckh, M., Lode, H., Deppermann, K. M., Grineisen, S., Shokry, F., Held, R., Wernicke, K., Koeppe, P., Wagner, J. and Krasemann, C. (1990). Pharmacokinetics and serum bactericidal activities of quinolones in combination with clindamycin, metronidazole, and ornidazole. *Antimicrob. Agents Chemother.* **34**(12): 2407–2414.

Brown, S.A. (1996). Fluorquinolones in animal health. *J.Vet.Pharm.Ther.* **19**:1–14.

Chaudhary, M., Tamta, A. and Sehgal, R. (2009). Sub-chronic toxicity study of fixed dose combination of ofloxacin ornidazole in *Mus Musculus* Mice. *Open Toxicol. J.* **3**: 24-29.

Kamali, F. (1994). Lack of a pharmacokinetic interaction between ciprofloxacin and fenbufen. *J. Clin. Pharm. Ther.* **19**(4): 257–259.

Khargharia, S., Barua, C.C., Nath, N. and Bhattachrya, M. (2007). Blood biochemical studies of enrofloxacin in Yak after intravenous administration. *Iranian J. Pharmacol. Therap.* **6**: 137-138.

Patel, J.H., Varia, R.D., Patel, U.D., Vihol, P.D., Bhavsar, S.K. and Thaker, A.M. (2009). Safety of levofloxacin following repeated oral administration in white leg horn layer birds. *Vet. World.* **2**(4): 137-139.

Werk, R. and Schneider, L. (1988). Ciprofloxacin in combination with metronidazole. *Infection.* **16**: 257-260.

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Short Communication

EVALUATION OF ANTISTRESSOR ACTIVITY OF PANCHGAVYA IN RATS

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ABSTRACT

The antistressor activity of panchgavya was evaluated by anoxia tolerance and swimming induced stress. The results indicated that oral administration of panchgavya @ 50 mg/ kg day for 30 consecutive days caused significant increase in anoxia tolerance time (ATT) and swimming endurance time (SET) suggesting antistressor activity of panchgavya. The anoxia tolerance time was 29.86±0.68 min on day 0 (pre treatment) which increased significantly with panchgavya as 47.71±1.05 min on day 15 and 53.35±1.10 min on day 30 (post treatment). The swimming time was 3.74±0.46 min on day 0 (pre treatment) which increased significantly with panchgavya as 10.31±0.93 min on day 15 and 13.13±1.10 min on day 30 (post treatment). It is concluded from this study that oral administration of panchgavya @ 50 mg/ kg day for 30 days produced antistressor activity in rats.

Keywords: Panchgavya, antistressor, anoxia tolerance time

Panchgavya is the combination of five major substances obtained from cow that includes milk, ghee, curd, urine and dung. All the five products possess medicinal properties against many disorders and are used for the medicinal purpose singly or in combination with some other herbs. This kind of treatment is called panchgavya therapy or Cowpathy (Chauhan and Dhama, 2010). Stress is the reaction of animal body to the forces of deleterious nature and various abnormal states that tend to disturb its normal physiological equilibrium. Cow urine has been claimed to act as an anti-oxidant, antistressor, anti-toxin, anti-allergic and as bioenhancer.

The study was conducted on 12 adult healthy albino rats of either sex weighing between 150-200 gm. Panchgavya obtained from Central Council for Research in Ayurveda and Siddha (CCRAS), New Delhi. Panchgavya was administered orally for 30 consecutive days @ 50 mg per rat in two groups of rats consisting of six rats in each group. Group I was used for anoxia tolerance test and group II for swimming endurance test.

The antistressor property of the panchgavya by ATT was studied as per the method described by Ali *et al.* (2010). The rats were made to swim in a tank of dimension 30 × 18 × 12 inches till vigorous escape attempt ceases. The rats were taken out, dried and replaced in cages. Time, for which rat swim before getting fatigue was noted, the training to the rats was given thrice a day for four days. After completion of training, the panchgavya was given orally daily for 30 consecutive days. Observations were recorded on day 0 (pre-treatment), day 15 and day 30 (post-treatment).

Experiment for SET was conducted by the method of Ali *et al.* (2010) by putting the rats in an air tight anoxia container containing CaO to absorb moisture and CO_2. Time, for which rat tolerates anoxia was noted down. Panchgavya was administered daily for 30 consecutive days. Observations were recorded on day 0 (pre-treatment), day 15 and day 30 (post-treatment). The data generated under various experiments were subjected to analysis of variance (ANOVA) using complete randomized design with interaction. Mean and standard error were obtained as per standard procedure. Each parameter was analyzed by using the method of complete randomized design with treatments allotted to the group of 6 animals each. Treatments were tested statistically at 5% level of significance (Snedecor and Cochran, 1994).

Table 1 shows the results ATT and SET of the study. The antistressor activity of panchgavya on anoxia tolerance in albino rats was evaluated on day 0 (pre treatment), day 15 and day 30 (post treatment) of the experiment. The anoxia tolerance time was 29.86±0.68 min on day 0 (pre treatment) which increased significantly with panchgavya as 47.71±1.05 min on day 15 and 53.35±1.10 min on day 30 (post treatment). The results indicated significant increase in anoxia tolerance time with panchgavya. Similarly the antistressor activity of panchgavya by swimming induced stress in albino rats was evaluated on day 0 (pre treatment), day 15 and day 30 (post treatment) of the experiment. The swimming time was 3.74±0.46 min on day 0 (pre treatment) which increased significantly with panchgavya as 10.31±0.93 min. on day 15 and 13.13±1.10 min on day 30 (post treatment). The results showed significant increase in swimming time with panchgavya.

Anoxia tolerance test employed in the study exhibited that the panchgavya produced significant increase in anoxia tolerance time from 29.86±0.68 minutes to 53.35±1.10 minutes. The ayurvedic preparations containing indigenous medicinal plants have been shown to enhance anoxia tolerance time suggesting antistressor activity. The

Table 1:
Mean values of anoxia tolerance time (minutes) in albino rats treated with Panchgavya

Groups	Treatment	Anoxia tolerance time (minutes)			CD at P<0.01
		Mean values ± SE			
		Day 0	Day 15	Day 30	
I	Panchgavya (50 mg/rat, daily for 30 days)	29.86^{c}±0.68	47.71^{b}±1.05	53.35^{a}±1.10	2.89
	Swimming endurance time (minutes)				
II	Panchgavya (50 mg/rat, daily for 30 days)	3.74^{c}±0.46	10.31^{b}±0.93	13.13^{a}±1.10	2.63

Values are mean of six observations.The mean values with different alphabets as superscript in a column differ significantly from each other SE: Standard Error; CD: Critical Difference

work of Sonkar and Mishra (2011) indicated antistressor activity of Triphala megaExt where anoxic tolerance time was increased from 21.5±3.44 minutes to 49.33±1.68 minutes which are almost similar to the findings of the present study. Similarly, Jain and Mishra (2011) also reported an increase in anoxic stress tolerance from 21.16±2.85 minutes to 58.5±2.88 minutes with oral administration of Trikatu megaExt. The ayurvedic preparations such as Triphala megaExt and Trikatu megaExt suggested the antistressor activity of ayurvedic formulations and similarly Panchgavya also exhibited antistressor effect by causing an enhancement of anoxic stress tolerance in rats. Swimming is frequently preferred as an exercise model for small laboratory animals and it has several advantages over other types of exercise. The intensity of labor during exercise is greater than running for equal periods and aversive stimulation used to promote running is not used in swimming (Yalcin *et al*., 2000). Finding of the study thus suggests that oral administration of panchgavya @ 50 mg/ kg day for 30 days produced antistressor activity in rats.

References

Ali. S., Roshan, S. and Khan, A. (2010). To study the effect of *Allium sativum* on swimming endurance, anoxia tolerance and cold stress. *J. Global Pharma Tech.,* **2**(7): 27-32.

Chauhan, R.S. and Dhama, K. (2010). Panchgavya (cowpathy): An ancient wisdom & modern science. *The Indian cow*. **23**: 2-22.

Jain, N. and Mishra, R. N. (2011). Adaptogenic activity of Trikatu megaExt *Int J. Res. in Pharmaceu. and Biomed. Sci.*, **2**(2): 570-574.

Snedecor, G. W. and Cochran, W. G. (1994). Statistical Method Publ., Oxford and IBH Publishing Co., New Delhi. 455p.

Sonkar, R. and Mishra, R. N. (2011). Adaptogenic activity of Triphala megaExt. *Int. J. Res. in Pharmaceu. and Biomed. Sci.*, **2**(2): 570-574.

Yalcin, O., Bor-Kucukatay, M., Senturk, U. K. and Baskurt, O. K. (2000). Effects of swimming exercise on red blood cell rheology in trained and untrained rats. *J. Appl. Physiol*., **88**(6): 2074-2080.

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ARTICLE TITLE: Heavy metals and macro- and micro-minerals levels in body tissues and fluids of buffaloes

AUTHORS: Nigam, B.R., Yadav, R.K.S., Choudhury, S., Garg Satish K. and Saxena, A

The values of lead (Pb) reported in body tissues and fluids of buffaloes in the above mentioned MS may be read as one tenth ($1/10^{th}$) of the values reported. There was inadvertent mistake in the dilution factor.

Journal of Veterinary Pharmacology And Toxicology

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Abbreviations and terminology: Spelling, units of measurement, symbols and standard abbreviations should conform to those recommended by the International Union of Biochemistry (IUB) and the International Union of Biochemistry (IUB) and Applied Chemistry (IUPAC). Metric measurements are preferred and dosages should be expressed entirely r metric units.

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Laskowski, R.A., Hutchinson, E.G., Michie, A.D., Wallace, A.C., Jones, M.L. and Thornton, J.M. (1997). PDBsum: a Web-based database of summaries and analyses of all PDB structures. *Trends Biochem. Sci.* **22**:488-490.

Bywater, R.J. (1991). Tetracycline. In Veterinary Applied Pharmacology and Therapeutics. 5th eds. Brander, G.C.. Puqh, D.M., Bywater, R.J. and Jenkins, W.L. pp. 467-473. Bailliere Tindall, London

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