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CONTENTS

Research Articles

1. **HEAVY METALS AND MACRO AND MICRO-MINERALS LEVELS IN BODY TISSUES AND FLUIDS OF BUFFALOES** 87-90
   B. R. NIGAM, R.K.S. YADAV, S.CHOUDHURY, S.K. GARG AND A. SAXENA

2. **EVALUATION OF THERAPEUTIC MODULE FOLLOWING SUBACUTE TOXICITY OF JATROPHA CURCAS SEED IN GOATS** 91-95
   AMIT SHUKLA AND S.P. SINGH

3. **EFFICACY OF *WITHANIA SOMNIFERA* AND *SWERTIA CHIRATA* ON LEAD INDUCED IMMUNOTOXICITY IN RATS** 96-98
   ABHA GAJBHIYE, Y. P. SAHNI, VIDHI GAUTAM AND SACHIN KUMAR JAIN

4. **DETECTION OF RESIDUAL CONCENTRATION OF LASALOCID, AN ANTICOCCIDIAL DRUG IN CHICKEN MEAT** 99-101
   R.P. SINGH, Y.P. SAHNI, M.A. QUADRI AND K. SHRMAN

5. ***IN VIVO* GENOTOXICITY OF METRONIDAZOLE IN ICR MICE (*MUS MUSCULUS*)** 102-105
   M. P. POSHIYA, S.K. MODY, H.B. PATEL, D.T. VALANI, C.K. KAJAVADARA AND K.A. SADARIYA

6. **SAFETY OF LONG ACTING MOXIFLOXACIN FOLLOWING INTRAMUSCULAR ADMINISTRATION IN GOATS** 106-108
   KUMARI ANJANA, SHAILESH K. MODY, HITESH B.PATEL, R. D. SINGH, NAVIN S. RAJGOR AND VIPUL A. PATEL

7. **PHARMACOEPIDEMIOLOGY OF CLINICAL USAGE OF VETERINARY ANTIMICROBIALS IN NORTHERN GUJARAT** 109-111
   H. A. PATEL, S. K. MODY, H. B. PATEL AND R. D. SINGH

8. **BIOCHEMICAL ALTERATIONS INDUCED BY TEFLUTHRIN AND ALUMINUM IN RATS** 112-114
   ARSHAD HUSSAIN MIR, MUDASIR SULTANA, MUNEER AHMAD DAR, SAHRISH CHIRAG, RASHMI SAGAR, RAJINDER RAINA AND N K PANKAJ

9. **STUDIES ON HEMATOLOGICAL AND HISTOPATHOLOGICAL CHANGES FOLLOWING SUBACUTE EXPOSURE OF ACETAMIPRID IN MALE MICE** 115-117
   PREETI, S. K. JAIN AND GAURI A. CHANDRATRE

10. **STUDIES ON ACUTE TOXICITY AND NEUROBEHAVIOURAL PROFILES OF IMIDACLOPRID IN MICE** 118-123
   RAJEEV SHARMA, J. S. PUNIA AND S. K. JAIN

11. **PHARMACOKINETIC AND PLASMA PROTEIN BINDING ANALYSIS OF CEFTIOFUR AND ITS ACTIVE METABOLITE AFTER SUBCUTANEOUS ADMINISTRATION IN BUFFALO CALVES** 124-127
   DAUNDKAR PRASHANT SUDAMRAO AND SURESH KUMAR SHARMA

12. **EFFECT OF *MORINDA CITRIFOLIA* ON HEMATO-BIOCHEMICAL PROFILE ON DIMETHOATE TOXICITY IN RATS** 128-130
   SWATI UMAP, C. R. JANGDE, VIJEYTA TIWARI, AFROZ JAHAN, R.P. LIMSAY AND ALKA SAWARKAR

13. **HEMATO-BIOCHEMICAL AND HISTOPATHOLOGICAL EVALUATION FOLLOWING REPEATED ORAL ADMINISTRATION OF ATORVASTATIN IN HYPERLIPIDEMIC RATS** 131-135
   J. B. PATEL, K. A. SADARIYA, K. A. VIHOL AND A. M. THAKER

14. **IN-VITRO ANTIBACTERIAL ACTIVITY OF ALKALOIDS ISOLATED FROM CHLOROFORM EXTRACT OF *PROSOPIS JULIFLORA* LEAVES** 136-139
   U.D. PATEL, S.K. BHAVSAR, A.M. THAKER, K.S. DAMLE, I.H. KALYANI, D.J. GHODASARA, D.S. NAURIYAL AND D. SWARUP

CONTENTS

15. **COMPARISON OF PESTICIDE RESIDES VARIOUS DISTRICTS OF KUMAON REGION OF UTTARAKHAND** 140-142
WASIF AHMAD AND A.H. AHMAD

16. **EVALUATION OF DETOXIFIED JATROPHA CURCAS SEED CAKE AS FEED SUPPLEMENT ON IMMUNOLOGICAL PARAMETERS IN WHITE LEGHORN COCKERELS** 143-145
DEEPTI SINGH, S.P. SINGH AND DISHA PANT

17. **EVALUATION OF PROTECTIVE EFFECT OF ERYTHRINA VARIEGATA AND SPIRULINA PLATENSIS IN COCKERELS INTOXICATED WITH IMIDACLOPRID** 146-151
MAYANK GUPTA, S.P. SINGH AND N.K. PANKAJ

18. **MOLECULAR CHARACTERIZATION OF SALMONELLA (RARE SEROVARS) ISOLATED FROM GANGETIC WATER BASED ON ERIC-PCR** 152-155
BALWANT, AMIT TYAGI, S.P. SINGH, M.K. SAXENA AND D.P. MISHRA

19. **HAEMATOLOGY AND PLASMA BIOCHEMISTRY INFLUENCED BY ADMINISTRATION OF MARBOFLOXACIN IN SHEEP: SAFETY IMPACT** 156-160
H. B. PATEL, S. K. MODY, RATN DEEP SINGH, S. H. RAVAL AND H. A. PATEL

20. **ANTHELMINTIC ACTIVITY OF *CLERODENDRON INFORTUNATUM* LEAVES AGAINST COMMON G.I. NEMATODES IN CALVES** 161-164
M. KUMARI, R. PRASAD, B.K. ROY AND S. PANIGRAHI

21. **EFFECT OF PRETREATED TRIKATU ON THE PHRMACOKINETICS OF LEVOFLOXACIN IN CROSSBRED COW CALVES** 165-168
MONIKA AGRAWAL AND NITESH KUMAR

Short Communications

22. **EFFECT OF EMBLICA OFFICINALIS ON EGG CHOLESTEROL AND PRODUCTION PERFORMANCE TRAITS IN BIRDS** 169-170
SONAL PANDEY AND Y.P. SAHNI

23. **EFFECT OF KETOPROFEN ON PHARMACOKINETICS OF LEVOFLOXACIN IN BUFFALO CALVES** 171-172
SANTOSH KHUTALE, R.K. SHARMA, VARSHA SHARMA AND ARPITA SHRIVASTAV

HEAVY METALS AND MACRO AND MICRO-MINERALS LEVELS IN BODY TISSUES AND FLUIDS OF BUFFALOES

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ABSTRACT

Concentrations of toxic heavy metals (lead, arsenic and mercury) and major nutritional and trace elements (copper, iron, calcium, magnesium, zinc and selenium) were determined in acid-digested samples of blood, tissues and body fluids of buffaloes (*Bubalus bubalis)* (n=25) using atomic absorption spectrophotometer. Liver, kidneys, ovarian stroma, ovarian follicular fluid, ruminal ingesta and ovarian follicular fluids from non-pregnant and pregnant animals and amniotic and allantoic fluids contained varying concentrations of heavy metals and macro- and micro-minerals. Comparison of levels of toxic metals in blood and body tissues and fluids of buffaloes suggested that blood had high levels of arsenic, lead, mercury than the permissible levels and there was deficiency of zinc. Apart from blood, liver, kidneys, and other tissues and fluids can be considered as good bio-indicators of exposure to different metallic pollutants.

Key words: Buffaloes, biomonitoring, toxic heavy metals, blood, tissues, body fluids.

INTRODUCTION

The entry heavy metals and non-metals into food chain due to anthropogenic activities including contamination of soil and water is of great concern as their accumulation in vital organs such as kidneys, liver and bones results in serious health disorders such as mutagenicity, carcinogenicity, teratogenicity, immuno-suppression, impaired reproduction etc. (Lehmann *et al.* 2011). High concentrations of heavy metals have been reported to cause reproductive tract disorders in farm animals (Maraeek *et al.* 1998; Akan *et al.* 2010).

Higher levels of heavy metals have been reported in blood and milk of cows and/or buffaloes from certain urban areas in India around industrial units. Higher levels of metals and non-metals while depletion of sodium, potassium, chloride, copper, manganese and phosphorus have been reported in ruminal fluid of buffalo from region of Mathura (Garg *et al.* 1996). The present study was undertaken as meagre information is available on levels of heavy metals and major nutritional an trace elements in blood and body tissues and fluids of buffaloes in this region.

MATERIALS AND METHODS

Collection, digestion of blood and other biological samples

Blood samples were collected into heparined tubes from external jugular vein while liver, kidneys, ovaries, ovarian follicular fluid, allantoic fluid, amniotic fluid and ruminal contents in air tight polythene packs/in plastic vials without adding any preservative from buffaloes slaughtered in abattoir of Mathura and stored at -20C up till digestion as per the procedure described by Kolmer *et al.* (1951). Rumen liquor was filtered using Whatman filter paper No.42 for estimation of metal content. Filtered rumen liquor (500 ml), ovarian follicular fluid, and allantoic fluid and amniotic fluids were preserved with 2 ml nitric acid to prevent precipitation of metals. Both the samples were concentrated ten-fold on a water bath and subjected to nitric acid digestion. Ruminal ingesta (1.0 g) was taken into a 100 ml conical flask and digested using nitric acid and perchloric acid.

Estimation of metals and minerals

Estimation was different toxic metals and macro- and micro-minerals was done with using atomic absorption spectrometer (AAS 400; Perkin Elmer, USA) by using flame, furnace and mercury hydride techniques.

Statistical analysis of data

Data generated is presented as mean ± SE and appropriate statistical analysis of data was performed using SPSS10 version applying one way analysis of variance (Tukey B[a]), paired “t” test and unpaired “t” test as per requirements.

RESULTS AND DISCUSSION

Levels of toxic heavy metals (arsenic, lead and mercury) and macro- and micro-minerals (copper, iron, zinc, selenium, calcium and magnesium) in blood and some of the body tissues and body fluids of buffaloes slaughtered in Mathura abattoir are summarized in Tables 1 and 2, respectively.

Levels of arsenic, lead and mercury are summarized in Tables 1.Arsenic was detected in liver, kidneys, rumen ingesta, rumen liquor, ovarian stroma of pregnant and non-pregnant, ovarian follicular fluid of pregnant and non-pregnant animals), and allantoic and amniotic fluids of buffaloes. Data revealed highest arsenic levels in liver and kidneys and these were even more than in blood. Presence of high concentrations of arsenic in

Table 1:
Levels of heavy metals in different body fluids and tissues of buffaloes

Biological Sample(s)	Arsenic (ppb)	Lead (ppm)	Mercury (ppb)
Blood	228.11±11.30 (98.0 – 320.4)	11.20±0.17 (8.8 – 12.82)	160.87±10.40 (88.4 – 265.8)
Liver	288.62±13.40 (134.4 – 409.2)	10.01±0.15 (8.92 – 13.16)	277.62±23.90 (73.2 – 498.6)
Kidneys	287.89±11.61 (169.6 – 438.0)	12.34±0.24 (10.22 – 15.24)	226.75±26.66 (68.4 – 514.2)
Ruminal ingesta (solids after squeezing liquid)	184.01±5.12^{b} (123.0 – 231.0)	2.54±0.19^{b} (1.25 – 5.00)	69.82±1.28^{b} (45.0 – 85.25)
Ruminal liquor (after removal of solids)	74.42±2.67^{a} (47.36 – 98.56)	2.08±0.02^{a} (1.89 – 2.28)	22.29±0.51^{a} (15.64– 25.24)
Ovarian stroma from pregnant animals	255.98±14.21^{a} (176.8 – 360.8)	13.08±0.63^{a} (5.44– 16.20)	253.01±22.91^{b} (119.0– 404.2)
Ovarian stroma from non-pregnant animals	280.88±12.24^{a} (210.8 – 370.0)	12.20±0.67^{a} (6.68– 16.44)	171.75±16.59^{a} (75.2– 284.60)
Follicular fluid from ovary of pregnant animals ovaries	125.74±5.16^{a} (100.8 – 156.8)	11.84±0.30a (9.98 – 12.66)	94.20±7.49^{a} (54.9 – 123.6)
Follicular fluid from ovary of non-pregnant animals	110.80±5.37^{a} (66.6 – 128.8)	11.21±0.30^{a} (9.61 – 12.32)	65.61±9.12^{a} (27.0 – 128.5)
Allantoic fluid	37.58±0.97^{a} (31.18 – 43.84)	1.82±0.02^{b} (1.71 – 1.93)	20.91±1.99^{a} (12.28– 34.15)
Amniotic fluid	41.63±1.37^{b} (33.11 – 48.11)	1.29±0.10^{a} (0.68 – 1.80)	26.83±0.53^{b} (23.17–29.48)

Figures in parentheses indicate the range, Different superscripts in a column indicate statistically significant ($P<0.05$) differences between 4 and 5, 6 and 7, 8 and 9, and 10 and 11

Table 2:
Levels of macro- and micro-minerals in different body fluids and tissues of buffaloes

Biological Sample(s)	Copper (ppm)	Iron (ppm)	Calcium (ppm)	Magnesium (ppm)	Zinc (ppm)	Selenium (ppb)
Blood	1.19 ±0.03 (0.92 – 1.56)	540.50±18.10 (299.2–690.40)	101.78±3.41 (66.88–128.24)	24.38±1.32 (16.4 – 39.6)	0.43±0.01 (0.36 – 0.48)	243.83±29.18 (76.56–524.60)
Liver	29.63±4.12 (3.16 – 65.76)	179.66±6.87 (121.2 – 261.2)	110.14±1.77 (98.08 – 131.68)	44.78±2.66 (27.2 – 67.2)	3.65±0.09 (2.0 – 4.28)	228.67±17.14 (125.4 – 427.4)
Kidneys	4.43±0.19 (3.24 – 8.0)	75.88±2.51 (64.0 – 121.92)	119.68±2.39 (102.12 – 153.4)	50.43±3.49 (22.4 – 80.4)	2.45±0.06 (1.96 – 3.64)	609.76±43.94 (249.4 – 1046.2)
Ruminal ingesta (solids after squeezing liquid)	7.74±0.64^{b} (4.50 – 18.0)	460.50±39.63^{b} (159.0 – 856.0)	94.63±3.23^{b} (67.18 – 129.55)	78.15±4.31^{b} (39.0 – 129.0)	0.08±0.01^{a} (0.05 – 0.15)	105.87±4.80^{b} (57.75 – 157.0)
Ruminal liquor (after removal of solids)	0.35±0.01^{a} (0.24 – 0.53)	8.61±0.58^{a} (2.25 – 14.02)	31.18±2.23^{a} (10.14 – 52.38)	10.42±0.76^{a} (6.88 – 18.4)	0.12±0.00^{b} (0.08 – 0.16)	38.04±1.29^{a} (23.84 – 50.16)
Ovarian stroma from pregnant animals	3.09±0.15^{b} (1.96 – 4.2)	66.54±3.42^{a} (45.76 – 86.04)	114.56±4.78^{a} (81.52 – 154.72)	68.57±3.62^{a} (42.0 – 92.4)	1.84±0.09^{b} (1.0 – 2.48)	506.48±41.95^{a} (184.6 - 771)
Ovarian stroma from non-pregnant animals	2.39±0.11^{a} (1.92 – 3.28)	60.47±2.18^{a} (46.32 – 76.68)	126.64±1.79^{b} (110.48 – 138.44)	70.65±2.7^{a} (50.8 – 90.40)	1.61±0.04^{a} (1.24 – 1.92)	406.59±38.89^{a} (230 – 899.6)
Follicular fluid from ovary of pregnant animals ovaries	0.84±0.05^{b} (0.62 – 1.06)	15.91±0.44^{a} (14.2 – 17.9)	125.84±6.56^{a} (102.12 – 171.84)	22.04±3.07^{a} (10.4 – 42.0)	0.36±0.03^{a} (0.28 – 0.52)	323.59±34.78^{b} (110.7 – 464.5)
Follicular fluid from ovary of non-pregnant animals	0.65±0.02^{a} (0.5 – 0.74)	16.50±0.45^{a} (13.6 – 18.9)	111.10±2.92^{a} (91.24 – 124.32)	18.64±1.91^{a} (8.4 – 28.4)	0.35±0.02^{a} (0.28 – 0.48)	230.32±15.09^{a} (140.1 – 288.0)
Allantoic fluid	0.17±0.01^{b} (0.12 – 0.20)	6.55±0.18^{b} (5.47 – 7.23)	63.75±5.56^{b} (25.22 – 97.59)	15.54±1.04^{b} (10.36 – 20.93)	0.06±0.001^{b} (0.04 – 0.08)	130.87±8.73^{a} (83.78 – 178.28)
Amniotic fluid	0.11±0.01^{a} (0.09 – 0.15)	5.47±0.18^{a} (4.71 – 6.49)	49.10±2.08^{a} (38.19 – 58.14)	13.34±0.91^{a} (9.18 – 16.77)	0.04±0.001^{a} (0.03 – 0.05)	130.79±13.03^{a} (83.41 – 209.81)

Data presented are mean±SE of 25 animals, Figures in parentheses indicate the range, Different superscripts in a column indicate statistically significant ($P<0.05$) differences between 4 and 5, 6 and 7, 8 and 9, and 10 and 11.

rumen ingesta suggests the possibility of its entry into body of buffaloes either through fodder or drinking water. High arsenic levels in buffaloes indicate that it traverses across different body organs and systems. Although, Jones (2007) reported that arsenic does not appreciably bio-accumulate or bio-magnifies in food chain but detection of high levels in buffaloes suggest its tendency to bioaccumulate in buffaloes, and, therefore, possibility of toxicity in animals or human beings consuming meat and milk of such animals cannot be ruled out. Arsenic content in buffalo blood in the present study was much above the levels reported earlier in animals from Nadia District in West Bengal which is identified as arsenic-problem area of the state and country (Rana *et al.* 2009). Therefore, arsenic problem seems to be on rise and requires complete mapping in different agro-climatic zones of not only of UP

state but whole of the country in an endeavour to identify the hot-spots of such emerging dangers.

Blood lead level in buffaloes was much higher as reported by Wardrope and Graham (1982). Blood lead levels above 0.35 ppm have been reported to produce toxic effects and >1 ppm to result in death of animals (Radostits *et al.* 2000). In the present study, blood lead in buffaloes ranged from 7.53 to 14.91 ppm, but there were no apparent toxic symptoms in animals, therefore, either buffaloes are tolerant to lead or it produces subtle effects on reproduction and macro- and micro-minerals or enzymes status (Patra, 2006). Lead levels in liver (10.01±0.15 ppm) and kidneys (12.34±0.24 ppm) were almost comparable to those in blood and also ovarian stroma of pregnant and non-pregnant animals. However, rumen ingesta and rumen liquor and allantoic and amniotic fluids contained comparatively lower concentrations of lead, thus suggesting deposition of lead in liver, kidneys, ovarian stroma and other tissues along with bones (Gulson, 2003).

Maximum permissible concentration of mercury in food is 30ppb (Sanitary and Food norms 2.3.2.1078-01). Blood, liver, kidneys, ovarian stroma of pregnant and non-pregnant buffaloes revealed mercury levels of >100ppb. But levels in rumen ingesta, rumen liquor, follicular fluid of pregnant and non-pregnant buffaloes and allantoic fluid and amniotic fluid levels were comparatively lower. Mercury accumulates more in kidneys than in other tissues (Radostits *et al.* 2000) but our results suggest that apart from kidneys, hepatic and ovarian stroma can also be considered as excellent bio-indicator of mercury exposure.

Levels of macro- and micro-minerals-copper, iron, zinc, selenium, calcium and magnesium in blood and some of the body tissues and body fluids of buffaloes are summarized in Table 2. Selenium is an essential micronutrient having several biological functions (Brown and Arthur, 2001). Puls (1994) reported that 200.00 – 1200.00 ppb selenium is adequate and 10.00 – 14.00 ppm is considered to produce toxic effects. Compared to 120.00 ppb selenium in blood of cattle (Kommisrud *et al.* 2005), it was 243.83±29.18 ppb in blood of buffaloes. Liver, kidneys, rumen ingesta, ovarian stroma and follicular fluids of pregnant and non-pregnant, and allantoic and amniotic fluids revealed quite high levels of selenium. But rumen liquor had low levels. Data on selenium levels in different body tissues and fluids of buffaloes indicated that kidney had the highest tendency to accumulate selenium followed by ovarian stroma of pregnant and non-pregnant buffaloes, follicular fluid of pregnant and non-pregnant buffaloes, blood, allantoic and amniotic fluid. Presence of considerable amount of selenium in rumen ingesta indicates that fodder and/or water are the possible sources of entry of selenium into body of buffaloes.

Copper is an essential for reproductive function (Prasad *et al.* 1989). Blood copper in buffaloes (1.19±0.03 ppm) was almost comparable to that in cows. But much higher levels of copper were observed in liver, kidneys, rumen ingesta, ovarian stroma of pregnant and non-pregnant buffaloes. Ovarian follicular fluid of pregnant and non-pregnant buffaloes had almost comparable or slightly lower levels of copper than in blood with traces in allantoic and amniotic fluids. From these observations, it is apparent that copper maximally accumulates in liver as has been reported by several other workers.

Iron is an essential component of myoglobin, heme enzymes and some metallo-flavoprotein enzymes. Presence of varying concentrations of iron in blood, rumen ingesta, liver, kidneys, rumen liquor, ovarian stroma and follicular fluids of pregnant and non-pregnant buffaloes, and allantoic and amniotic fluids and especially in ruminal ingesta suggested entry of iron into body of animals primarily through ingestion of fodder having iron in or on fodder or drinking iron-contaminated water.

Calcium is a major extracellular divalent cation which promotes blood coagulation and involved in several physiological functions. Blood calcium levels in cattle ranged between 97 and 104 ppm (Radostits *et al.* 2000). In buffalo blood too, almost similar level was observed (101.78±3.41 ppm). Level of calcium in liver, kidneys, rumen ingesta, ovarian stroma and follicular fluid of pregnant and non-pregnant buffaloes were also almost comparable to blood levels while these were comparatively lower in rumen liquor (31.18±2.23 ppm), allantoic (63.75±5.56 ppm) and amniotic fluids (49.10±2.08 ppm); thus suggesting varied distribution of this macro-mineral in different body systems of buffaloes and blood seems to be the best bio-indicator of calcium status in body.

Magnesium is an active component of several enzyme systems, cardiac muscles, skeletal muscles and nerve tissues and it depends on the proper balance between calcium and magnesium ions. Normal magnesium level in cattle ranges between 18.00 and 26.00 ppm (Radostits *et al.* 2000). Almost similar values of magnesium were detected in blood of buffaloes (24.38±1.32 ppm). Compared to blood levels, liver, kidneys, rumen ingesta, ovarian stroma of pregnant and non-pregnant buffalo had much higher levels of magnesium. These observations suggested that magnesium accumulates in these tissues and these tissues/organs can be considered as bio-indicators of magnesium status in animals. However, rumen liquor, follicular fluid of pregnant and non-pregnant animals and allantoic and amniotic fluids contained comparatively lower levels of magnesium.

Zinc is functional component of several enzymes and necessary for synthesis of RNA and maturation of spermatozoa while its deficiency results in abnormal development of bones and cartilages, severe dermatitis and breaks in skin around hoof of animals (Swensson, 2000). Zinc level has been reported to be between 0.66

and 0.91 ppm in cows (Akar and Yildiz, 2005). Compared to cattle, buffaloes had much lower blood levels of zinc (0.43±0.01 ppm). On the contrary, liver, kidneys, ovarian stroma of pregnant and non-pregnant buffaloes had much higher levels while rumen ingesta, rumen liquor, follicular fluids of pregnant and non-pregnant buffaloes and allantoic and amniotic fluids contained only trace levels. Our results evidently indicate apparent zinc deficiency in buffaloes, which may be due to excess of copper and/or cadmium (Radostitis *et al.* 2000) or even iron which in turn might be responsible for production and reproduction losses in these animals.

It is concluded from our study that arsenic, lead and mercury levels in blood and different body tissues and fluids of buffaloes were above the permissible levels. But iron, selenium, calcium and magnesium were not significantly altered except for apparent deficiency of zinc. Liver, kidneys and other body tissues and fluids appeared be good bio-indicators of the levels of these toxic heavy metals and other minerals in buffaloes.

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Research Article

EVALUATION OF THERAPEUTIC MODULE FOLLOWING SUBACUTE TOXICITY OF *JATROPHA CURCAS* SEED IN GOATS

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ABSTRACT

The present study was designed to evaluate sub acute toxicity of *Jatropha curcas* seed and to develop its therapeutic module in goats. Nine healthy goats, weighing 28-32 kg and 16-18 months old, equally and randomly divided into three groups I, II and III. Group I served as control. Group II and III were given *Jatropha curcas* seed @ 1 seed / 4 kg bodyweight, po.for 28 days. Group II was also followed by the treatment with the therapeutic module comprising of sodium thiosulphate (50mg/kg body weight, IV) and glutathione (0.25 mg/kg body weight; IM) for 28 days. Mild to moderate diarrhoea, dullness, depression and letharginess were observed in all animals in without treatment group III, whereas, mild clinical signs were observed in few cases in treatment group II. A significant ($P<0.05$) decrease in Hb, PCV, TEC ,TLC, total serum protein, albumin and globulin values were observed in groups II and III in comparison to the control group I.Treatment group II showed amelioration in these parameters in comparison with without treatment group III indicating the therapeutic efficacy of the therapeutic module. A significant ($P<0.05$) increase in serum creatinine, serum urea, cholesterol, AST , ALT and ALP was observed in all groups in subacute study. Antioxidative parameters as lipid peroxidation (LPO) and reduced glutathione (GSH) increased in the without treatment group III in comparison to the control and group II. It is concluded from the above study that therapeutic module comprising of sodium thiosulphate (50mg/kg; IV) and glutathione (0.25 mg/kg; IM) revealed therapeutic value following subacute toxicity of *Jatropha curcas* seed @ 1 seed / 4 kg bodyweight, po. for 28 days in goats.

Key words: Curcin, goat, haematobiochemical, *Jatropha*, lipid peroxidation, phorbol esters, toxicity.

INTRODUCTION

Jatropha is a hardy and drought resistant crop which can be grown in marginal lands with minimum input. It has high tolerance to temperature but is highly susceptible to frost .It is quick growing and survives in poor soil and is drought resistant (Ishii *et al.,* 1987 and Munch *et al.,*1989). The oil com-prises 40% by weight of the seed making the *Jatropha* bean desirable for biodiesel production (Berchmans and Hirata 2008; Patil and Deng 2009). Now, *Jatropha* production is being scaled up as a bio fuel crop, however the seeds having sweet taste may be hazardous to the people, children in particular, engaged in its agriculture (Agarual and Saxena 2011). The seeds from *J. curcas* have been reported to produce toxicity due to the toxin curcin, a ricin like toxalbumin, characterized by burning and pain in mouth and throat, vomiting, delirium, decrease of visual capacity and increased pulse with a high mortality rate in rodents and domestic animals (Rai and Lakhanpal 2008 ; Singh *et al.*2010). Toxicity of *Jatropha curcas* seeds occurs due to presence of toxic principles including Phorbol esters, curcin, saponins, phytates, protease inhibitors and curcalonic acid. As the report on toxicity and treatment of *Jatropha curcas* seed in goats are lacking, the present study was undertaken to develop therapeutic module for the treatment following its toxicity in goats.

MATERIALS AND METHODS

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

Experimental design

Nine healthy goats of 16 to 18 months of age and 28-32 kg weight, were divided randomly and equally into three groups. Group I was kept as control, Group II and III were given seed with treatment (T) and without treatment (WT), respectively. The single oral dose for sub acute toxicity of the seed in group II and III was 1 seed /4 kg body weight. Therapeutic module comprised of sodium thiosulphate @ 50 mg/kg body weight, IV, and glutathione @ 0.25 mg/kg body weight, IM, during the 28 days study (Table-1). After 14th and 28th day interval the blood samples (4.0 ml) were collected for haematological examination and antioxidant parameters and separation of serum for biochemical parameters. Experiment was conducted after the permission of the IAEC and adequate measures were taken to minimize pain or discomfort to animals.

Table 1:
Experimental design for sub acute toxicity of *Jatropha curcas* seed and its therapeutic module in goats (n=3).

Groups	Treatment	(Dose)	Days of administration
I	Control	-	-
II	Seed (T)	1seed/4kg	28 days
	Treatment*	-	2-28 days
III	Seed (WT)	1seed/4kg	28 days

* Treatment comprised of sodium thiosulphate @ 50 mg/kg bodyweight IV route and glutathione @ 0.25 mg/kg bodyweight via IM route.

Hematology

Blood (1ml) was collected from each goat in clean heparinised microcentrifuge tube and haematological parameters such as packed cell volume, haemoglobin, total erythrocyte count and total leucocytes count were estimated (Jain, 1986) immediately after the collection of blood samples. 0.1N-HCl was used for estimating the blood haemoglobin concentration while, Hayem's RBC diluting fluid and Thomas's WBC diluting fluid were used for TEC and TLC estimation, respectively.

Blood biochemical and enzyme profile

The serum total proteins , albumin , globulin, total cholesterol , creatinine and urea were estimated by standard methods as in ERBA diagnostic kits.Serum enzymes viz. aspartate aminotransferase, alanine aminotransferase and alkaline phosphatise were estimated using standard methods as in ERBA diagnostic kits.

Antioxidative parameters

Lipid peroxidation product malondialdehyde (LPO) (Rehman, 1984) and reduced glutathione (GSH) (Prins and Loos, 1969) were determined in erythrocytes. Separation of erythrocyte pellet from blood samples was done immediately after collection of the blood sample. Absorbance of all the estimations was read on UV-VIS spectrophotometer.

Statistical analysis

Statistical analysis of data was done by using ANOVA for significant difference. Comparisons among treated and untreated groups were made with help of student't' test at 5%level of significance.

RESULTS

There was mild to moderate dirrhoea, letharginess, inappetance and dullness in case of the without treatment group III whereas the mild clinical signs were recorded in group II.

A significant (P<0.05) decrease in the values of haemoglobin, packed cell volume, total erythrocytes count and total leucocytes count as compare to the control group I. It was observed in the study that the values of the above mentioned parameters were higher in the group II than the group III. The decrease in the above parameters was recorded after 14 th day of exposure. The higher values of the above parameters in with treatment group II showed that the therapeutic module was ameliorating the adverse effect produced by the seeds of *Jatropha curcas.*(Table 2). There was a significant (P<0.05) decrease in the total protein, albumin and globulin values in the group II and III as compare to the group I. A significant (P<0.05) increase in the value of the total protein, albumin and globulin was observed in with treatment group II in comparison to the without treatment group III. A significant (P<0.05) increase in the values of urea, creatinine, cholesterol , AST, ALT and ALP were observed in the treated groups II and III with respect to the control group I after around 14 days exposure. The values were in the lower side in with treatment group II than the without treatment group III (Table 3 & 4).

The value of LPO was measured in the form of malondialdehyde (nM MDA/ml) in RBCs. A significant

Table 2 :
Effect on hematological parameters following daily oral administration of Jatropha seed with and without treatment for 28 days in goats (Mean value ± S.E. , n=3).

Parameters		Groups		
		Control (group I)	Seed with treatment (group II)	Seed without treatment (group III)
Hb (g %)	0 Day	7.70±0.189	7.76±0.087 ^{a}	7.56±0.057 ^{a}
	14 days	7.73±0.039^{A}	7.03±0.120$^{a\ A}$	6.40±0.251$^{a\ b\ AG}$
	28 days	7.63±0.021^{A}	6.33±0.176 ^{aA}	5.60±0.110 $^{a\ b\ AG}$
PCV (%)	0 Day	28.00±1.154	28.18±1.781 ^{a}	28.54±0.381 ^{a}
	14 days	27.33±1.452^{A}	23.81±0.997 ^{bB}	20.09±1.154 $^{a\ b\ ABG}$
	28 days	29.00±1.527 ^{A}	17.93±1.156 ^{abB}	16.18±0.303$^{a\ b\ AG}$
TEC (X 10^6/µl)	0 Day	11.93±0.24	11.98±0.006^{a}	12.05±0.344 ^{a}
	14 days	12.00±0.118^{A}	7.80±0.23 $^{a\ AB}$	8.46±0.349 $^{ab\ A}$
	28 days	11.93±0.133 ^{A}	6.133±0.478 $^{a\ A}$	6.20±0.978 $^{ab\ A}$
TLC (X 10^3/µl)	0 Day	9.53±0.176	9.51±0.057	9.54±0.130^{a}
	14 days	9.50±0.090^{A}	8.73±0.240^{B}	7.66±0.569 ^{AB}
	28 days	9.52±0.018 ^{A}	8.40±0.305 ^{AB}	6.70±0.550 $^{a\ AB}$

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

Table 3:
Effect on biochemical parameters following daily oral administration of Jatropha seed with and without treatment for 28 days in goats (Mean value ± S.E. , n=3).

Parameters		Groups		
		Control (group I)	Seed with treatment (group II)	Seed without treatment (group III)
Total protein(g/dl)	0 Day	7.49±0.043	7.48±0.063^{a}	7.47±0.056^{a}
	14 days	7.49±0.024^{A}	3.92±0.005^{abAB}	8.40±0.005$^{ab\,AB}$
	28 days	7.45±0.003^{A}	4.52±0.023^{abAB}	4.23±0.012^{abAB}
Albumin(g/dl)	0 Day	2.96±0.005	2.94±0.029^{a}	2.93±0.034^{a}
	14 days	2.93±0.005^{A}	3.00±0.002^{abAB}	3.42±0.001^{abAB}
	28 days	2.91±0.040^{A}	2.03±0.000^{abAB}	1.81±0.00^{abAB}
Globulin(g/dl)	0 Day	4.64±0.017	4.61±0.002^{a}	4.66±0.057^{a}
	14 days	4.65±0.163^{A}	0.924±0.00$^{a\,bAB}$	4.98±0.00^{abAB}
	28 days	4.63±0.008^{A}	2.98±0.00$^{a\,bAB}$	2.41±0.00$^{ab\,AB}$
Urea (mg/dl)	0 Day	21.44±0.15	21.24±0.37^{a}	21.13±1.898^{a}
	14 days	21.32±0.008	31.73±0.42$^{ab\,AB}$	28.33±2.416$^{ab\,AB}$
	28 days	21.29±0.12	45.33±0.001^{abAB}	48.33±1.421^{abAB}
Creatinine (mg/dl)	0 Day	0.78±0.003	0.76±0.011^{a}	0.776±0.008^{a}
	14 days	0.77±0.010^{A}	1.51±0.001$^{ab\,AB}$	2.42±0.001^{abAB}
	28 days	0.75±0.020^{A}	1.81±1.005^{abAB}	3.21±0.009$^{ab\,AB}$
Cholesterol(mg/dl)	0 Day	58.67±0.019	58.44±0.115^{a}	58.65±0.065^{a}
	14 days	58.41±0.207^{A}	51.33±0.012^{bAB}	77.65±0.017^{abAB}
	28 days	58.66±0.356^{A}	68.37±0.122^{abAB}	96.78±0.110^{abAB}

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

Table 4:
Effect on serum enzymic and antioxidant activities following daily oral administration of *Jatropha* seed with and without treatment for 28 days in goats (Mean value ± S.E. , n=3).

Parameters		Groups		
		Control (group I)	Seed with treatment (group II)	Seed without treatment (group III)
AST (U/L)	0 Day	25.25±0.996	25.24±0.371^{a}	25.17±0.699^{a}
	14 days	24.80±0.012^{A}	38.55±0.008$^{a\,bAB}$	44.16±0.012$^{a\,bAB}$
	28 days	24.92±0.011^{A}	50.55±0.015^{abAB}	66.64±0.014^{abAB}
ALT(U/L)	0 Day	16.25±0.044	16.30±0.198^{a}	16.11±0.480^{a}
	14 days	16.44±0.220^{A}	25.80±0.014$^{a\,b\,AB}$	30.97±0.060$^{ab\,AB}$
	28 days	16.33±0.005^{A}	30.33±0.015^{a,bAB}	41.26±0.008^{abAB}
ALP (U/L)	0 Day	67.54±0.012	67.15±0.234^{a}	67.62±0.005^{a}
	14 days	67.18±0.005^{A}	71.30±0.342^{abAB}	65.94±0.08^{abAB}
	28 days	67.45±0.105^{A}	78.96±0.086^{a,bAB}	81.54±0.076^{abAB}
LPO (nM MDA/ml)	0 Day	19.66±0.025	19.48±0.243^{a}	19.62±0.155^{a}
	14 days	19.98±0.601^{A}	33.96±0.354$^{a\,bAB}$	28.80±0.095$^{a\,b\,AB}$
	28 days	19.18±0.596^{A}	43.46±0.371^{abAB}	41.70±1.041$^{a\,b\,A}$
GSH((µM/ml))	0 Day	2.65±0.006	2.69±0.008^{a}	2.63 ±0.011^{a}
	14 days	2.65±0.00^{A}	2.95±0.30^{aAB}	3.01±0.034$^{a\,A}$
	28 days	2.64±0.017^{A}	2.92±0.028^{aAB}	3.07±0.031$^{a\,AB}$

*Treatment with sodium thiosulphate (50 mg/kg bodyweight IV route) and glutathione (0.25 mg/kg bodyweight IM route)
*Mean values bearing common superscripts with small letters differ significantly (P< 0.05) when compared vertically with in the same column and mean values bearing common superscripts with capital alphabets differ significantly (P<0.05) when compared horizontally in the same row.

(P<0.05) increase in the MDA value in groups II and III was observed in comparison to group I on 28th day interval. The value of GSH was measured in the form of µM/ml in RBCs. A significant (P<0.05) increase in the GSH level was found in groups II and III, as compared to control. There was a significant (P<0.05) decrease in GSH value in seed with treatment group II in comparison to the seed without treatment groups III on both 14th and 28th day interval (Table 4).

DISCUSSION

The appearance of the clinical signs in seed intoxicated goats in sub acute study might be due to the higher concentration of active principles as phorbol esters and diterpenes in *Jatropha curcas* seed. Seed and seed oil contains phorbol esters known for its purgative effect (Gandhi *et al.,*1995) that occurs due to its stimulating effect on kinase-C enhancing intracellular signal transduction process (Makkar and Becker 1997). *Jatropha* seed also contain

various other toxic principles i.e. curcin, tannin and sterol (Lin *et al.,* 2010). Curcin is known to cause direct GIT irritation and cellular toxicity (Lin *et al.* 2003) which might be responsible for toxicity manifestation such as hepatotoxicity and nephrotoxicity in goats in this study. Phorbol esters are among the main toxins in *Jatropha curcas* seed (Lin *et al.,* 2010). Low level of clinical manifestation in groups treated with glutathione reveals the therapeutic potential of the therapeutic module. Glutathione plays a vital role in neutralizing the oxidative radical and remove the electrophiles and, thus diminishes their toxic effect on the cells.

Haematological parameters such as Hb, PCV, TEC and TLC decreased in seed intoxicated groups in sub acute toxicity. Decrease in Hb, PCV, TEC and TLC might be due to haemolytic activity of curcin. The anaemia might have been occurred due to the haemolytic action of curcin as evidenced in this study. Furthermore, damage to the GIT could also have resulted in maldigestion and malabsorption of nutrients required for erythropoiesis (Chivandi *et al.*,2006). Leucopoenia might be correlated with the stress caused by anti nutritional factors as tannins, saponins, phytates etc. present in the *Jatropha* meal (Feldman *et al.*, 2000). Alteration in protein profile indicates hepatorenal dysfunction and gastroenteritis which might have resulted due to presence of curcin and phytates in *Jatropha* seed and seed oil. Similar findings were reported following *Jatropha* intoxication in rats at the dose rate of 25% *Jatropha* seed protein on 7th, 14th and 22nd day (Awasthy *et al.*, 2011) and in rabbits receiving 5%, 7.5% and 10% *Jatropha* seed meal for 6 weeks (Abdel-safy *et al.,* 2011). There was an increase in urea and serum creatinine level in seed intoxicated groups indicating the nephrotoxic potential of *Jatropha* as urea and creatinine act as indicators of the kidney damage. Similar results were reported in goats following oral administration of *Jatropha* seed at the dose of 1 and 0.25g/kg/day (Gadir *et al.*, 2003) and in rats at the dose rate of following 25% and 50% levels seed protein for 21 days (Awasthy *et al.*2011) .A significant increase in serum cholesterol level was observed in seed intoxicated groups in sub acute toxicity study. Liver is the major site of cholesterol synthesis and metabolism. Hepatic cholesterol homeostasis is maintained by equilibrium between the activities of the hydroxyl methyl glutryl Coenzyme A reductase and acyl co enzyme A cholesterol acyl transferase. The rise in activity of serum AST, ALT and ALP enzymes has been attributed to the damaged structural and cellular integrity of the hepatocytes and there might be the likelihood of the centrilobular necrosis which in turn increases the leakage of the liver specific enzymes. Being cytoplasmic in location, these enzymes are released into systemic blood circulation after cellular damage of hepatocytes.

Oxidative stress is characterized by increased lipid peroxidation (LPO) and or altered non enzymatic and enzymatic antioxidant systems. LPO of membranes is regulated by the availability of substrate in the form of polyunsaturated fatty acids (PUFA), the availability of inducers such as free radicals and the excited state molecules to initiate propagation , the antioxidant defence status of environment and physical status of membrane lipids (Pipicelli *et al.*, 2005). The present study showed that exposure to toxicity stress resulted in increase in LPO levels in erythrocytes as evidenced by the increased production of malondialdehyde (MDA). Curcin, also an toxalbumin like ricin may induce DNA damage, oxidative stress and thyroid toxicity with necrosis in rats (Kumar *et al.*, 2007). The phorbol esters, present in both seed have been reported to interfere with the activity of protein kinase C that affects a number of processes including phospholipids and protein synthesis , enzyme activities, DNA synthesis, phosphorylation of proteins, cell differentiation and gene expression (Makkar and Becker, 2009) . There was significant reduction in the GSH value in seed with treatment group in comparison with the seed without treatment group on 14th and 28th day interval .The exogenously given glutathione in the therapeutic module itself served as an oxidizing agent which might have reduced the free radical formation and in turn decreased the endogenous generation of the GSH. It indicated that the glutathione provided a better relief in terms of oxidative stress to the goats.

It is concluded from the above study that the clinical manifestations of the sub acute toxicity appeared after 14th post exposure in seed without treatment groups. The therapeutic module comprising of sodium thiosulphate (50 mg/kg, IV) and glutathione (0.25 mg/kg, IM)) revealed therapeutic value following subacute toxicity of *Jatropha curcas* seed @ 1 seed / 4 kg bodyweight, po. for 28 days in goats.

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EFFICACY OF *WITHANIA SOMNIFERA* AND *SWERTIA CHIRATA* ON LEAD INDUCED IMMUNOTOXICITY IN RATS

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ABSTRACT

Indigenous medicinal plants; *Withania somnifera* and *swertia chirata* were administered at the dose rate of 500mg per kg body weight to determine their efficacy on lead-induced immunotoxicity. Lead acetate reduced the levels of serum gamma globulin; however serum gamma globulin concentration increased significantly with *S. chirata* followed by *W. somnifera*. Similarly, *S. chirata* and *W. somnifera* also reduced neutrophil adhesions count significantly which indicated the protective effect of indigenous plants. Oral administration of *S. chirata* for 30 consecutive days counteracted the immunosuppression of lead acetate on absolute lymphocyte count and produced a significant increase in absolute lymphocyte count. However *W. somnifera* did not show any significant change on absolute lymphocyte count in albino rats.

Key words: Immunotoxicity, Immunosuppression, Lead acetate

INTRODUCTION

Lead is one of the ubiquitous pollutant which have been associated primarily with growth of industry and the expanding use of motor vehicle in transport. Immunosuppressive effect of lead was reported as early as 1966, when it was noted that lead increased the sensitivity of rats to bacterial endotoxins. Since then a substantial literature has demonstrated adverse effect of lead on the immune system in a variety of laboratory animals. *Withania somnifera* commonly known as 'Ashwagandha' has been reported to posses anti-inflammatory, antitumor and immunomodulatory activity (Krishan *et al.*, 2000). *Swertia chirata* is a medicinal plant of the family Gentinaceae and it has been used as a bitter tonic in treatment of fever and for curing various skin diseases (Joshi and Dhawan, 2005).

MATERIAL AND METHODS

The study was conducted on 36 adult male albino rats weighing between 100-150 gm. The indigenous medicinal plants; *Withania somnifera* (root powder) and *Swertia chirata* (whole plant) were procured from the Dept. of Aromatic and Medicinal Plant, College of Agriculture, JNKVV, Jabalpur. Lead was used in the form of Lead acetate trihydrate (Certified ACS) supplied by M/s Fisher Scientific, Mumbai.

Assaying of humoral immune response in rats

Preparation of goat RBC for immunization

Goat blood was collected in Alsever's solution in the proportion of 1:1 of Alsever's solution for each ml of blood collected. It is an anticoagulant used during collection of blood for preparation of RBC.

The samples were autoclaved at 10 lbs pressure for 10 min. and then stored at 4^0C in refrigerator. Erythrocyte were washed three times in large volume pyrogen free 0.89% normal saline at 1500 rpm for 10 min. and adjusted to a concentration for immunization and challenge and stored at 4^0C for further use.

Experimental design

Group	Treatment
I	Lead acetate (7.5 mg/rat/day) orally for 30 consecutive days
II	Lead acetate (7.5 mg/ rat/ day) + *W. somnifera* (500mg/kg),orally for 30 consecutive days
III	Lead acetate (7.5 mg/rat/day) + *S. chirata* (500mg/kg), orally for 30 consecutive days
IV	Lead acetate (15 mg/rat/day) orally for 30 consecutive days
V	Lead acetate (15mg/rat/ days) + *W. somnifera* (500mg/kg), orally for 30 consecutive days
VI	Lead acetate (15mg/rat/day) + *S. chirata* (500mg/kg), orally for 30 consecutive days

Neutrophil adhesion test

On the 14 day of drug treatment, blood samples were collected (before challenge) by puncturing retro-orbital plexus into heparinized vials and analysed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smear and staining with field stain - I and II - Leishman's stain. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 15 min. at 37^0C. The incubated blood samples were again analysed for TLC and DLC. The product of TLC and percent neutrophil gives neutrophil index (NI) of blood sample.

Percent neutrophil adhesion was calculated as shown below.

$$\text{Neutrophil adhesion (\%)} = \frac{NI_u - NI_t}{NI_u} \times 100$$

Where, NI_u = Neutrophil index of untreated blood sample

NI_t = Neutrophil index of treated blood sample

Estimation of γ-globulin

0.3 ml of serum was added to a test tube containing 5.7 ml of ammonium sulfate – sodium chloride solution. After mixing it was kept on ice bath for 15 min, then centrifuged at 3000 rpm for 10 min and the supernatant was discarded. The process was repeated twice and finally the content was dissolved in 2 ml of normal saline. 5 ml of biuret reagent was added and kept for 10 min at room temperature. Blank was made with 2.0 ml of 0.15% bovine serum albumin and 5.0 ml biuret reagent. The absorbance or transmittance of standard and test was read against blank set at zero at 555 nm wavelength and gamma globulin was calculated by the following equation.

$$\text{Serum gamma globulin (Gram/100ml)} = \frac{\text{OD of test}}{\text{OD of standard}}$$

Absolute lymphocyte count

Absolute lymphocyte count was calculated by using the data of differential and total leukocyte counts. The following formula was used.

$$\text{Absolute lymphocyte count (per cumm of blood)} = \frac{\text{\% lymphocyte x total leukocyte count}}{100}$$

Statistical analysis

The data was analyzed and tested statistically for significant difference by analysis of variance (ANOVA) using complete randomized design (Snedecor and Cochran, 1994).

RESULTS

Parameters on immunomudulatery activity of *W. somnifera* and *Swertia chirata* following subchronic lead administration in rats have been presented in Table 1. Neutrophil adhesion count was reduced significantly from 23.61 percent to 15.9 percent and 17.44 percent on treatment with lead acetate (7.5 mg/rat) and *W. somnifera* (500mg/kg); and lead acetate (7.5 mg/rat) and *S. chirata* (500mg/kg), respectively, in comparision to neutrophils adhesion count as 23.61 percent with lead acetate alone. Neutrophil adhesion count was also reduced significantly from 26.88 percent to 19.02 and 22.50 percent with lead acetate (15 mg/rat) and *W. somnifera* (500 mg/kg) and lead acetate (15 mg/rat) and *S. chirata* (500 mg/kg), respectively, as compared to lead acetate alone.

The increased level of serum gamma globulin were recorded as 0.78 g/dl and 2.89 g/dl in groups of lead acetate (7.5 mg/rat) and *W. somnifera* (500 mg/kg); and lead aceate (7.5 mg/rat) and *S. chirata* (500 mg/kg), respectively. However, lead acetate (15 mg/rat) with *W. somnifera* (500 mg/kg) increased serum gamma globulin level to 1.76 g/dl from 0.74 g/dl which was found to be significant. Similarly, lead acetate (15 mg/rat) with *S. chirata* (500 mg/kg) caused significant increase in level of serum gamma globulin from 0.74 g/dl to 2.59 g/dl which was to the extent of maximum increase level of gamma globulin.

The mean values of absolute lymphocyte count was recorded as 6.65 and 6.3 per cumm with the doses of 7.5 and 15 mg/rat of lead acetate. The values of absolute lymphocyte count were reduced significant to the extent of 4.37 and 3.97 per cumm with the daily doses of 7.5 and 15 mg/rat of lead acetate administered for 30 consecutive days. The simultaneous administration of lead acetate (7.5 mg/rat) and *W. somnifera* (500 mg/kg) caused an elevation in absolute lymphocyte count from 4.12 to 5.14 per cumm. Similarly the concurrent administration of lead acetate (15 mg/rat) and *S. chirata* (500 mg/kg) also increased the count of absolute lymphocyte from 4.68 to 6.39 per cumm. which was calculated to be significant.

DISCUSSION

Lead is a multiple source pollutant causing a great risk for environment and health system of human and animal body. The main target sites for lead toxicosis are the haemopoetic, renal and immune systems (Durgut *et al.* 2008). Immunosuppressive effect of lead has been reported and basic toxicological immunological effects of lead were reported by Koller (1981) and Sharma (1981). On other hand, natural products of plant origin have been claimed to possess immunomodulatory activity by causing stimulation of both specific and non specific immune responses. Many plants of traditional medicine have been used for immunomodulatory properties due to stimulation of humoral and cell mediated immunity.

Indigenous medicinal plants *W. somnifera* and

Table 1:
Immunomodulatory activity of *Withania somnifera* and *Swertia chirata* on subchronic lead administration in albino rats.

Groups	Serum Gamma Globulin (g/dl)	Neutrophil Adhesion Count (%)	Absolute Lymphocyte count (per cumm)			SEM	CD at P<0.05
			Day 0	Day 15	Day 30		
I	1.77[a]	23.61[a]	6.65[a]	6.33[b]	4.37[b]	0.48	1.46
II	2.78[b]	15.9[b]	4.12	4.33	5.14	0.37	1.14
III	2.89[b]	17.44[b]	5.21	5.15	5.26	0.22	0.68
IV	0.74[a]	26.88[a]	6.83[a]	5.29[ab]	3.97[b]	0.60	1.86
V	1.76[b]	19.02[b]	5.28	5.16	5.27	0.30	0.93
VI	2.59[c]	22.50[b]	4.68[a]	6.30[b]	6.39[b]	0.29	0.88

Values are mean of six observations. The mean values with different alphabets as superscript in a column differ significantly from each other, SEM: Standard Error Mean; CD: Critical Difference; NS: Non Significant

S. chirata have been claimed to possess immunomodulatory property, however their scientific validation as immunostimulant against the lead induced immunosuppression is lacking in literature or elsewhere. The study undertaken by Dhote *et al.* (2005) investigated immunomodulatory efficacy of a herbal preparation: "Immuplus" that contains various herbs including *W. somnifera* . They further recorded an increase in absolute lymphocyte count with *W. somnifera* which substantiates our findings whereas *W. somnifera* exhibited a significant increase in absolute lymphocyte count. The values of Absolute lymphocyte count were found significant on day 16th of the experiment as reported by Dhote *et al.* (2005) whereas in the present study an elevated absolute lymphocyte count was observed on day 30 of the experiment. The difference of day schedule for absolute lymphocyte count in both the studies may due to variation in the doses of *W. somnifera.* An investigation carried out by Kuttan (1996) also showed immunostimulant activity of *W. somnifera* against immunosuppression caused by radiation therapy, suggesting *W. somnifera* to exhibit immunostimulant property. The aforesaid reports of various researchers are in close conformity to the findings of present study.

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Research Article

DETECTION OF RESIDUAL CONCENTRATION OF LASALOCID, AN ANTICOCCIDIAL DRUG IN CHICKEN MEAT

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ABSTRACT

In the present study, surveillance regarding use of anticoccidials at poultry farms located in and around district Jabalpur was conducted. The data on use of various anticoccidials study was extended shortlisted and found that lasalocid is one of the most commonly used anticoccidials and therefore to detect lasalocid residues in chicken meat .Total number of 180 chicken meat samples were analyzed including 36 samples each from five area of Jabalpur. Out of the 180 samples, 27 samples were found positive for residual concentration of lasalocid in chicken meat. Six samples (22.22 per cent) were found lasalocid residue at violative levels while 21 samples (77.77%) were found below maximum residue limits recommended by Commission Regulation of European Union for lasalocid in chicken muscle, liver and kidney samples. The study was further extended to determine the effect of boiling on residual concentration of lasalocid in chicken meat samples positive for drug residue. Mean residual concentration of positive samples was detected on pre and post boiling.

Key words: Lasalocid, anticoccidial, chicken, residue analysis

INTRODUCTION

Coccidiosis is a continuing problem in the intensive chicken industry and chicken meat infected with coccidia (*Eimeria* or *Isospora spp.*) causes high morbidity, reduced weight gain and less frequently and destruction of the intestinal epithelium which may lead to mortality (Dowling, 1992). Coccidiostats are considered feed additives for poultry and must meet a number of legislative criteria of Regulation 1831/2003/EC. Various antibiotics, sulfonamides and coccidiostats are usually administered in feed or drinking water for prevention and treatment of various infectious diseases in poultry birds. They enhance feed efficiency, promote growth and improve productivity (Gaudin *et al.*, 2004; Bergwett, 2005). Anticoccidials are used as feed additives in poultry for prophylactic control of coccidiosis. Ionophore antibiotics (polyether compounds) are commonly used for treatment and prophylaxis of coccidiosis.

The uncontrolled and unlimited use of anticoccidial drugs may, however, lead to the accumulation of undesirable residues in the animals treated and their products. These residues may have adverse effects on both the animals and human beings (McCracken *et al.*, 2005). Keeping in view, the present investigation was carried out to know the status of lasalocid anticoccidial residues in chicken meat samples.

MATERIALS AND METHODS

Sample collection

Information was gathered by conducting a surveillance study pertaining to use of anticoccidials in various poultry farms located in and around Jabalpur district. On the basis of surveillance study, lasalocid was short listed for determination of residual concentration in a total of 180 broiler meat samples including 36 each of muscle (12), liver (12) and kidney (12) from five target areas as Jabalpur.

Chemical and standards

The chemicals and standards used for extraction, detection and quantification of residual concentration of laslocid, were Ethyle acetate (Sigma – Aldrich), Hexane (HiMedia), Methanol (Sigma – Aldrich), Sulphuric acid (Human Diagnostic & Surgichem), Water (Sigma - Aldrich) and Standard Lasalocid A sodium salt (Fluka- analytical).

Anticoccidial extraction

Five gram of muscle/liver/kidney of chicken meat was extracted with 20 ml methanol, mixed for 30 min. and centrifuged at 3000 rpm for 10 min. Extraction was repeated twice each time. The supernatants were evaporated to dryness in a rotary evaporator. 100µL methanol was added and used directly for HPTLC analysis.

Detection of lasalocid

The silica gel 60 F_{254} TLC plates (Merck, Germany) was activated at 120°C for 2 hr in hot air oven before use. Standard was prepared by weighing lasalocid 10 mg in 100 ml acetonitrile and diluted to the mark with methanol to obtain a standard stock solution of lasalocid (0.1 mg/ml) (Thangadu *et al.,* 2002). The standard (2-10µl) and samples were spotted on TLC plates in the form of narrow bands of length 8 mm with 8 mm distance from bottom and 15 mm from left margin and the solutions were sprayed as 6×0.45 mm bands. Silica plates were developed using mobile phase consisting of ethyl acetate/hexane 8/3 (v/v). Linear ascending development was carried out in 20 X 10 cm twin trough chamber equilibrated with mobile phase. The optimized chamber saturation time for mobile phase

was 20 min. After development, the TLC plates were dried completely. Visualization of lasalocid was done with vanillin spray (vanillin 2% in methanol and sulfuric acid 99.5/0.5). The plates were heated at 90 ^{0}C for 5–15 min. Lasalocid was appeared as a thick yellowish band. Densitometric scanning was performed on camag TLC scanner 4 in absorbance mode and operated by winCATS planar chromatography version 1.3.4. at 512 nm wave length. The slit dimension used in the analysis was length and width of 6 mm and 0.45 mm, respectively, with a scanning rate of 20 mm/sec.

RESULTS

The surveillance data on the use of anticoccidial was obtained by questionnaire from the poultry farmers (n=50). The results showed that supplementation of anticoccidials mainly in feed was 64 percent (n=32). Fifty six percent (n=28) poultry farmers did not consult the veterinarian for addition of anticoccidials and antibiotics in the feed. Eighty six percent poultry farmers (n=43) were unaware of rules and regulations for the use of anticoccidials and antibiotics and their residue in relation to public health.

The analysis of lasalocid by HPTLC method was developed by using the solvent system comprising of ethyl acetate/hexane 8/3 (v/v). This mobile phase exhibited good separation of lasalocid from its matrix with a mean Retardation Factor (R_F value) of 0.79 with max absorbance of 512 nm. The percent recovery was consistently around 80-90% for lasalocid in meat samples.

Total number of 180 chicken meat samples were analyzed including 36 samples each from five target areas of Jabalpur. Among the 180 samples, 27 samples (15.0 per cent) were found positive for lasalocid residues. Out of 60 muscle samples tested, 7 samples (11.67 per cent) were found positive for lasalocid residue. While, out of 60 liver samples tested, 10 samples (16.67 per cent) were found positive. Out of 60 kidney samples tested, 10 samples (16.67 per cent) were found positive (Table 1). Out of the 27 positive samples, 6 samples (22.22 per cent) were found with lasalocid residue at violative levels while 21 samples (77.77 per cent) were found below maximum residue limits recommended by Commission Regulation of European Union (2007) for lasalocid in chicken muscle, liver and kidney samples.

The mean residue concentrations of lasalocid in muscle, liver and kidney samples collected from Jabalpur-1 target area were found below detectable limit. The mean residue concentrations of lasalocid in muscle, liver and kidney samples collected from Jabalpur-2 target area were 15.4±0.70, 89.5±12.89 and 36.7±7.10 μg/kg, respectively. The samples collected from Jabalpur-3 target area contained 12.0±4.19, 68.7±13.70 and 46.7±1.50 μg/kg lasalocid in muscle, liver and kidney samples, respectively. Mean residue concentration of lasalocid in muscle, liver and kidney samples of Jabalpur-4 target area were 25.6±0.0, 98.6±9.38 and 50.8±6.82 μg/kg, respectively. Mean lasalocid residues concentration in the muscle, liver and kidney samples from Jabalpur-5 target area were 13.4±2.20, 93.87±17.16 and 44.0±1.79 μg/kg, respectively.

Table 1:
Mean residue concentration of lasalocid (μg/kg) in chicken muscle, liver and kidney samples of different target areas.

Target area	Mean residual concentration(μg/kg)		
	Muscle	Liver	Kidney
Jabalpur-1	Nil	Nil	Nil
Jabalpur-2	15.4±0.70	89.5±12.89	36.7±7.10
Jabalpur-3	12.0±4.19,	68.7±13.70	46.7±1.50
Jabalpur-4	25.6±0.0	98.6±9.38	50.8±6.82
Jabalpur-5	13.4±2.20,	93.87±17.16	44.0±1.79

Maximum residual limit of lasalocid in muscle, liver and kidney of chicken (Regulation of European Union), 20μg/kg=20ppb, 100μg/kg=100ppb and 50μg/kg=50ppb respectively

Effect of boiling on lasalocid residues

Mean residual concentration of lasalocid out of 27 positive samples was 15.31±2.07 µg/kg for muscle, 89.38±6.81 µg/kg for liver and 45.14±2.69 µg/kg for kidney samples. After boiling the same samples, the mean

Table 2:
Effect of boiling on lasalocid residual concentration in muscle, liver and kidney samples of chicken meat.

Tissue sample	Mean residual concentration (µg/kg)		Percentage reduction of residual concentration
	Raw samples	Boiled samples	
Muscle	15.31±2.07	13.12±1.89	14.32
Liver	89.38±6.81	68.89±4.42	22.92
Kidney	45.14±2.69	36.89±2.09	18.27

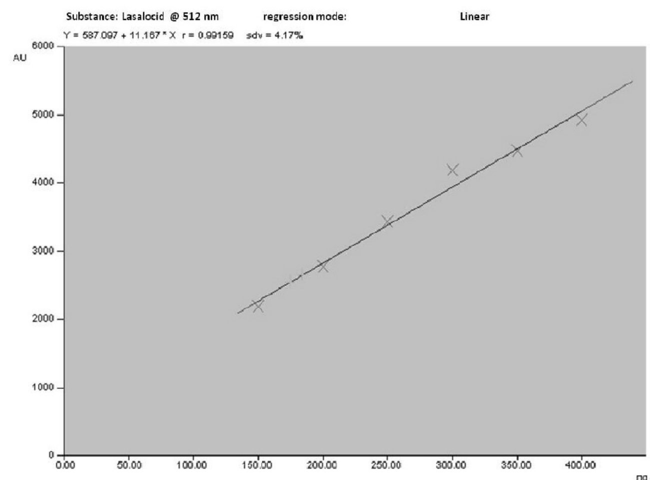

Fig. 1:
Linear relationship for lasalocid in spiked chicken meat samples.

residual concentration reduced to 13.12±1.89, 68.89±4.42 and 36.89±2.09 μg/kg for boiled muscle, liver and kidney samples respectively, showing a percentage reduction of 14.32, 22.92 and 18.27 percent respectively (Table 2).

DISCUSSION

On the basis of surveillance study the result showed that most of poultry farmers were unaware about the potential hazard associated with anticoccidial residues in foods of meat origin.

Out of 180 samples, 27 samples had detectable level of lasalocid residue by HPTLC analysis in chicken meat in which liver samples had more concentration than kidney and muscle samples found positive for lasalocid residues. This study revealed that out of 180 samples, 27 samples had detectable level of lasalocid residue, however among 27 positive samples, 6 samples had residue above than MRLs. Lasalocid residue concentration was higher than the study conducted by Rokka and Peltonen (2006). However, Danaher *et al.* (2008) confirmed that eggs and poultry were still commonly contaminated with coccidiostat residues

As depicted in Fig. 1, linearity was excellent for spiked chicken meat samples (r2>0.99) with standard deviation near to 4.0 %. The percent recovery was consistently around 85% (80-90%) in meat samples. These values are quite good and satisfactory as compare to other studies (Hormazabal and Ostensvik., 2005; Rokka and Petonen, 2006). In fact our method was one of the few quantitative techniques for the determination of ionophore antibiotics in chicken meat samples.

On boiling positive samples revealed that the mean residual concentration were reduced to 14.32, 22.92 and 18.27 percent for muscle, liver and kidney samples respectively. The findings were similar to the study conducted by Rose (1997) who also found reduction in residual level of lasalocid on boiling. It may be concluded that the process of boiling cannot annihilate the total amounts of drug residues however boiling may decrease the amount of residues in boiled meat as compared to raw meat.

In this study, a sensitive and simple method based on HPTLC was developed and validated for the estimation of common anticoccidial drug lasalocid. The simplicity, reliability and economy of this method make it suitable for large scale screening of this drug in poultry meat. Presence of chemical like lasalocid can affect animals as well as human being in numerous ways and could leading to short and long term untoward effects on animals and public health.

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IN VIVO GENOTOXICITY OF METRONIDAZOLE IN ICR MICE (*MUS MUSCULUS*)

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ABSTRACT

Metronidazole (MTZ) is an antibacterial and antiprotozoal drug that has been in use for over 35 years. Study was conducted to evaluate the genotoxic effect of MTZ against mitomicin C induced micronuclei frequency in ICR mice. A total of five groups, each group comprised of 5 mice per sex. Group I served as vehicle control and received only vehicle (0.2% CMC), while group II, III, IV received metronidazole at doses of 130 mg/kg (low dose), 390 mg/kg (mid dose), 780 mg/kg (high dose) orally by gavaging for two days at 24 hours interval respectively and group V received mitomicin C (2 mg/kg) intra peritoneally 24 hours prior to sacrifice served as a positive control. Animals were observed daily for clinical signs, behavioral changes and any mortality. Live body weights of each group of male and female mice were recorded daily. The mean number of micronuclei/1000 polychromatic erythrocytes (PCE) found in groups I, II, III, IV and V were 0.80 ± 0.37, 1.60± 0.40, 1.00 ± 0.45, 3.60 ± 0.75 and 39.8 ± 1.28 in male while 1.20 ± 0.73, 2.20 ± 0.37, 1.40 ± 0.51, 2.20 ± 0.37 and 34.6 ± 3.70 in female respectively. The result of the present work indicated that there was significantly increase MN frequency in male from high dose group however, which was absent in female. There was no significant change PCE/NCE ratio of male and female treated with metronidazole and mitomicin C. The finding of the present study gave evidence that metronidazole is genotoxic in male animals at high dose level.

Keywords: Genotoxicity, metronidazole (MTZ), , micronucleus test (MNT), mice, mitomicin C

INTRODUCTION

Metronidazole (MTZ) is an antibacterial and antiprotozoal drug that has been in use for over 35 years. Nowadays, it is one of the most used drugs worldwide; it is among the top 100 most prescribed drugs in the US (data by IMS Health) and one of the 10 most used drugs during pregnancy. Metronidazole is a prodrug; it requires reductive activation of the nitro group by susceptible organisms. Its selective toxicity toward anaerobic and microaerophilic pathogens such as the amitochondriate protozoa *T. vaginalis, E. histolytica,* and *G. lamblia* and various anaerobic bacteria derives from their energy metabolism, which differs from that of aerobic cells (Land and Johnson, 1997; Samuelson, 1999; Upcroft and Upcroft, 1999). Increasing levels of O_2 inhibit metronidazole-induced cytotoxicity because O_2 competes with metronidazole for electrons generated by energy metabolism. Thus, O_2 can both decrease reductive activation of metronidazole and increase recycling of the activated drug. In humans, oxidative metabolism is the major route for biotransformation of MTZ and two major oxidative metabolites are found: the hydroxy metabolite 1-(hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (HM) that represents the principal metabolite found in urine (50%) and in minor degree, the acetic metabolite 2-methyl-5-nitroimidazole-1-acetic acid (AAM) (Loft, 1990). MTZ is a potent mutagen in bacterial systems (Voogd, 1981); the main DNA lesions being base pair substitutions (Vanelle *et al*., 1990). In addition, several reports have demonstrated that HM and AAM also have mutagenic potential (Connor *et al*., 1977; Koch *et al*., 1981; Mohtashamipur and Norpoth, 1986). Genotoxic effects of MTZ have been studied in humans and rodents using different endpoints, however controversial results have been reported (Bendsky *et al*., 2002; Dobias *et al*., 1994). According to the International Agency for Research on Cancer (IARC), the evidence is sufficient to consider MTZ as an animal carcinogen, but insufficiently so for humans. The micronucleus test is one of the most widely applied short term test used in genetic toxicology and has become one of the most important tests implemented by the regulatory entities of different countries to evaluate mutagenicity and sensitivity to xenobiotics (OECD, 1997; EPA, 1998). Breakage of chromatids or chromosomes can result in micronucleus formation. Micronuclei can also result from lagging of one or more whole chromosome(s) at anaphase leading to genotoxicity. The present study is therefore aimed to investigating the possibility of genotoxicity and cytotoxicity with fact about carcinogenic potential of metronidazole in different sex of ICR mice.

MATERIALS AND METHODS

Drugs and chemicals

Metronidazole technical grade (99.8% pure) obtained from Aarti Drugs Limited, Mumbai, India was used for inducing toxicity in mice. Mitomicin C (Sigma Aldrich),

fetal bovine serum, Giemsa and May Gruenwald stain obtain from Himedia, Mumbai.

Experimental animals

This study was carried out in the Toxicology Department of Zydus Research Centre (ZRC), Moraiya, Ahmedabad in collaboration with Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Sardarkrushinagar. ICR mice of 6-8 weeks old, 25 males and 25 females were obtained from the animal research facility at Zydus Research Centre (ZRC), Moraiya, Ahmedabad. All the animals were examined for health status and acclimatized to experimental room condition for 5 days prior to use. Nulliparous and non pregnant female mice were selected. All the animals were housed in individually ventilated cage system (three animals per cage). The cage was designed to maintain temperature 22 ± 3^0C, relative humidity 30-70%, light intensity 130-400 Lux and a 12 h light:12 h dark photoperiod. The mice were provided with Standard 'NIN' brand pelleted diet and clean R.O. water *ad libitum* throughout the study period. The Institutional Animals Ethical Committee (IAEC) has approved the experimental protocol.

Experimental design

The 0.2 % Carboxymethyl cellulose (CMC) was used to formulate the formulation. The formulation was prepared fresh daily before dosing. Vehicle/test compound was administered directly in to the esophagus by graduated syringe with intubation cannula at an approximate constant dosage volume of 10 ml/kg of body weight based on their daily body weight. Experimental design is depicted in Table 1.

A total of five groups, each group comprised of 5 mice per sex. Group I served as vehicle control and received only vehicle (0.2% CMC), while group II, III, IV received metronidazole at doses of 130 mg/kg (low dose), 390 mg/kg (mid dose), 780 mg/kg (high dose) orally by gavaging for two days at 24 hours interval respectively and group V received Mitomicin C (2 mg/kg) intra peritoneally 24 hours prior to sacrifice served as a positive control. Animals were observed daily for clinical signs, behavioral changes and any mortality. Live body weights of each group of male and female mice were recorded daily.

Micronuclei assay

Micronuclei assay was carried out in bone marrow cells as per the method suggested by Hayashi, (1994). For Micronuclei assay mice were sacrificed 24 h after administration of the last dose of the metronidazole by Co_2 gas. The femur of mice was cut at the both ends with bone snips. The bone marrow was aspirated from the body of femurs in the fetal bovine serum (FBS). The cell suspension was centrifuged for 10 min at 1000 rpm. Supernatant was discarded and the cells in the sediment were mixed carefully. A drop of cell suspension was taken and smeared on the clean slides. The smears were fixed with methanol for 2 min and stained with a combination of May Gruenwald stain and Giemsa working solution in succession. The fixed smears were stained with undiluted May Gruenwald stain for 3 min and 1:1(v/v in distilled water) diluted May Gruenwald stain for 2 min. Slides were washed in distilled water and stained with Giemsa 1:6 (v/v in distilled water) diluted for 10 min. Slides were air dried after washing with distilled water. For the micronuclei (MN) assay scoring 1000 polychromatic erythrocytes (PCEs) per animal were scored to determine MN frequencies and 200 erythrocytes were examined to calculate the ratio of PCEs to normochromatic erythrocytes (NCEs).

Statistical analysis

The data obtained for pharmacokinetic parameters, NCE: PCE ratio, no. of MN per 1000 PCE subjected to statistical analysis. The statistical procedure used for analysis of above data by ANOVA and t test. Where p d" 0.05 was considered as statistically "significant" and p d" 0.01 was considered as statistically "highly significant". The data were described using statistical parameters viz. arithmetic mean, standard error of mean (SEM) and % change.

RESULTS

Animals from all the groups did not reveal any observable symptoms and no mortality were observed in ICR mice. Live body a weight of all mice has been presented in Table 2. There was no significant reduction in body weights in the metronidazole treated mice. Bone marrow micronuclei assay was performed immediately after humanly sacrificing of animals on 3rd day. The mean number of PCE/200 total erythrocytes (TE) in bone marrow cells of animals has been given in Table 3. There was no significant change PCE/NCE ratio of male and female mice treated with metronidazole.The mean number of micronuclei/1000 polychromatic erythrocytes (PCE) has been presented in Table 4. Group treated with mitomycin C showed significantly higher micronucleus than metronidazole and vehicle treated groups. Significantly increase micronuclei frequency was observed in male from high dose group however, which was absent in female mice. The vehicle treated negative control groups showed lower micronuclei values than metronidazole and mitomycin C treated groups.

DISCUSSION

Genotoxic effects of MTZ have been studied in humans and rodents using different endpoints, however controversial results have been reported (Bendsky *et al*., 2002; Dobias *et al*., 1994). According to the International Agency for Research on Cancer (IARC), the evidence is sufficient to consider metronidazole as an animal carcinogen, but insufficiently so for humans. Male and

Table 1:
Experimental design for micronucleus test.

Group No.	Treatment	Dose(mg/kg)	No. of Animals		Route
			Male	Female	
I (Vehicle control)	0.2% (CMC)	0	5	5	Oral
II (Low dose)	Metronidazole	130	5	5	Oral
III (Mid dose)	Metronidazole	390	5	5	Oral
IV (High dose)	Metronidazole	780	5	5	Oral
V (Positive control)	Mitomicin C	2	5	5	i.p.

Table 2:
Comparison of daily body weight (g) of male and female of different experimental groups (n=5).

Dose group	Male (Mean ± SE)			Female (Mean ± SE)		
	Day 1	Day 2	p value	Day 1	Day 2	p value
I (VC)	28.78 ± 1.85	30.34 ± 1.20	NS	28.78 ± 1.85	23.28 ± 0.95	NS
II (Low)	30.04±1.48	29.86 ± 1.21	NS	30.04 ±1.48	24.14 ± 1.13	NS
III (Mid)	30.20 ± 1.53	30.14 ± 1.26	NS	30.2 ±1.53	23.78 ± 0.79	NS
IV (High)	30.28 ± 1.01	30.02 ± 0.83	NS	30.28 ±1.01	22.06 ± 0.44	NS
V (PC)	28.60 ± 1.64	28.48 ± 1.64	NS	28.60 ±1.64	22.48 ± 0.56	NS

VC = Vehicle Control and PC = Positive Control

Table 3:
Comparison of PCE/NCE ratio of male and female (Mean ±SE) of different experimental groups (n=5).

Dose group	Male (PCE/NCE Ratio)		Female(PCE/NCE Ratio)	
	Mean ± SE	p value	Mean ± SE	p value
I (VC)	1.05 ±0.02	NS	0.82± 0.11	NS
II (Low)	1.15 ±0.10	NS	0.92± 0.09	NS
III (Mid)	0.98 ±0.03	NS	1.16±0.10	NS
IV (High)	1.00 ±0.14	NS	1.01±0.04	NS
V (PC)	1.00 ±0.02	NS	0.93±0.01	NS

VC = Vehicle Control, PC = Positive Control, PCEs = Polychromatic Erythrocytes, NCE = Normochromatic Erythrocytes, NS = Non Significant

Table 4:
Comparison of micronuclei frequency in PCE of male and female mice (Mean ± SE) of different experimental groups (n=5).

Dose Group	Male (Mean ± SE)			Female (Mean ± SE)		
	MN PCE	% MN PCE	P value	MN PCE	% MN PCE	P value
I (VC)	0.80± 0.37	0.08±0.04	NS	1.20±0.73	0.12±0.07	NS
II (Low)	1.60± 0.40	0.16±0.04	NS	2.20±0.37	0.22±0.04	NS
III (Mid)	1.00±0.45	0.10±0.05	NS	1.40±0.51	0.14±0.05	NS
IV (High)	3.60±0.75	0.36±0.08	S*	2.20±0.37	0.22±0.04	NS
V (PC)	39.8±1.28	3.98±0.13	S**	34.6±3.70	3.46±0.38	S**

VC = Vehicle Control, PC = Positive Control, MN PCEs = Micronucleated Polychromatic Erythrocytes, NS = Non Significant, S = Significant (* = $p \leq 0.05$ and ** = $p \leq 0.01$)

female animals of all treatment and control group did not reveal any symptoms attributable to oral administration of metronidazole for 2 days. No sex related difference was noticed. (Chaudhary *et al*., 2009). Investigate safety/ toxicity profile of fixed dose combination of ofloxacin-ornidazole injection in *Mus musculus* mice for 30 days. There were no signs of toxicity and mortality obsereved at any dose level used in these studies. Metronidazole and ornidazole are nitroimidazole derivatives. Thus, present short term study coincides with this ornidazole toxicity studies.

The daily body weight (g) of all the male and female animals was measured till day 3 of the experiment. The relative weights of spleen as well as the relative body weight gain decreased in mice treated with metronidazole at dose rate 14, 28, 42, 57 and 114 mg/kg intraperitoneal (Fararjeha *et al*., 2008). In present study revealed that there is no effect of metronidazole short term toxicity on body weight which may occur in long term studies.

Tolga and Serap (2005) evaluated genotoxic potential of metronidazole in fish *(Oreochromis niloticus)*. They observed that the frequencies of micronucleated PCEs was increased at both dose (10 and 15 mg/L) and time dependently while PCE/NCE ratio decreased. The results revealed that metronidazole has cytotoxic and genotoxic effects on fish. Ximena *et al*. (2007) show that metronidazole was possible genotoxic agent in three mouse strains (CFW, BALB/cJ and NIH). In his study NIH strain always showed the highest micronucleus frequency. The result of the present study indicated that no induction

of micronuclei frequency in male and female at low and mid dose of metronidazole and it concord with *in vitro* studies of metronidazole where metronidazole did not produce any cytotoxicity (in short term and long term exposure) and did not alter the micronuclei frequency (Buschini, *et al.*, 2009). Hartley-Asp (1981) was examined cytogenicity of metronidazole *in vivo* and *in vitro* in mice and humans. In the micronucleus test in mice metronidazole was given either as 2 oral doses 24 h apart or as a daily dose for 7 days. Neither forms of treatment produced any increase in the micronucleus frequency at doses up to 4000 mg/kg but in present studies higher dose significantly increased micronuclei frequency in male ICR mice while there was no induction of micronuclei frequency in female ICR mice. Present study in disagreement with previous study. Our finding showed that metronidazole might be possible genotoxic at higher dose level and male are more susceptible than female.

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Research Article

SAFETY OF LONG ACTING MOXIFLOXACIN FOLLOWING INTRAMUSCULAR ADMINISTRATION IN GOATS

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ABSTRACT

In this study, moxifloxacin, a fourth generation fluoroquinolone, was made long acting by addition of L-arginine, N-butyl alcohol and benzyl alcohol in parent compound. Study was planned to evaluate safety of long acting moxifloxacin (LAM) by investigating hematological and blood biochemical changes if any induced after single intramuscular administration of 10 % formulation of LAM given at the dose rate of 7.5 mg/kg in male Mehsana goats. A total of 9 blood samples per animal collected from 0 day (control or pre-drug sample) up to 7th day of treatment. Hematological (TLC, DLC, HB, HCT, MCV, MCH, MCHC, RDW and MPV) and blood biochemical (ALT, AST, ACP, ALP, Creatinine, CK, Billirubin, Blood Glucose and BUN) parameters were evaluated before and after drug administration. All above hematological and blood biochemical parameters were found trivially varied but within normal range and the mean values were not significantly differed ($p<0.05$) from corresponding control values. In present study, LAM was found safe and well tolerated antibacterial agent to be used in goats.

Keywords: Biochemical, long acting moxifloxacin, goats, hematological, safety

INTRODUCTION

Moxifloxacin is a latest fourth generation fluoroquinolone having 8 - methoxy - quinolone ring and bulky C_7 side chain with extended spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria, anaerobes and atypical organisms such as *Mycoplasma* spp. and *Chlamydia* spp. It is well absorbed; the distribution volume is large and less than 50% of bound to plasma protein. It exhibits good penetration into tissues and macrophages. Safety of moxifloxacin was assessed by scientists worldwide in human (Fogarty *et al*., 1999; Lode *et al*., 2003) and in rat and dog (Keutz and Schluter, 1999), albeit safety data of moxifloxacin in ruminant animals are deficient. As this drug is made long acting formulation for the first time for animal use, it is inevitable to investigate safety of LAM by studying its influence on hematology and blood biochemistry in animal body before its clinical use. Thus, the present study was conducted to investigate alteration in hematological and blood biochemical parameters induced by intramuscular (IM) administration of LAM in male Mehsana goats (n=6) given at the dose rate of 7.5 mg/kg body weight.

MATERIALS AND METHODS

Experimental Animals

Six healthy male Mehsana goats (local Indian breed) of body weight between 25-35 kg and of 2-3 year of age were selected for the study. Animals were kept in well ventilated house with adequate space and fed with concentrate, green, dry fodder and *ad libitum* fresh water supplied to the animals. This study was approved by the Institutional Animal Ethics Committee (IEAC) and all the measures of animal welfare were taken as per direction given in CPCSEA guidelines.

Experimental protocol

LAM (formulation of 10 % pure moxifloxacin powder in solution, with L-arginine, N-butyl alcohol and benzyl alcohol, procured from Intas Pharma Ltd, Ahmedabad, India) was given at the dose rate of 7.5 $mg.kg^{-1}$ IM in Mehsana goats (n=6). Approximately 3 ml of blood samples were collected from right jugular vein of goats before administration of drugs (0 day) and were considered as control in this study. Similarly, remaining blood samples were collected into K_2EDTA vacutainer tubes and sterile heparinized test tubes at 6 h, 12 h, 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day for hematological and plasma biochemical analysis, respectively. Plasma was separated after refrigerated centrifugation of blood samples at 1600 *g* for 10 minutes. The plasma samples were transferred to cryo-vials and then stored at 2-8°C until assayed for enzyme estimation.

Biochemical analysis

Biochemical parameters *viz*; alanine aminotransferase (ALT), aspartate aminotransferase (AST), acid phosphatse (ACP), alkaline phosphatase (ALP), creatinine, creatine kinase (CK), total bilirubin (conjugated and unconjugated), blood glucose and blood

urea nitrogen (BUN) were examined by clinical analyzer (Systronic-635, Ahmedabad) using analytical kits (Merck Specialties Pvt. Ltd., Mumbai.).

Hematological analysis

Hematological parameters *viz*; total leukocyte count (TLC), differential leukocyte count (DLC), total erythrocyte count, hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW) and mean packed volume (MPV) were assayed by automated hematology analyzer (Cell Dyne 3700 ,Abbott diagnostic, U.S.A) using ready to use suitable kits.

Statistical analysis

Descriptive statistical analysis of hematological and blood biochemical parameters were performed by using software program SPSS 11.0.

RESULTS

Values of biochemical parameters including ALT, AST, ACP, ALP, BUN, creatinine, CK, total bilirubin, blood glucose and BUN evaluated for safety study are presented in Tab. 1. There were no significant ($p<0.05$) differences found in the values of serum biochemistry between pre-dosing (0 day or control) and post-dosing time period.

Results of various hematological parameters after IM administration of LAM including WBC count and DLC counts (lymphocytes, monocytes, eosinophils, neutrophils and basophils) are presented in Tab. 2 and RBC count, HB concentration, HCT, MCV, MCH, MCHC, RDW and MPV count are presented in Tab. 3. Average value of all these hematological parameters observed after post LAM

Table. 1:

Effect of single dose IM administration of LAM (7.5 mg/kg b. wt.) on values of biochemical parameters in goats (Mean ± SE, N=6).

Blood collection schedule	Biochemical parameters								
	ALT (U/liter)	**AST** (U/liter)	**ACP** (U/liter)	**ALP** (U/liter)	**Creatinine** (mg/dl)	**CK** (U/liter)	**Total bilirubin** (mg/dl)	**Blood glucose** (mg/dl)	**BUN** (mg/dl)
0 day	52.84 ± 1.86	47.25 ± 2.10	5.70 ± 0.78	121.42 ± 3.46	1.07 ± 0.13	10.94 ± 0.96	0.44 ± 0.04	92.17 ± 1.27	17.86 ± 3.00
6 h	50.95 ± 1.31	44.64 ± 1.94	6.69 ± 0.45	134.52 ± 5.92	1.24 ± 0.12	11.91 ± 0.33	0.38 ± 0.02	95.74 ± 1.73	18.85 ± 2.75
12 h	51.32 ± 1.08	47.27 ± 1.64	6.25 ± 0.59	121.81 ± 6.58	0.85 ± 0.11	10.43 ± 0.61	0.41 ± 0.01	94.49 ± 1.20	19.28 ± 3.00
2nd day	51.24 ± 1.73	48.85 ± 1.50	6.07 ± 0.75	123.14 ± 6.69	0.73 ± 0.04	10.11 ± 0.26	0.46 ± 0.02	95.73 ± 2.78	18.26 ± 2.14
3rd day	51.54 ± 1.28	47.60 ± 1.67	5.92 ± 0.63	110.91 ± 4.45	0.85 ± 0.08	10.39 ± 0.74	0.49 ± 0.02	94.25 ± 2.08	19.71 ± 1.57
4th day	56.04 ± 2.19	51.13 ± 2.80	5.57 ± 0.59	121.80 ± 4.20	1.42 ± 0.15	11.73 ± 0.56	0.46 ± 0.02	85.10 ± 3.05	18.40 ± 0.80
5th day	50.04 ± 1.80	49.99 ± 1.93	5.98 ± 0.78	123.13 ± 2.57	1.47 ± 0.15	11.74 ± 0.47	0.51 ± 0.02	84.44 ± 2.82	18.04 ± 1.55
6th day	54.09 ± 1.79	47.13 ± 1.60	5.79 ± 0.42	124.25 ± 2.19	1.03 ± 0.06	11.58 ± 0.59	0.52 ± 0.02	83.66 ± 4.11	17.78 ± 1.45
7th day	52.72 ± 2.25	43.60 ± 2.24	6.15 ± 0.45	126.23 ± 2.50	0.94 ± 0.02	10.33 ± 0.32	0.54 ± 0.01	87.88 ± 3.26	17.69 ± 1.94

Table. 2:

Effect of single dose IM administration of LAM (7.5 mg/kg b. wt.) on WBC and DLC in goats (Mean ± SE, N=6).

Blood collection schedule	Hematological parameters					
	WBC($x10^3$ /µL)	**Neutrophils**(%)	**Lymphocytes**(%)	**Monocytes**(%)	**Eosinophils**(%)	**Basophils**(%)
0 day	12.13 ± 0.80	36.82 ± 1.14	57.78 ± 1.55	2.642 ± 0.331	2.63 ± 0.48	0.15 ± 0.09
6 h	12.73 ± 0.65	40.17 ± 2.44	51.42 ± 3.35	2.918 ± 0.514	1.97 ± 0.59	0.37 ± 0.23
12 h	12.07 ± 0.75	39.40 ± 3.51	56.00 ± 3.90	2.668 ± 0.396	1.90 ± 0.63	0.13 ± 0.03
2nd day	13.28 ± 1.05	37.03 ± 3.06	56.72 ± 3.19	2.970 ± 0.398	3.10 ± 0.84	0.18 ± 0.06
3rd day	11.21 ± 1.26	34.22 ± 2.46	61.30 ± 3.43	3.358 ± 0.355	3.21 ± 0.98	0.53 ± 0.12
4th day	12.49 ± 1.33	38.23 ± 2.48	57.00 ± 3.42	3.655 ± 0.344	2.60 ± 0.64	0.20 ± 0.07
5th day	13.00 ± 0.94	35.50 ± 3.83	58.38 ± 3.81	3.220 ± 0.394	2.78 ± 1.12	0.19 ± 0.06
6th day	13.58 ± 0.79	36.30 ± 3.23	61.60 ± 4.19	2.423 ± 0.121	2.69 ± 0.74	0.33 ± 0.04
7th day	12.32 ± 0.73	34.37 ± 3.89	56.63 ± 4.47	2.535 ± 0.379	2.70 ± 0.70	0.43 ± 0.13

Table.3:

Effect of single dose IM administration of LAM (7.5 mg/kg b. wt.) on RBC, HB, HCT, MCV, MCH, MCHC, RDW and MPV in goats (Mean ± SE, N=6).

Blood collection schedule	Hematological parameters							
	RBC($x10^{12}L^{-1}$)	**HB**($g.dl^{-1}$)	**HCT**(%)	**MCV**(fL)	**MCH**(pg)	**MCHC**($g.dl^{-1}$)	**RDW**(%)	**MPV**(fL)
0 day	6.44 ± 0.29	7.91 ± 0.15	13.67 ± 0.71	21.27 ± 0.13	12.40 ± 0.61	55.77 ± 2.12	55.77 ± 2.12	32.02 ± 1.16
6 h	6.61 ± 0.30	8.69 ± 0.25	14.28 ± 0.73	21.60 ± 0.13	13.28 ± 0.69	58.73 ± 2.42	59.90 ± 2.61	33.82 ± 1.46
12 h	6.70 ± 0.33	7.92 ± 0.24	14.30 ± 0.74	21.35 ± 0.08	11.99 ± 0.76	56.13 ± 3.59	56.13 ± 3.59	30.75 ± 1.95
2nd day	6.48 ± 0.33	7.69 ± 0.23	13.73 ± 0.76	21.17 ± 0.14	12.02 ± 0.66	53.47 ± 2.43	53.47 ± 2.43	31.35 ± 1.52
3rd day	6.38 ± 0.32	7.30 ± 0.17	13.35 ± 0.79	20.87 ± 0.18	11.58 ± 0.61	55.65 ± 3.31	55.65 ± 3.31	29.00 ± 0.91
4th day	6.38 ± 0.32	7.58 ± 0.22	13.43 ± 0.69	21.08 ± 0.15	12.01 ± 0.62	57.02 ± 2.99	57.02 ± 2.99	31.02 ±2.05
5th day	6.48 ± 0.32	7.66 ± 0.13	13.77 ± 0.73	21.20 ± 0.12	11.96 ± 0.67	56.53 ± 3.38	56.53 ± 3.38	31.48 ± 1.24
6th day	6.55 ± 0.35	8.16 ± 0.22	13.98 ± 0.82	21.33 ± 0.15	12.62 ± 0.62	57.50 ± 2.78	57.50 ± 2.78	32.33 ± 2.10
7th day	6.33 ± 0.32	7.94 ± 0.21	13.48 ± 0.72	21.27 ± 0.13	12.68 ± 0.64	58.03 ± 2.52	58.03 ± 2.52	31.48 ± 1.21

dosing when compared with control values (0 day), have been found non-significant ($p<0.05$).

DISCUSSION

Generally fluoroquinolones are well tolerated, safe and efficacious antimicrobial agents, most adverse effects are mild in severity, self-limiting and rarely result in treatment discontinuation (Ball *et al.,* 1999). With fluoroquinolone treatment, the most commonly occurring class effects are GI upset (nausea, vomiting, diarrhea, constipation and abdominal pain; less than 7% total) and less common effects may include effect on central nervous system (less than 5%), blood disorders (approximately 5%), renal disturbances (approximately 4.5%) and skin hypersensitivity and photosensitivity effects (approximately 2%) (Childs, 2000). Moxifloxacin is as safe and well tolerated as other commonly prescribed antibiotics (Andriole, *et*. *al*., 2005).

In present study, various blood plasma enzymes including ALT, AST, ALP and total bilirubin were determined during the course of study to evaluate potency of liver functions following single dose LAM administered (7.5 $mg.kg^{-1}$) in goats. Similarly, creatinine and BUN levels were determined to monitor the potency of renal function. Additionally levels of ACP and blood glucose were also measured. Comparison of results indicated insignificant differences in the values of any of the blood biochemical parameters estimated in control and treated animals. Safety of single dose LAM administered (7.5 $mg.kg^{-1}$ b.wt.) in goats was also monitored by studying various hematological parameters. The results of the present study have indicated insignificant differences between the values of all the hematological parameters estimated at 0 day (pre- treatment) and post- treatment days. All parameters observed were within normal ranges.

Results of the present study were similar to reports of moxifloxacin safety in human (Stass *et al*., 1998; Iannini *et al*., 2001; Ball *et al*., 2004). Results of present study were also supported by report of non significant change in hematological and biochemical parameters observed following repeated intramuscular administration of moxifloxacin in Wistar rats (Sadariya *et al.*, 2010). In another study, significant change in hematology and blood biochemical parameters following repeated administration of moxifloxacin in rats, mice and dog were reported (Keutz and Schluter, 1999).

In present study LAM was found safe and well tolerated antibacterial agent in goats as evidenced by lack of clinical signs of adverse reactions and presence of non-significance hematological and blood biochemical alterations. Strive of development of safe LAM formulation would enroll a new entity in an antimicrobial armament for treating bacterial infections in goats.

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Research Article

PHARMACOEPIDEMIOLOGY OF CLINICAL USAGE OF VETERINARY ANTIMICROBIALS IN NORTHERN GUJARAT

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ABSTRACT

The study was conducted to determine usage trend, adverse drug reactions and pattern of resistance of antimicrobial drugs used for the treatment of infectious diseases of animals. It involved survey of seventy veterinarians in five districts of Northern Gujarat by the means of pre-structured questionnaire. It was observed that tetracyclines were most extensively used antimicrobial drugs among all groups. Tetracycline (oxytetracycline), penicillins (amoxicillin with cloxacillin), cephalosporin (ceftriaxone), fluoroquinolone (enrofloxacin), aminoglycoside (gentamicin) were found to be most used antimicrobial drugs. The nature and frequency of adverse drug reactions reported in the present study clearly indicated that their occurrence including mortality was not an uncommon event. Antimicrobial resistance was experienced by 47.14% of respondents particularly in the treatment of mastitis (81.25% of respondents).

Key word: Adverse drug reactions, antimicrobial resistance, North Gujarat , pharmacoepidemiology,

INTRODUCTION

Pharmacoepidemiology is defined as study of use and effect of drugs in large number of people. It provides valuable information about clinical and economic outcome of drugs and biologicals (Strom, 2006) and covers two essential disciplines *viz.* pharmacology, defining both beneficial as well as adverse drug effects, and epidemiology, the response of the population to these effects (Lawson, 1984). A pharmaco-epidemiological (PE) survey involves sourcing for information about the effects of drug use which includes post-marketing assessment and drug surveillance, risk-benefit ratio, beneficial and adverse drug reactions with methodologies to study the outcomes of drug therapy in man and animal(Strom, 2006). In veterinary profession, the incidences of adverse drug reactions/side effects are not uncommon but lack of proper documentation and record keeping system makes them hidden or unreported (Dogan, 2011). Such studies are essentially needed to frame guidelines on the use of antimicrobials and to explore possible outcomes of antimicrobial therapy. Thus, present study was undertaken to determine usage trend, adverse drug reactions (ADRs) and resistance of antimicrobials drug used for the treatment of infectious diseases in animals.

MATERIALS AND METHODS

The study was conducted in five districts of northern Gujarat area *viz*. Banaskantha, Patan, Mehsana, Sabarkantha and Kutch. Pharmaco-epidemiological data were collected from a total of 70 veterinarians working in public and private sectors by the means of pre-structured questionnaire (Table 1). Out of 70 respondents 29, 18, 15 and 8 belonged to government animal husbandry department, co-operative societies, agricultural university employee and private practitioners, respectively, withB.V.Sc.& A.H. (42), M.V.Sc.(22) and Ph.D. (06) qualification. Most of respondents (71.4 %) were having more than five years of clinical practice experience in veterinary field.

RESULTS AND DISCUSSION

Among major antimicrobial groups, tetracyclines were most extensively used group as reported by 85.71% of respondents followed by aminoglycosides (52.86%) and penicillins (38.57%). Fluoroquinolones (54.29%) and cephalosporins (42.86%) were used moderately and whereas sulphonamide (51.43%) and amphenicol (48.57%) were used rarely and macrolides were never used in animals (Table 2). Usage pattern of individual drugs within particular antimicrobial group is presented under Table 3. Commonly used penicillins were combination of amoxicillin and cloxacillin (61.43%) followed by amoxicillin alone, whereas long acting penicillinswere used moderately by 40.00% of the respondents.

Individual antimicrobials reported as most extensively used drugs were ceftriaxone (45.71%), oxytetracycline (82.86%), enrofloxacin (57.14%), gentamicin (67.14%), erythromycin (7.14%) and chloramphenicol (14.29%), respectively. Higher frequency of usage of oxytetracycline observed in the survey might have been due to low cost, broad spectrum property and easy availability of wide range of its formulation with proven efficacy. Arowolo *et al.* (2012) also reported oxytetracycline (74.1%) as most frequently used tetracyclines but they found procaine penicillin (long acting penicillin) as most used penicillins (41.5%). Many respondents (62.86%) also

Table 1:
Type of questions included in questionnaire survey form

Sr. No.	Theme of question
1	Most used group of antimicrobials (in order of frequency) for treating infectious diseases of animal.
2	Preference of individual drugs in respective antimicrobial groups.
3	Modes for selecting particular antimicrobial drugs.
4	Sources of information for use of antimicrobial drugs.
5	Knowledge on Withdrawal period and MRL (Maximum Residue Limit) of drugs
6	Preferable routes of administration for antimicrobial drug.
7	Preferable site of administration for antimicrobial drug.
8	Observed adverse drug reactions (ADRs) of antimicrobials in animals.
9	Observed antimicrobial resistance (AMR).
10	Occurrence of infectious diseases in practices area.
11	Types and frequency of antimicrobials used for important infectious diseases.

Table 2:
Frequency of usage of antimicrobial groups

Extensively used		Moderately used		Rarely used		Never used	
1st Rank	Tetracyclines (85.71)	1st Rank	Fluoroquinolones (54.29)	1st Rank	Sulphonamides (51.43)	1st Rank (80.00)	Macrolides
2nd Rank	Aminoglycosides (52.86)	2nd Rank	Cephalosporins (42.86)	2nd Rank	Amphenicols (48.57)	2nd Rank	Amphenicols (37.14)
3rd Rank	Penicillins (38.57)	3rd Rank	Penicillins (40.00)	3rd Rank	Cephalosporins (28.57)	3rd Rank	Cephalosporins (20.00)

Values in parenthesis () represents % respondents

Table 3:
Usage pattern of individual drugs within an antimicrobial group

Group	Most extensively used	Most moderately used
Penicillins	Amoxicillin + Cloxacillin (61.43)	Long acting Penicillins (40.00)
Cephalosporins	Ceftriaxone (45.71)	Ceftriaxone + Sulbactam (50.00)
Tetracyclines	Oxytetracycline (82.86)	Long acting oxytetracycline (55.71)
Fluoroquinolones	Enrofloxacin (57.14)	Ciprofloxacin (35.71)
Aminoglycosides	Gentamicin (67.14)	Gentamicin (31.43)
Macrolides	Erythromycin (7.14)	Erythromycin (8.57)
Amphenicols	Chloramphenicol (14.29)	Chloramphenicol (17.14)

Values in parenthesis () represents % respondents

Table 4:
Antimicrobials of choice in the treatment of various infectious diseases

Name of disease	First Choice	Second Choice
Actinomycosis & Actinobacillosis	Strepto-penicillin (48.98)	Long acting penicillin (20.41)
Anthrax	Strepto-penicillin (25.71)	Long acting penicillin (22.86)
Arthritis	Long acting penicillin (18.75)	Oxytetracycline (16.96)
Black-Quarter	Long acting penicillin (29.27)	Strepto-penicillin (24.39)
Brucellosis	Oxytetracycline (54.76)	Strepto-penicillin (13.10)
Ephemeral fever	Oxytetracycline (57.89)	Ampicillin (8.77)
Gastro-enteritis	Sulphonamide +Trimethoprim (28.36)	Enrofloxacin (24.63)
Haemorrhagic septicaemia	Sulphonamide +Trimethoprim (31.62)	Oxytetracycline (17.65)
Mastitis	Ceftriaxone + sulbactam (23.81)	Amoxicillin+Cloxacillin (16.40)
Metritis	Oxytetracycline (33.33)	Gentamicin (17.39)
Pyometra	Oxytetracycline (31.88)	Strepto-penicillin (15.22)
Tetanus	Long acting penicillin (40.00)	Strepto-penicillin (22.22)
Urinary tract infection	Enrofloxacin (22.62)	Gentamicin (22.02)

Values in parenthesis () represents % respondents

reported the use of combination of streptomycin and penicillin (strepto-penicillin). It was found that combination of sulphonamides with trimethoprim (25.71%) was more commonly usedthan sulphonamides alone (7.14%) indicating that potentiated sulpha-drugs are more popular rather than their alone use.

Different antimicrobials of choice for different disease conditions are summarized in Table 4. It is clearly evident from survey results that in spite of availability of newer generation drugs, conventional antimicrobials like streptopenicillin, long acting penicillin, oxytetracycline and potentiated sulpha-drugs are still most preferred

Table 5:
Frequency and type of ADRs observed with the use of antimicrobials in animals

Name of antibacterial	Frequency of ADR (in %)	Type of ADR (no. of major events)
Enrofloxacin	n= 9(21.95)	Colic (8),Hyper-salivation (1),
Strepto-penicillin	n=9 (21.95)	Allergic reaction (8), Hyper-salivation (1)
Oxytetracycline	n=8 (19.51)	Allergic reaction (3), Local inflammation (3), Diarrhoea (1), Death (1)
Sulphonamides	n=8 (19.51)	Allergic reaction (4), Hyper-salivation (2), Death (2)
Long acting penicillin	n=3 (7.32)	Allergic reaction (3)
Ciprofloxacin	n=1(2.44)	Skin lesion (1)
Ampicillin + cloxacillin	n=1 (2.44)	Sweating (1)
Cephalexin	n=1 (2.44)	Skin lesions (1)
Chloramphenicol	n=1 (2.44)	Anaemia (1)

antimicrobials for treatment of animal diseases.Clinical mastitis is the disease condition which requires highest consumption of antimicrobials at field level.The variation in the pattern of antimicrobial usage over different geographical area may be evident due to variation in pathogen susceptibility, complex etiological factors involved, severity of diseases and paying capacity of livestock owners (Manimaran *et al.*, 2013).

Basis for selection of antimicrobials by veterinary practitioners mainly included their own experience (50.39%), laboratory findings (18.90%), empirical approach (14.96%) and recommendation or guidelines issued from state government (14.96%). Books including drug-index (40.15%) were emerged to be main source of information for use of antimicrobials followed by information provided by veterinary sales representatives or pharmaceutical company literatures (31.06%), consultation from academic persons (15.91%) and internet (12.88%). Descending order of preference was noticed as intramuscular followed by intravascular, subcutaneous and oral route. For intramuscular injections, 95.71% of clinicians opted neck region as preferred site.

About 34.3% of respondents reported ADRs associated with the use of different antimicrobials while treating animals and observed mostly in crossbred cattle. Use of enrofloxacin and strepto-penicillin were reported to produce more ADRs followed by oxytetracycline and sulfonamides (Table 5). Death of one camel due to oxytetracycline and two other animals (buffalo and HF cross cattle) with sulphonamides were noted in present study. A veterinary pharmacovigilance survey conducted by Sarathchandra *et al.* (2013) in Tamil Nadu state of India including 172 field veterinarians revealed that 37% of ADRs of veterinary medicinal products were due to antimicrobials only during year 2011-12. Deaths reported in their study too, mainly due to oxytetracycline,strepto-penicillin, streptomycin and procaine penicillin.In a surveyinvolving 250 practicing clinical veterinarians of Turkey (Dogan, 2011), it was noted that 81% of veterinarians had experienced at least oneadverse drug reaction. Most ADRs occurred due to vitamin-mineral-amino acid preparations (39%) followed by antibacterials (26%). In agreement to our findings, most common ADRs were anaphylaxis or allergic reactions. Arowolo *et al.* (2012) also reported many cases of reddening and rashes due to procaine penicillin and a death due to long acting oxytetracycline.

Resistance of antimicrobial drugs was observed by 47.14% of respondents and most of incidences of drug resistance wereobserved during the treatment of mastitis (81.25%) followed by wound management (4.17%). Overall, it was observed that most extensive used antimicrobials like tetracyclines, aminoglycosides and penicillins also showed highest degree of resistance during veterinary practice whereas rarely used antimicrobials like chloramphenicol (off-label use) showed least resistance.

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Research Article

BIOCHEMICAL ALTERATIONS INDUCED BY TEFLUTHRIN AND ALUMINUM IN RATS

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ABSTRACT

The aim of present study was to evaluate single and interactive toxic potential of tefluthrin and aluminum in wistar rats after their oral administration for a period of 28 days. The animals were divided into four groups with six rats in each group. Group I served as control and rats were orally administered with corn oil. Group II animals were orally exposed to tefluthrin @ (1/20 LD_{50}) after its dissolution in corn oil. Animals in group III were orally administered with aluminum chloride @ 34mg/kg (1/25 LD_{50}) in water while as animals in group IV were administered with both tefluthrun @ 1.1mg/kg (1/20 LD_{50}) and aluminum chloride @ 34mg/kg (1/25 LD_{50}). Significant ($P<0.05$) increase in enzymes like ALT, AST, AKP, ACP and LDH was observed in group II and III as compared to control group I after 28th day of oral treatment. More significant increase in these enzymes were observed in group IV receiving both tefluthrin and aluminum chloride as compared to groups I, II and III. Biochemical variables like total plasma protein and albumin decreased significantly ($P<0.05$) in group II, III and IV as compared to control with changes in group IV even differing significantly ($P<0.05$) from group II and III. Significant increase in urea and creatinine levels were observed in group II, III and IV as compared to group I with significantly ($P<0.05$) increased creatinine concentration in group IV even from group II and III. The present study, therefore, draw an inference that the toxicity of tefuthrin is enhanced in the present of aluminum and together can cause severe health implications in animals.

Key words: Tefluthrin, aluminum chloride, enzymes, wistar rats.

INTRODUCTION

Tefluthrin, a fluorinated synthetic type-I pyrethroid, owe its insecticidal activity to the ability to disrupt electrical signaling in the nervous system by prolonging the opening of voltage-gated sodium channels which are also considered to be the primary targets for the central neurotoxic effects of pyrethroids in mammals (Soderlund *et al*., 2002).

The widespread occurrence of aluminum has so heavily contaminated the environment that human and animal exposure to it seems virtually inescapable. The daily ingestion of aluminum by humans is estimated to be 30 to 50 mg and is known to cause toxic effects to a variety of organ systems including brain, bone, kidney and blood (Oteiza *et al.,* 1993). The assessment of deleterious or toxic effects helps in comprehensive management of untoward effects produced by these chemicals. Therefore, present study was an attempt to assess the degree of hazard posed by simultaneous exposure to tefluthrin and aluminum in wistar rats after oral administration for a period of 28 days.

MATERIALS AND METHODS

Experimental animals and design

Wistar rats (200-250 gm b. wt) of either sex procured from Indian Institute of Integrtaed Medicine (CSIR Lab) Jammu were maintained under standard environmental conditions. The experiment was conducted strictly in accordance to the Institutional Animal Ethics committee. The rats were divided randomly into four groups consisting of 6 rats each and were provided with free access to feed and water. The animal room was maintained at 21–24°C and 40–60% relative humidity with 12-h light–dark. After 2 weeks of acclimation, the groups were assigned at random to different treatments. The animals of Groups A served as control and received corn oil 1 ml daily. Animals in group B were given Tefluthrun @1.1mg/kg (1/20 LD_{50}) daily in corn oil (Abdou. *et.al,* 2009). Animals in group C were orally administered with Aluminum chloride @ 34mg/kg (1/25 LD_{50}) (Fatma M and El-Demerdash, 2004) in water while as animals in group D were administered with both Tefluthrun @ 1.1mg/kg (1/20 LD_{50}) and aluminum chloride @ 34mg/kg (1/25 LD_{50}). All the doses were administered in the morning continuously for 28 days and body weight recorded at 7 days interval to adjust the dosage of application according to body weight.

Chemicals

Tefluthrin (PESTANAL®), analytical standard obtained from Sigma Aldrich and Aluminum chloride ($AlCl_3$ x 6 H_2O), analytical grade purchased from Hi-Media Labs Mumbai were used in the present study.

Blood collection and analysis

On the 29th day rats were anaesthetized with diethyl ether and blood samples of about 5-6 ml was collected from retro-orbital fossa and heart of anaesthetized rats in a set of tubes containing heparin @ 5-10 IU/ml of blood. The collected blood samples were centrifuged at 3000 rpm for 15 min and the plasma was harvested in clean sterile glass test tubes and stored at -20^{0}C till further analysis. The activities of alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), acid phosphatase (ACP), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and levels of total protein, albumin, blood urea, creatinine (CR) in plasma were determined by standard kits from Transasia Bio-Medicals by using chemistry analyzer (Kayto).

Statistical analysis

The data was subjected to analysis of variance applying completely randomized design (CRD) at 5% level (P<0.05) of significance (Duncan, 1955).

RESULTS AND DISCUSSION

In the present study the effect of repeated administration of aluminium chloride and tefluthrin alone and in combination for 28 days on various biochemical parameters were studied. The results of repeated administration of tefluthrin and aluminum alone and in combination on various enzyme activities is shown in Table 1 while the results of total protein, albumin, creatinine and blood urea are shown in Table 2. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alakaline phosphatase (AKP), acid phosphatase (ACP) and lactate dehydrogenase (LDH) was significantly (P<0.05) increased after 28th day in tefluthrin and aluminum chloride treated rats (Group II & III) as compared to control (Group I). Group IV receiving both tefluthrin and aluminum showed more significant (P<0.05) increase in the activities of these enzymes not only against control group I but also in comparison to groups II and III.

Plasma total protein and albumin levels showed significant (P<0.05) decline in tefluthrin and aluminum chloride treated rats (Group II & III) as compared to control grou while as urea and creatinine levels showed significant increase as compared to control. In the co-adminstered groups total protein and albumin levels showed significant (P<0.05) decline while as significant (P<0.05) increase of creatinine level as compared to groups I, II and III.

Determination of aspartate aminotransferases (AST) and alanine amino transferases (ALT) activities are very common in evaluating the liver and muscle damage in laboratory animals (Hall, 1992). AST is found in high levels in heart and liver while ALT is more active in liver and their levels are elevated following tissue damage in which cellular enzymes are dumped into the blood. Liver is a predominant organ for metabolism of xenobiotics and also the first organ exposed to ingested toxicants via portal blood supply. Alterations of these enzymes are the indicators of the status of the liver. Increase in AST and ALT levels in tefluthrin and aluminium chloride treated rats is in accordance with studies of Ahrar *et al*. (2010).

Alkaline phosphatase is a zinc containing enzyme (Clampitt and Hart, 1978) which occurs in duodenum, liver, biliary tracts, lung and kidney. Raised levels often indicate non-specific tissue irritation but pathological elevations are most common in bone and liver diseases (Forbes, 2001). Increased Alkaline phosphate levels in the present study might be suggestive of possible damage in liver. Acid phosphatase is a lysosomal enzyme that gets stimulated in cases of imminent or prevelant tissue damage (Wilson *et al.,* 1970). Increase in AKP and ACP in aluminium chloride treated group which could be attributed to the damage of liver, kidney and bone resulting into liberation of AKP and ACP.

Lactate dehydrogenase is a cytosolic enzyme that

Table 1:
Effect of repeated oral administration of aluminium, tefluthrin and their combination on various enzyme activities

Group	ALT (IU/L)	AST(IU/L)	ACP(IU/L)	ALP(IU/L)	LDH(IU/L)
I(Control)	59.68±1.42^a	66.90±2.71^a	8.18±0.69^a	124.70±7.73^a	584.77±9.91^a
II(Tefluthrin)	75.82±3.02^b	80.74±2.86^b	10.75±0.79^b	231.04±12.06^b	658.65±6.91^b
III(Aluminum)	71.44±2.66^b	77.37±3.09^b	9.90±0.61 --^{b}-	198.52±7.12^c	634.56±9.57^b
IV(Aluminum+tefluthrin)	81.13±2.00^c	89.78±3.37-^{c}	15.49±0.69^c	268.73±11.38^d	705.47±9.57^c

Values given are mean±SE of the results obtained from 6 animals.
Values with at least one common superscript do not differ significantly at 5% (P<0.05)

Table 2:
Effect of repeated oral administration of aluminium, tefluthrin and their combination on levels of various biochemical parameters.

Group	Protien(g/dl)	Urea(mg/dl)	Albumin(g/dl)	Creatinine(mg/dl)
I(Control)	6.86±0.31^a	58.53±3.59^a	3.60±0.10^a	0.521±0.033^a
II(tefluthrin)	4.87±0.34^b	71.61±3.82^b	2.87±0.17^b	0.688±0.031^b
III(Aluminum)	5.30±0.32^b	70.32±3.60^b	2.59±0.09^b	0.871±0.165^b
IV(Aluminum+trefluthrin)	3.88±0.30^c	77.59±5.01^b	1.91±3.45^c	1.026±0.139^c

Values given are mean±SE of the results obtained from 6 animals.
Values with at least one common superscript do not differ significantly at 5% (P<0.05)

plays an important role in the intermediary metabolism by acting as a link between amino acid metabolism and the citric acid cycle where it converts lactate into pyruvate. During cellular damage it gets released from important organs like liver, lung, muscle, kidney, testicles or heart into systemic circulation (Bhargava *et al*., 1978). An increase in serum LDH activity following tefluthrin treatment (orally) may be due to the hepatocellular necrosis leading to leakage of the enzyme into the blood stream (Yousef *et al*., 2004).

Creatinine is present in tissues i.e., muscles, brain, blood etc as a high energy compound phospho-creatine and is excreted in small quantities in urine. Increase in creatinine concentration might be due to impairment of kidney function. The high levels of blood urea results from either increased breakdown of tissue or dietary protein or impaired excretion (Bush, 1991). The increased urea levels could be attributed to the decreased feed intake during the stress period which in turn can lead to an increased catabolism of body proteins and hence, resulting in increased levels of urea (Rana and Soni, 2008).

Decreased protein in aluminium treated group may be due to increased catabolism of proteins and their decreased synthesis. Decrease in total protein and albumin in tefluthrin and aluminium chloride treated rats in present study is in accordance with the studies of Shah and Gupta (2001) and Amal (2007).

In the present study, co-administered group receiving both tefluthrin and aluminum showed more significant alterations in biochemical parameters as compared to other groups but nodirect *in vivo* relation between tefluthrin and aluminum could be established.

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Research Article

STUDIES ON HEMATOLOGICAL AND HISTOPATHOLOGICAL CHANGES FOLLOWING SUBACUTE EXPOSURE OF ACETAMIPRID IN MALE MICE

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ABSTRACT

The present study was carried out to assess the toxicological effect of acetamiprid in mice. Eighteen mice were divided into three groups of six animals each, first group served as control. Treated groups were given acetamiprid @ 2.3 & 4.6 mg/kg b.wt., i.p. for 30 days, respectively, in 3% aqueous solution of gum acacia (vehicle). Vehicle control group animals were given 3% aqueous solution of gum acacia i.p. for 30 days. There was no significant decrease in value of Hb in treated groups as compared to control group. Other hematological parameters were non-significantly decreased with increase in treatment level except MCHC which non-significantly increased. Various histopathological lesions were observed in liver, kidney and testis. Central lobular necrosis with congestion of central vein, formation of new bile duct, diffused hemorrhages, infiltration of mononuclear cells around the central vein were observed in the liver. In kidney, tubular necrosis surrounded by infiltration of mononuclear cells, hydropic degeneration and presence of various casts and hemorrhages and atrophy of glomerulus were observed. In testes, there was detachment and degeneration of spermatogonial from seminiferous tubules and reduced concentration of sperms in seminiferous tubules were observed.

Key Words: Acetamiprid, mice, hematology, histopathology.

INTRODUCTION

Acetamiprid, a new member of neonicotinoid synthetic chlorinated insecticide family has been recently introduced in the market and is highly effective for controlling aphids, beetles, moth, leafhopper, pests on crops and leafy vegetables, along with fleas infesting livestock and pet animals. It is a systemic insecticide with translaminar action which has a contact and stomach action (Zhang *et al.*, 2010). Acetamiprid is used against insects that have gained resistance to organophosphate, carbamate and synthetic pyrethroid (Si *et al.*, 2005). Although acetamiprid is widely used in India and abroad but, there is paucity of data on its toxicity in animals. Therefore, the present study was carried out to evaluate influence of subacute acetamiprid exposure on hematological and histopathological changes in adult male mice.

MATERIALS AND METHODS

Experimental animal

Adult Swiss albino male mice weighing between 17-27 g were procured from Disease Free Small Animal House of the Institute and acclimatized in laboratory conditions for a period of 7 days prior to experimentation. Animals were housed in polyacrylic cages in group of six per cage at room temperature with a natural light-dark cycle and provided balanced feed and clean water *ad libitum*. Animal house temperature varied between 22 to 27°C throughout the experiment. The prior approval of Institutional Animal Ethics Committee was obtained for use of the animals in this study. The experiments were conducted as per guidelines of CPCSEA. Technical grade Acetamiprid (ACE) (96.8% pure) used in this study was procured from Tropical Agrosystem (India) Pvt. Limited, Chennai.

Experimental design

Adult male Swiss albino mice were divided into three groups comprising of six animals each. Group I (vehicle control) animals were administered 3% aqueous solution of gum acacia and Group II and III (treatments) animals were administered acetamiprid 2.3 and 4.6 mg/kg body weight in 3% aqueous solution of gum acacia (1/20 and 1/10 of MTD), respectively. Vehicle and acetamiprid were administered to the Swiss albino mice by i.p. daily for 30 days. The desired concentration of acetamiprid was prepared in 3% aqueous solution of gum acacia, which served as vehicle. At the end of experiment, mice were sacrificed using mild anesthesia. Blood from individual animal was collected from heart at the time of sacrifice using dry sterilized vials containing anticoagulant, sodium EDTA. Various hematological parameters viz. Hb, PCV, TEC, MCV, MCH and MCHC were determined using automated hematology analyzer.

Patho-morphological Studies

At the end of experiment mice were sacrificed, macroscopic lesions in liver, kidney and testis were recorded and representative pieces of tissues from these organs were collected in 10% formal saline solution. Preserved samples were processed in ascending grade of alcohol and cleared in xylene, embedded in paraffin, sectioned at 4-5 ìm thickness and stained with

haematoxylin and eosin (H&E) 16 (Luna, 1968).

Statistical analysis

The data was expressed as Mean ± SEM and analyzed by using SPSS statistics 17 software. Data were compared by employing one way ANOVA with Duncan's multiple comparisons as post hoc test. A p-value of <0.05 was considered as statistically significant.

RESULTS

The results are presented in Table 1 and 2. The results showed non-significant decrease in value of Hb content in treated groups as compared to control group. A dose dependent decrease in other hematological parameters was also recorded in treated groups, but MCHC was found increased in treated groups as compared to control group. The value of TLC revealed dose dependent non-significant decrease as compared to control.

The histopathological examination of control mouse liver tissue revealed radially arranged hepatic cords around the central vein, while the liver of the mice treated with acetamiprid showed single cell necrosis in centrilobular region and single cell necrosis of hepatocytes and focal area of hemorrhage (Fig. 1). Kidney of acetamiprid treated mice showed cellular swelling of distal convoluted tubules, mild degeneration of proximal convoluted tubules and glomerular atrophy (Fig. 2). Testis of acetamiprid treated mice revealed detachment of seminiferous tubules with reduced spermatogenic activity and showed degeneration of spermatogonia and edematous changes in seminiferous tubules (Fig. 3 & 4). Examination of liver, kidney & testicular tissue of control rats revealed normal histological structures.

DISCUSSION

The results of the present study revealed that i.p. administration of acetamiprid for a period of 30 days exhibited a non-significant decrease in Hb, RBCs count and PCV of acetamiprid treated mice in comparison to control mice. Reduction in hemoglobin content could be due to impaired biosynthesis of heme in bone marrow (Shakoori *et al.*, 1992). Further, the reduction in Hb could also be due to increased rate of destruction of RBCs or reduction in the rate of formation of RBCs. This premise is supported by low TEC values observed in acetamiprid

Table 1:
Effect of toxicity of acetamiprid on various hematological parameters in mice.

Expt.	Treatment	Hb(g/dl)	TEC($x10^6$ /cmm)	PCV(%)	MCV(fl)	MCH(pg)	MCHC(g/dl)
30days	Control	14.10±0.44[a]	9.54±0.57[a]	47.42±0.61[a]	51.52±1.91[a]	15.25±0.46[a]	29.65±0.31[a]
	ACE(2.3mg/kg)	13.95±0.33[a]	9.39±0.35[a]	47.02±1.44[a]	50.13±0.48[a]	14.92±0.26[a]	29.7±0.28[a]
	ACE(4.6mg/kg)	13.62±0.17[a]	9.10±0.18[a]	45.8±1.22[a]	48.88±1.04[a]	14.72±0.24[a]	30.14±0.39[a]

Values are Mean ± SEM; n=6; Values bearing common superscripts within a column do not differ significantly at 5% level of significance; ACE: Acetamiprid

Table 2:
Effect of toxicity of acetamiprid on various hematological parameters in mice.

Expt.	Treatment	TLC($x10^3$/cmm)	DLC (No. of cells/100 leukocyte)				
			N	L	M	E	B
30days	Control	10.68±1.32[a]	24.41±4.36[a]	70.93±3.36[a]	2.45±1.36[a]	0.43±011[a]	1.76±0.42[a]
	ACE(2.3mg/kg)	9.11±0.61[a]	33.36±4.26[a]	63.86±4.26[a]	1.3±0.51[a]	0.42±0.11[a]	1.06±0.25[a]
	ACE(4.6mg/kg)	7.68±1.07[a]	29.34±3.73[a]	65.54±4.98[a]	3.28±2.32[a]	0.68±0.30[a]	1.16±0.48[a]

Values are Mean ± SEM; n=6; Values bearing common superscripts within a column do not differ significantly at 5% level of significance; ACE: Acetamiprid

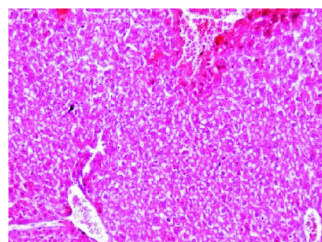

Fig.1: Liver (4.6 mg/kg b.wt.) showing single cell necrosis of hepatocytes and focal area of hemorrhage (20X).

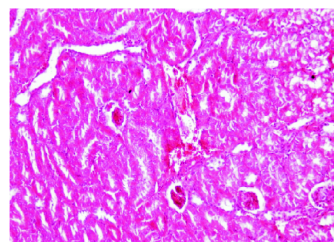

Fig.2: Kidney (4.6 mg/kg b.wt.) showing cellular swelling of distal convoluted tubules, mild degeneration of proximal convoluted tubules and glomerular atrophy (20X).

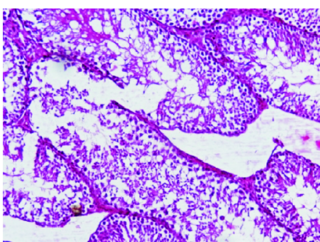

Fig.3: Testis (2.3 mg/kg b.wt.) showing detachment of seminiferous tubules with reduced spermatogenic activity (20X)

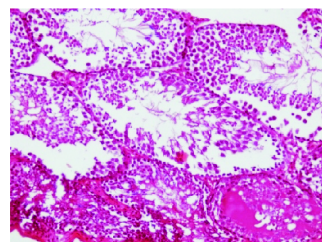

Fig.4: Testis (4.6 mg/kg b.wt) showing degeneration of spermatogonia and edematous changes in seminiferous tubules (20X).

treated mice. The reduction in blood parameters (PCV, TEC and Hb) may be attributed to a hyperactivity of bone marrow (Tung *et al.*, 1975) leading to production of red blood cells with impaired integrity which were easily destructed in the circulation. The decrease in PCV is obviously contributed by the decreased cellular count in blood after pesticide treatment. In this study, alterations in PCV, MCV and MCHC indicated that acetamiprid produces adverse effect on hematopoietic system of mice after subacute exposure. The value of TLC revealed dose dependent non-significant decrease as compared to control. However, a significant decrease in total leukocyte count (thousands/ cu.mm) was observed in female rats after oral administrations of acetamiprid for 28 days (Mondal *et al.*, 2009). Results of present study revealed that exposure to acetamiprid resulted in abnormal histological alterations in liver, kidney and testis of mice. Liver sections displayed moderate degenerative changes, single cell necrosis in centrilobular region and single cell necrosis of hepatocytes and focal area of hemorrhage. Similar observations were also made after repeated exposure of high dose of another neonicotinoid, imidacloprid (20 mg/ kg/d) for 90 days in female rats wherein the hepatocytes of liver showed mild focal necrosis with swollen cellular nuclei and cytoplasmic lesions (Bhardwaj *et al.*, 2010). Kidney of acetamiprid treated mice showed cellular swelling of distal convoluted tubules, mild degeneration of proximal convoluted tubules and glomerular atrophy. Histopathological alteration in kidney showed that acetamiprid produces nephrotoxicity in addition to hepatotoxicity. Testis of acetamiprid treated mice revealed detachment of seminiferous tubules with reduced spermatogenic activity and showed degeneration of spermatogonia and edematous changes in seminiferous tubules. This is in agreement with the similar finding in albino mice reported earlier by Chawsheen (2011).

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STUDIES ON ACUTE TOXICITY AND NEUROBEHAVIOURAL PROFILES OF IMIDACLOPRID IN MICE

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ABSTRACT

The maximum tolerated dose (MTD) of imidacloprid, a neonicotinoid insecticide was found to be 55 mg/kg, i.p. in male Swiss albino mice by conducting pilot dose range finding studies. Various gross behavioural profiles viz. spontaneous motor activity, forced locomotor activity, cocaine induced increased locomotor activity, amphetamine induced increased locomotor activity were determined. Imidacloprid decreased spontaneous motor activity and forced locomotor activity in a dose dependent manner and antagonised the cocaine and amphetamine induced locomotor behaviour. These findings indicated that imidacloprid possesses the moderate toxic potential and alters the normal behaviour of mice.

Key words: Imidacloprid, amphetamine, cocaine, spontaneous motor activity

INTRODUCTION

Last decade has seen the development of a new major class of highly effective and widely used insecticides i.e. neonicotinoids for crop protection and veterinary pest control. Imidacloprid, a neonicotinoid insecticide is used extensively in agricultural practices. It has been suggested to act in a specific manner by inhibiting nicotinic acetylcholine receptor channel (Nagata *et al.*, 1997) in insects. Interruption of synaptic transmission by imidacloprid thus leads to sustained contractions of respiratory muscles and death of the insects (Bai *et al.*, 1991). Imidacloprid has been shown to be highly selective and provide outstanding control over sucking pests like aphids, leaf hoppers, thrips, leaf miners, beetles and white flies in vegetables. It has shown excellent activity against citrus leaf miners and apple leaf miners and highly effective for flea control in cats and dogs (Tomizawa and Casida, 2005). Due to its widespread usage in agriculture and veterinary medicine, there is every possibility that the persons handling it or animals may accidently be exposed to it. Moreover reports of homicidal tendencies are also available with insecticides.

Therefore, the present study was planned to investigate the acute toxicity and neurobehavioural changes induced by imidacloprid in mice. Acute toxicity studies give insights into the mechanisms of action and biologic effects of the compounds. Behaviour is considered as the final expression of integrated nervous functions and locomotor activities as behavioural end point (Lawrence, 1978).

MATERIALS AND METHODS

Experimental animals

Male swiss albino mice weighing 20-25 g were procured from Disease Free Small Animal House of the University and maintained in the Departmental Small Animal House. The animals were housed in polyacrylic cages in a group of five animals per cage and kept at room temperature with a natural light-dark cycle. The animals were provided feed and water *ad libitum*. All the experiments were conducted in noise free laboratory conditions. The prior approval of Institutional Animal Ethical Committee for the protocol of this study was obtained and formulation of imidacloprid (Confidor, 200 SL) was used for this study.

Maximal tolerated dose

Maximal tolerated dose (MTD) of imidacloprid by intraperitoneal route was determined by conducting pilot dose range finding studies. Initially small groups of animals (n = 5/dose) were administered a single dose of imidacloprid and observations were recorded at various time intervals. A range of doses was used initially, including a few lethal doses and then several iterations were selected to determine a dose that would produce clear signs of toxicity but not result in lethality i.e. maximal tolerated dose (MTD). To verify the MTD, additional groups (n=5/dose) were administered that dose and were tested to define more closely the time course of effects.

Gross observable behaviour

The effect of imidacloprid on gross observable behavioural profiles was studied using Irwin schedule as described by Turner (1965). The mice were distributed randomly in three groups (n=5). The control group was administered 10 ml/kg of gum acacia solution (2%) and treatment groups received imidacloprid at the dose rate of $2/5^{th}$ or $4/5^{th}$ of MTD by intra-peritoneal route. The procedure involved manipulative phase in which animals were subjected to least provoking stimuli. The animals were observed for behavioural activity profiles at various time intervals (2, 10, 20, 40, 60, 120, 240, 360 and 1440 minutes) and scores were assigned as per Irwin schedule, as described by Turner (1965). The onset, duration of effect

and time of peak effect was determined.

Spontaneous motor activity

Spontaneous motor activity (SMA), a measure of exploratory behaviour of animals was monitored using a computerised activity meter (Opto verimex, Columbus Instruments, USA) as described by Matsuoka *et al.* (1993). It consisted of transparent cage of dimensions 43 cm X 43 cm. The cage space was transcended by perpendicular horizontal and vertical infra-red beams such that the movements of the animal intercepted the infra-red beams. These interceptions were monitored by a computer based system. The system was programmed to count the interceptions at 5, 15, 30, 45 and 60 minutes for a period of five minutes. Animals were divided randomly in three groups of five animals each. Control group received 10 ml/kg of gum acacia solution (2%), while treatment groups received 2/5th or 4/5th of MTD of imidacloprid intraperitoneally. Immediately after the treatment, the animals were placed in the activity cage/chamber. Various components of locomotor behaviour viz. distance travelled, resting time, stereotypy time, ambulatory time, bursts of stereotypy, horizontal counts, ambulatory counts were noted.

Forced locomotor activity

It is a measure of strength and co-ordinated movements of animal. The effect of imidacloprid on forced locomotor activity was studied using rota rod (Acceler, Rota rod 7750 UGO Basile, Italy) as described by Dunham and Miya (1957). The mice were randomly divided in three groups of five animals each and trained to remain on the horizontal rod of rota rod apparatus rotating at twenty five revolutions per minute. As soon as mice fell off the rota rod, they were immediately placed back on the rod. The training session was terminated when mice remained on the rod continuously for two minutes. Next day, the effect of imidacloprid was studied thirty minutes after the treatment. Control group received 10 ml/kg of gum acacia solution (2%), while treatment groups received 2/5th or 4/5th of MTD of imidacloprid intraperitoneally. Thirty minutes after the treatment, the mice were given three consecutive trials of two minutes each on the rota rod. The cumulated time spent on the rota rod was recorded with a cut off of two minutes.

Cocaine induced increased locomotor activity

The effect of imidacloprid on cocaine induced increased locomotor activity was studied in mice. Mice were randomly divided in three groups, each having five animals. Control group received 10 ml/kg of gum acacia solution (2%), while treatment group received 2/5th or 4/5th of MTD of imidacloprid intraperitoneally thirty minutes prior to cocaine (15 mg/kg, s.c.) administration and observed for various components of locomotor behaviour viz. distance travelled, resting time, stereotypy time, ambulatory time, bursts of stereotypy, horizontal counts, ambulatory counts using a computerised activity meter (Opto verimex, Columbus Instruments, USA). The system was programmed for observation of various components of locomotor behaviour at five minutes interval for a period of five minutes. Five observations for various components of locomotor behaviour were recorded at 5, 10, 15, 20, and 25 minutes after treatment.

Amphetamine induced increased locomotor activity

The effect of imidacloprid on amphetamine induced increased locomotor activity was studied in mice. Mice were randomly divided in three groups, each having five animals. Control group received 10 ml/kg of gum acacia solution (2%), while treatment groups received 2/5th or 4/5th of MTD of imidacloprid intraperitoneally thirty minutes prior to amphetamine (0.7 mg/kg, s.c.) administration. The animals were then observed for various components of locomotor behaviour viz. distance travelled, resting time, stereotypy time, ambulatory time, bursts of stereotypy, horizontal counts, ambulatory counts using a computerised activity meter (Opto verimex, Columbus Instruments, USA). The system was programmed to note various components of locomotor behaviour at five minutes interval for a period of five minutes. Five observations for various components of locomotor behaviour were recorded at 5, 10, 15, 20, and 25 minutes after treatment.

RESULTS

For determining initially the range for calculation of MTD, various iterations of imidacloprid (80, 75, 70, 65, 60, and 55 mg/kg, i.p.) were used and MTD was found to be 55 mg/kg body weight by intraperitoneal route. It produced a dose related onset and severity of toxic symptoms. Symptoms of toxicity were seen as early as two min following intraperitoneal administration of imidacloprid. The major gross observable symptoms induced by imidacloprid were decreased alertness and grooming, prolonged restlessness, tremors, convulsions and ataxia. In addition, open mouth breathing, flexion of head followed by death in some animals was also observed. However, stereotypy, writhing and straub tail were not observed at any of the iterations used in the study.

Mortality, if any, took place as early as 10-25 min and no mortality was observed after 24 h. No muscarinic effects viz. salivation, lacrymation, defaecation, urination were observed at any of the dose levels of imidacloprid studied in this investigation.

Effect of 22 mg/kg and 44 mg/kg, i.p. of imidacloprid on gross observable behaviour was studied (Tables 1 and 2). Lower dose (22 mg/kg, i.p.) produced no effect on the profiles observed. Higher dose caused decreased alertness and grooming, restlessness, tremors, decreased snout withdrawal response. On the basis of these observations the onset, peak effect and duration of

Table 1:
Effect of imidacloprid (22 mg/kg, i.p.) on gross observable behavioural profiles in mice Activity profiles Normal score Scores at various time intervals (min) after drug administration

Activity profiles	Normal score	Scores at various time intervals (min) after drug administration								
		2	10	20	40	60	120	240	360	1440
Alertness	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Stereotypy	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Grooming	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Restlessness	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Touch response	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Pain response	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Startle response	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Straub tail	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Tremors	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Twitches	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Convulsions	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Body posture	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Limb position	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Ataxia	0	0.0±0.00	0.00±0.0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Grip strength	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Pinnal reflex	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Corneal reflex	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Writhing	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Snout withdrawal	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00

Values are mean ± SEM of five observations.

Table 2:
Effect of imidacloprid (44 mg/kg, i.p.) on gross observable behavioural profiles in mice Activity profiles Normal score Scores at various time intervals (min) after drug administration

Activity profiles	Normal score	Scores at various time intervals (min) after drug administration								
		2	10	20	40	60	120	240	360	1440
Alertness	4	3.7±0.25	3.5±0.50	3.5±0.02	2.7±0.60	1.5±0.14	3.7±0.25	4.0±0.00	4.0±0.00	4.0±0.00
Stereotypy	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Grooming	4	4.0±0.00	4.0±0.00	4.0±0.00	3.5±0.00	3.5±0.02	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Restlessness	0	1.0±0.57	1.2±0.47	2.2±0.25	2.0±0.70	2.2±0.75	2.0±0.70	0.0±0.00	0.0±0.00	0.0±0.00
Touch response	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Pain response	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	3.7±0.25	3.7±0.25	4.0±0.00	4.0±0.00	4.0±0.00
Startle response	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Straub tail	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Tremors	0	0.0±0.00	0.0±0.00	0.5±0.50	0.7±0.75	1.0±0.57	0.5±0.50	0.0±0.00	0.0±0.00	0.0±0.00
Twitches	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Convulsions	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Body posture	4	3.5±0.28	3.5±0.28	3.0±0.40	3.5±0.50	3.0±0.57	3.5±0.05	4.0±0.00	4.0±0.00	4.0±0.00
Limb position	4	3.5±0.28	3.5±0.28	3.0±0.40	3.5±0.50	3.0±0.57	3.5±0.05	4.0±0.00	4.0±0.00	4.0±0.00
Ataxia	0	0.2±0.25	0.7±0.25	1.0±0.57	1.5±0.28	1.2±0.25	1.5±0.28	0.0±0.00	0.0±0.00	0.0±0.00
Grip strength	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Pinnal reflex	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Corneal reflex	4	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00	3.5±0.00	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00
Writhing	0	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Snout withdrawal	4	4.0±0.00	4.0±0.00	4.0±0.00	3.5±0.02	3.5±0.02	4.0±0.00	4.0±0.00	4.0±0.00	4.0±0.00

Values are mean ± SEM of five observations.

imidacloprid effect was found to be 2 minutes, 30 minutes and 2 h respectively. The observations at peak effect of imidacloprid on various behavioural profiles irrespective of time are shown in Table 3.

Spontaneous motor activity in mice was decreased by imidacloprid. Various components of locomotor behaviour were affected by lower as well as higher dose levels of imidacloprid (Table 4). There was decrease in locomotor activity. However, forced locomotor activity was decreased only at higher dose (Table 5).

Imidacloprid antagonised the cocaine and amphetamine induced effect on various components of locomotor behaviour in a dose dependent manner. However, the antagonistic effect was significant for cocaine induced behaviour (Tables 6 and 7).

DISCUSSION

Rapid absorption of imidacloprid from peritoneum of the mice could be the probable cause of rapid onset of severe toxic symptoms and death. It has been suggested

Table 3:
Peak Effect of imidacloprid on gross observable behavioural profiles in mice

Activity profiles	Normal score	Peak scores after imidacloprid (mg/kg, i.p.) administration		
		22	44	55
Alertness	4	4.0±0.00	0.0±0.00	0.0±0.00
Stereotypy	0	0.0±0.00	0.0±0.00	0.0±0.00
Grooming	4	4.0±0.00	0.0±0.00	0.0±0.00
Restlessness	0	0.0±0.00	2.7±0.12	4.0±0.00
Touch response	4	4.0±0.00	4.0±0.00	1.0±0.00
Pain response	4	4.0±0.00	3.7±0.12	1.5±0.28
Startle response	4	4.0±0.00	4.0±0.00	4.0±0.00
Straub tail	0	0.0±0.00	0.0±0.00	0.0±0.00
Tremors	0	0.0±0.00	2.0±1.00	4.0±0.00
Twitches	0	0.0±0.00	0.0±0.00	0.0±0.00
Convulsions	0	0.0±0.00	0.0±0.00	0.0±0.00
Body posture	4	4.0±0.00	2.0±0.00	1.0±0.00
Limb position	4	4.0±0.00	2.0±0.00	1.0±0.00
Ataxia	0	0.0±0.00	2.0±0.00	2.0±0.00
Grip strength	4	4.0±0.00	4.0±0.00	4.0±0.00
Pinnal reflex	4	4.0±0.00	4.0±0.00	4.0±0.00
Corneal reflex	4	4.0±0.00	4.0±0.00	4.0±0.00
Writhing	0	0.0±0.00	0.0±0.00	0.0±0.00
Snout withdrawal	4	4.0±0.00	3.0±0.00	4.0±0.00

Values are mean ± SEM of five observations.

by Tomizawa *et al.* (2000) that imidacloprid acts at nicotinic acetylcholine receptor site. It causes complete and irreversible blockade of post synaptic nAchR in central nervous system of insects (Abbink, 1991). The gross observable signs in this study showed that imidacloprid acts as agonist on nAchR. Respiratory failure in mice treated with imidacloprid might be due to both central paralysis and peripheral blockade of muscles of respiration

Table 5:
Effect of imidacloprid on forced locomotor activity in mice

Dose (mg/kg, i.p.)	Time (sec) spent on Rotarod
Control	120.0±0.00^{A}
22	120.0±0.00^{A}
44	96.1±12.70^{B}

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

(Hardman *et al.*, 1996). Tomizawa and Casida (2005) reported that the mammalian toxicity of neonicotinoids is centrally mediated since the symptoms of poisoning are similar to that of nicotine and agonist action in the vertebrate á4â2 nAchR, the primary target in brain.

Activation of mesencephalic dopamine pathway results in increase in locomotor activity (Robbins and Everitt, 1982). Disruption of normal activity of dopamine cells in the ventral tegmental area or substantia nigra, with lesions or pharmacological manipulations, can inhibit locomotor activity (Koob *et al.*,1981). Nicotine is known to pass through the blood brain barrier readily (Oldendorf, 1971). Acute administration of nicotine activates the cortical region and diminishes locomotor activity in animals (Schechter and Rosecrans, 1972). Similar motor incoordination and decrease in SMA have been reported in isoproturon toxicity in mice (Sarkar, 1990) suggesting that reduction in SMA and effect on motor coordination may be through involvement of distinct inhibitory action on motor performances.

Administration of cocaine leads to increased ambulatory activity and dopaminergic system plays an important role in mediating the ambulatory and rewarding

Table 4:
Effect of imidacloprid on spontaneous motor activity (SMA) in mice

Parameter	Dose	Time (min) after treatment with imidacloprid				
	(mg/kg, i.p.)	5	15	30	45	60
Distance travelled (cm)	Control	560.0±91.70 ^{A}	456.2±172.70 ^{A}	398.8±175.30 ^{A}	436.8±143.80 ^{A}	347.2±151.00^{A}
	22	244.4±55.00 ^{B}	140.4±85.20 ^{AB}	154.4±67.30 ^{AB}	239.2±74.70 ^{AB}	94.6±36.00^{AB}
	44	6.2±3.30 ^{C}	14.6±14.60 ^{B}	6.6±3.40 ^{B}	0.0±0.00 ^{B}	0.6±0.60^{B}
Resting time (sec)	Control	152.6±41.80 ^{A}	161.8±53.00^{A}	165.2±53.70 ^{A}	152.8±47.20 ^{A}	164.0±48.60 ^{A}
	22	237.2±26.30 ^{AB}	271.0±15.20 ^{B}	260.6±18.20 ^{AB}	266.4±9.20 ^{B}	267.6±13.50 ^{B}
	44	297.2±1.40 ^{B}	299.2±0.80 ^{B}	297.6±1.10 ^{B}	300.0±3.2 ^{B}	299.6±0.40 ^{B}
Stereotypy time (sec)	Control	88.6±30.00 ^{A}	90.2±37.30 ^{A}	92.8±38.10 ^{A}	98.0±39.60 ^{A}	101.2±35.50 ^{A}
	22	41.8±26.80 ^{AB}	11.4±8.20 ^{B}	24.0±14.00 ^{AB}	14.0±4.10 ^{B}	23.4±10.90 ^{B}
	44	2.0±1.10 ^{B}	0.0±0.00 ^{B}	1.6±1.02 ^{B}	0.0±0.00 ^{B}	0.2±0.20 ^{B}
Ambulatory time (sec)	Control	58.8±12.12 ^{A}	48.0±16.80 ^{A}	42.0±17.00 ^{A}	48.0±15.00 ^{A}	34.5±15.20 ^{A}
	22	20.8±5.80 ^{B}	12.6±7.20 ^{B}	15.4±4.70 ^{AB}	19.6±6.00 ^{AB}	9.0±3.00 ^{B}
	44	0.8±0.30 ^{B}	0.8±0.80 ^{B}	0.8±0.30 ^{B}	0.0±0.00 ^{B}	0.2±0.20 ^{B}
Bursts of stereotypy	Control	40.2±12.50 ^{A}	37.4±15.13 ^{A}	35.4±14.50 ^{A}	36.0±11.90 ^{A}	36.7±13.30 ^{A}
	22	15.6±5.80 ^{AB}	7.4±5.40 ^{B}	10.8±4.70 ^{AB}	8.8±2.00 ^{B}	10.2±3.70 ^{B}
	44	1.4±0.60 ^{B}	0.0±0.00 ^{B}	0.8±0.50 ^{B}	0.0±0.00 ^{B}	0.2±0.20 ^{B}
Horizontal counts	Control	469.4±164.90 ^{A}	585.4±242.00 ^{A}	501.8±210.30 ^{A}	631.2±276.90 ^{A}	566.5±206.80 ^{A}
	22	302.2±135.10^{AB}	115.6±91.80 ^{B}	154.8±96.20 ^{AB}	113.4±65.300 ^{B}	378.2±212.50 ^{AB}
	44	4.2±2.37 ^{B}	1.4±1.40 ^{B}	6.8±3.40 ^{B}	0.0±0.00 ^{B}	0.2±0.20 ^{B}
Ambulatory counts	Control	255.8±90.10 ^{A}	254.0±1.50 ^{A}	256.4±112.00 ^{A}	236.0±110.00 ^{A}	204.2±104.40 ^{A}
	22	73.4±20.60 ^{B}	25.0±16.50 ^{B}	39.8±22.80 ^{B}	27.8±7.10 ^{B}	24.8±10.90 ^{B}
	44	2.8±1.46 ^{B}	0.8±0.80 ^{B}	0.8±0.48 ^{B}	0.0±0.00 ^{B}	0.2±0.20 ^{B}

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

Table 6:
Effect of imidacloprid on cocaine (15.0mg/kg, s.c.) induced locomotor activity in mice

Parameter	Dose (mg/kg, i.p)	Time (min) after treatment with cocaine				
		5	10	15	20	25
Distance travelled (cm)	Control	1420.0±389.00 ^{A}	1654.0±654.58 ^{A}	1506.2±591.20 ^{A}	1828.0±472.5 ^{A}	1874.64±459.1 ^{A}
	22	457.0±287.20 ^{B}	787.6±383.60 ^{AB}	1145.4±346.40 ^{AB}	934.2±460.01 ^{AB}	712.6±354.6 ^{B}
	44	58.0±39.00 ^{B}	86.0±23.90 ^{B}	48.0±30.50 ^{B}	32.0±13.90 ^{B}	51.2±23.00 ^{B}
Resting time (sec)	Control	98.8±45.60 ^{A}	116.2±67.37	115.4±65.71 ^{A}	68.8±44.60 ^{A}	51.0±34.47 ^{A}
	22	216.8±36.00 ^{B}	190.8±47.80	181.2±50.60 ^{AB}	175.4±58.50 ^{AB}	198.2±49.20 ^{B}
	44	287.4±6.40 ^{B}	255.2±8.04	277.6±6.80 ^{B}	279.0±6.30 ^{B}	277.2±3.10 ^{B}
Stereotypy time (sec)	Control	67.0±17.58 ^{A}	40.0±16.90	40.4±15.48	64.0±15.92 ^{A}	66.0±12.08 ^{A}
	22	41.8±15.83 ^{AB}	48.0±19.26	47.6±19.40	44.2±19.80 ^{AB}	35.0±15.43 ^{AB}
	44	6.4±2.80 ^{B}	30.4±6.90	14.4±4.20	15.2±4.70^{B}	15.8±2.40 ^{B}
Ambulatory time (sec)	Control	134.2±38.70 ^{A}	143.8±55.67 ^{A}	140.2±59.80 ^{A}	167.2±41.70 ^{A}	183.0±58.70 ^{A}
	22	41.0±21.40 ^{B}	61.2±30.60 ^{AB}	71.2±34.40 ^{AB}	80.4±40.20 ^{B}	66.8±34.47 ^{B}
	44	6.4±3.60 ^{B}	14.4±2.40 ^{B}	8.0±3.90 ^{B}	5.8±2.00 ^{AB}	7.0±2.40 ^{C}
Bursts of stereotypy	Control	41.8±8.77 ^{A}	21.4±10.30	30.6±10.50	42.8±7.90 ^{A}	44.8±7.60 ^{A}
	22	24.6±8.00 ^{AB}	28.6±11.00	31.2±12.10	32.0±13.70 ^{AB}	24.8±10.88 ^{AB}
	44	5.0±2.40 ^{B}	19.0±3.30	8.4±2.40	11.0±3.00 ^{B}	10.4±1.00 ^{B}
Horizontal counts	Control	1099.2±406.90 ^{A}	1249.0±491.80 ^{A}	1225.0±490.00 ^{A}	1465.8±363.30 ^{A}	1575.0±335.00 ^{A}
	22	342.4±199.00 ^{AB}	452.6±257.2 ^{AB}	592.6±314.70 ^{AB}	570.6±311.70 ^{B}	500.6±284.60 ^{B}
	44	45.6±28.90 ^{B}	155.4±44.70 ^{B}	115.0±42.24 ^{B}	65.4±22.60 ^{B}	76.6±16.70 ^{B}
Ambulatory counts	Control	820.6±317.10 ^{A}	977.2±390.00 ^{A}	946.4±387.00 ^{A}	1110.8±295.80 ^{A}	1204.4±282.30 ^{A}
	22	226.0±148.70 ^{AB}	328.6±191.60 ^{AB}	427.2±332.50 ^{AB}	417.0±229.10 ^{B}	365.0±205.70 ^{B}
	44	28.0±18.80 ^{B}	78.0±17.70 ^{B}	72.0±32.29 ^{B}	37.4±13.70 ^{B}	34.0±13.11 ^{B}

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

Table 7:
Effect of imidacloprid on amphetamine (0.7mg/kg, s.c.) induced locomotor activity in mice

Parameter	Dose (mg/kg, i.p)	Time (min) after treatment with amphetamine				
		5	10	15	20	25
Distance travelled (cm)	Control	123.2±43.50	286.6±114.50	112.0±71.80	160.0±85.00	241.0±75.60^{A}
	22	74.0±59.11	135.6±131.60	130.4±129.40	44.6±40.20	37.6±36.80^{B}
	44	29.7±18.00	28.0±11.60	14.2±8.30	1.7±1.75	1.2±1.25^{B}
Resting time (sec)	Control	262.8±14.00	239.8±25.00	269.6±14.70	266.4±19.18	243.2±21.90^{A}
	22	278.0±14.60	268.8±27.00	265.0±32.70	279.2±17.40	289.4±9.60^{AB}
	44	297.0±1.70	296.2±1.60	298.5±0.86	299.7±0.25	299.7±0.25^{B}
Stereotypy time (sec)	Control	25.8±11.20	39.2±18.12	20.6±9.30	19.6±11.80	32.2±12.60^{A}
	22	16.2±11.20	17.2±13.80	23.2±21.20	17.2±14.30	7.6±6.80^{AB}
	44	1.0±1.00	1.5±0.95	0.5±0.50	0.0±0.00	0.0±0.00^{B}
Ambulatory time (sec)	Control	11.2±3.20	21.0±7.40	9.8±5.70	14.0±7.50	24.6±9.70^{A}
	22	5.8±3.40	14.0±13.20	11.8±11.50	4.0±3.03	3.2±2.70^{B}
	44	1.75±1.03	2.2±0.85	1.0±0.70	0.2±0.25	0.2±0.25^{B}
Bursts of stereotypy	Control	12.2±4.20	21.0±8.10	12.8±5.90	12.2±7.20	20.4±7.40^{A}
	22	9.2±6.50	13.4±7.50	12.6±11.60	7.4±6.40	4.4±3.90^{AB}
	44	1.0±0.70	1.5±0.95	0.5±0.50	0.0±0.00	0.0±0.00^{B}
Horizontal counts	Control	209.4±133.30	319.0±185.20	103.4±60.50	187.6±125.80	247.6±134.20^{A}
	22	116.6±97.10	235.2±198.30	330.8±294.12	262.0±215.00	61.8±57.40^{AB}
	44	10.5±7.30	12.5±6.50	4.5±3.50	0.2±0.25	0.2±0.25^{B}
Ambulatory counts	Control	29.8±12.17	68.8±25.90	28.4±17.50	39.4±23.10	63.8±27.40^{A}
	22	26.2±17.60	39.0±35.30	53.6±52.30	33.6±32.10	7.8±6.60^{B}
	44	2.2±1.40	2.5±1.19	1.7±1.03	0.2±0.25	0.2±0.25^{B}

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

activities of cocaine (Roy *et al.*, 1978). One of the neural substrate mediating this ambulation-acceleration activities of cocaine like psychostimulants as well as amphetamine is the increase in the dopamine level in the nucleus accumbens (Bradberry and Roth, 1989; Kuczenski and segal, !989). Therefore, the phenomenon of enhancement of dopamine receptor sensitivity can be regarded as a kind of possible mechanism of behaviour sensitization to psychomotor stimulatory drugs. Imidacloprid acts on nicotinic receptors in insects. Nicotine is known to potentiate the effect of amphetamine induced behaviour (Bhatwadekar *et al.*, 1999) which has been attributed to dopamine releasing effect of nicotine. However, in this study imidacloprid acted in antagonistic manner indicating that it does not behave exactly like nicotine in mammals.

Results of the present investigation indicate that

imidacloprid affects the normal behaviour in mice and also it does not behave exactly like nicotine in mice, having toxic potential and is a moderate risk insecticide.

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Research Article

PHARMACOKINETIC AND PLASMA PROTEIN BINDING ANALYSIS OF CEFTIOFUR AND ITS ACTIVE METABOLITE AFTER SUBCUTANEOUS ADMINISTRATION IN BUFFALO CALVES

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ABSTRACT

Pharmacokinetics of ceftiofur was studied in healthy buffalo calves following single subcutaneous injection (2 mg.kg^{-1}). Plasma concentrations of ceftiofur and its active metabolite DFC were determined using HPLC-DAD. Calibration curves were prepared in the range of 0.1–10 µg.ml^{-1} with good linearity (r^2= 0.9999). LOD and LOQ were 0.03 and 0.1 µg.ml^{-1}, respectively. Precision and accuracy were within the acceptable limits. The values of C_{max}, T_{max}, and $t_{½ka}$ were 2.96 ± 0.05 ìg.ml^{-1}, 0.92 ± 0.05 h and 0.13 ± 0.02 h, respectively. The value of AUC (25.2 ± 0.6 µg.h.ml^{-1}) indicated good antibacterial activity. $V_{d(area)}$ of 1.90 ± 0.12 L.kg^{-1} and $t_{½á}$ of 3.15 ± 0.09 h reflected better tissue distribution of the drug. The longer elimination half life (16.5 ± 0.79 h) along with smaller Cl_B (0.08 ± 0.002 L.kg^{-1}.h^{-1}) showed slow excretion of the drug from the animal body. Protein binding of ceftiofur was 40.9%. % T > MIC value gave an indication that selected dose can be repeated safely for 24 h interval for the effective treatment of the diseases caused by the pathogens having MIC d” 0.5 ìg.ml^{-1}.

Keywords: Pharmacokinetics, Ceftiofur, Buffalo calves, HPLC.

INTRODUCTION

Ceftiofur belongs to third generation cephalosporin antibiotic used in the treatment of bovines (Sawant *et al.*, 2005). It has good spectrum of activity against Gram-negative as well as Gram-positive bacteria, including beta lactamase producing strains because of presence of additional methoxyimino side-chain to the aminothiazole group (Yancey *et al.*, 1987). Ceftiofur is effective against *Pasteurella multocida*, *Mannheimia hamemolytica, Escherichia coli*, *Arcanobacterium pyogenes, Fusobacterium necrophorum, Prevotella melaninogenica* (Dore *et al.*, 2010; Witte *et al.*, 2011).

During recent few years, studies based on the systemic administration and efficacy of broad spectrum antibiotics has increased tremendously. Following intramuscular (i.m.) injection, ceftiofur undergoes rapid hydrolysis to the microbiologically active protein bound metabolite called desfuroylceftiofur (DFC) which is having much longer elimination half-life than parent ceftiofur (Salmon *et al.*, 1996). To avoid muscle soreness following IM administration, some practitioners have administered ceftiofur via subcutaneous (s.c.) route (Salmon *et al.*, 1996).

The pharmacokinetics of ceftiofur has been conducted in cattle (Brown *et al.*, 1996, 2000; Okker *et al.*, 2002). But the literature in buffalo species- one of the greatest contributor in the livestock sector in India is not available. It is also not recommended to extrapolate the data of one species to other species of animal without conducting the detailed pharmacokinetic study (Sharma and Srivastava, 2006). The studies in cattle and buffalo species have underlined the differences in the pharmacokinetics of cephalosporin antibiotic (Joshi and Sharma, 2009). The present study was therefore planned to investigate pharmacokinetic profile of ceftiofur after s.c. administration and its plasma protein binding in healthy buffalo calves.

MATERIALS AND METHODS

The experiment was conducted on six male buffalo calves of 6-12 months age (weight 90-120 kgs) after obtaining permission from Ethics Committee. Ceftiofur (Ceftivet, Pfizer India Ltd.) was administered subcutaneously in the neck region at a dose of 2 mg.kg^{-1} body weight. Blood samples (4-5 ml) were collected in tubes containing 8-10 units heparin per ml. of blood via jugular venipuncture at 0, 2.5, 5, 10, 15, 30, 45 min.,1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 18, 24, 36 and 48 hours following ceftiofur administration. Subsequently, plasma was separated and stored at -20°c until further analysis. Ceftiofur (Vetranal) standard was purchased from Sigma Aldrich. All the reagents used for the analysis were of HPLC grade. The assay determined ceftiofur as well as its metabolite i.e. DFC (both in bound as well free form).

Perkin Elmer system with UV/VIS detector (Series 200) set at 265 nm was used for analysis. The reverse-phase chromatography was performed with an analytical C_{18} column (Lichrosphere®, Particle size 5µ, 4.6 × 150 mm, Merck, Germany). Mobile phase conditions were similar to that of Jacobson *et al.* (2006). TotalChrom software® (version 6.1) was used for instrument control and data analysis. The samples were processed as per the method of Baere *et al.* (2004) with modification to

evaporate elute under vacuum concentrator (Eppendorf 5301) at 30 ^{0}C. The dry residue was dissolved in 500 µl of 0.1 % TFA in water, vortexed and filtered through 0.45 µm nylon filter (Millipore, Bedford, MA) and 50 µl aliquot was injected.

The retention time of desfuroylceftiofuracetamide (DCA) was 23 ± 0.5 minutes with total run time of 27 minutes. The DCA was absent in the blank plasma samples of buffalo calves not administered with ceftiofur sodium. The linearity of the method was evaluated in the range of 0.1 to 10 µg.ml^{-1} concentration with correlation coefficient (R^2) 0.9999. Plasma concentrations from treated animals were computed from the equation of the calibration curve y = 36224x + 1180 in the proposed range. The limit of detection (LOD) and quantification (LOQ) determined by signal-to-noise ratio were 0.03 µg.ml^{-1} and 0.1 µg.ml^{-1} respectively. The precision (interday and intraday) was more than 95% and the bias was within 5%. Recovery was in the range of 90 – 105%.

RESULTS

The best suited model for pharmacokinetic analysis was determined by visual examination of individual concentration–time curves. Various pharmacokinetic determinants were calculated according to standard equations given by Gibaldi and Perrier (1982). The time for which the plasma drug levels remain above or equal to minimum inhibitory concentration (MIC) value was calculated as per the method of Turnidge (1998). *In vitro*

Table 1.
Pharmacokinetic parameters of ceftiofur and desfuroylceftiofur after single subcutaneous administration of ceftiofur sodium (2 mg.kg^{-1})in healthy buffalo calves.

Parameter	Unit	Mean ±SE
$t_{½ka}$	h	0.13 ± 0.02
$t_{½á}$	h	3.15 ± 0.09
$t_{½β}$	h	16.49 ± 0.79
AUC	µg.h ml^{-1}	25.20 ± 0.60
AUMC	µg. h^{2}ml^{-1}	614.8 ± 25.6
$V_{d(area)}$	L.kg^{-1}	1.90 ± 0.12
$V_{d(B)}$	L.kg^{-1}	1.85 ± 0.12
Cl_B	L.kg^{-1}.h^{-1}	0.08 ± 0.002
MRT	h	24.47 ± 1.15
C_{max}	µg.ml^{-1}	2.96 ± 0.05
T_{max}	h	0.92 ± 0.05

$t_{1/2ka}$, absorption half-life; $t_{½á}$, distribution half life; AUC, area under concentration–time curve; AUMC, area under the first moment curve; Vd(area), apparent volume of distribution based on AUC; $V_{d(B)}$, volume of distribution based on dose; Cl_B, total body clearance; MRT, mean residence time; C_{max}, plasma drug concentration; T_{max}, time at which Cmax is attained.

Table 2:
Time above minimum inhibitory concentration (%T > MIC) expressed as percentage of the inter-dose interval (8, 12, 24, 48 h) for the suncutaneous administration of ceftiofur sodium (2 mg.kg^{-1}) to healthy buffalo calves.

Interdose interval (h)	MIC (µg.ml^{-1})					
	0.1	0.13	0.2	0.25	0.4	0.5
8	700	634	493.9	428	288	221
12	467	422	329.2	285	192	148
24	233	211	164.6	143	95.9	73.8
48	117	106	82.31	71.3	47.9	36.9

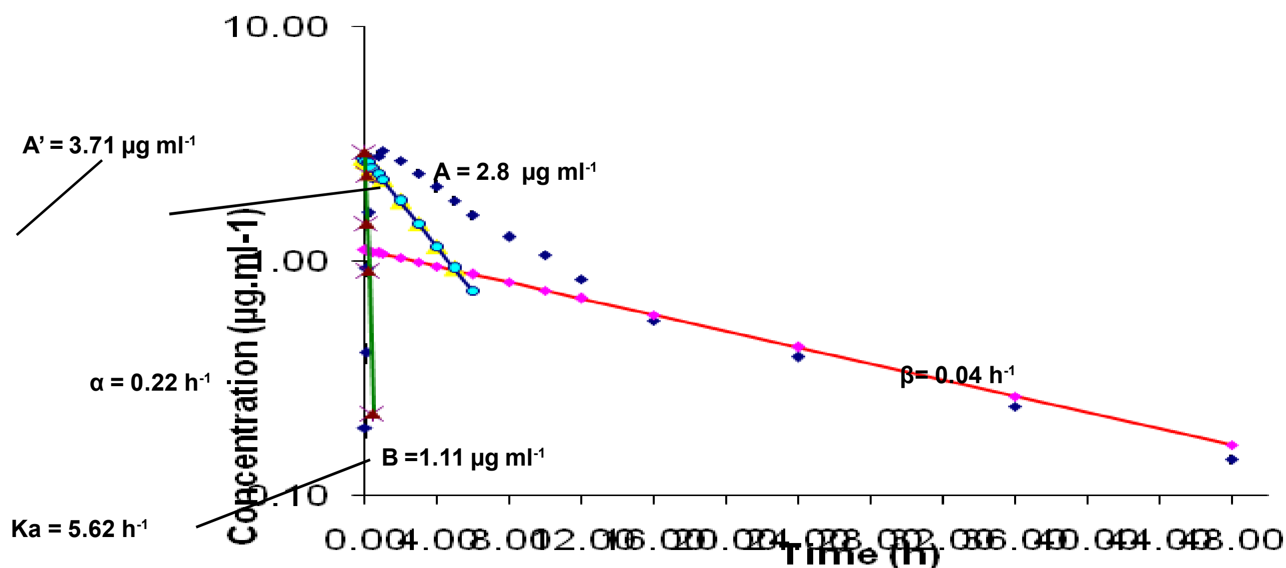

Fig. 1.
Semilogarithmic plot of plasma concentration-time profile of ceftiofur following single subcutaneous administration (2 mg.kg^{-1}) in healthy buffalo calves. Absorption (Ka), distribution (á) and elimination (â) phases are represented by least square regression lines. The calculated points of distribution phase were obtained by feathering technique. Constants A', A and B are zero-time intercepts of absorption, distribution and elimination phases, respectively. Symbols ×, Ä and Ê% indicate regression line of absorption, distribution and elimination phase respectively.

binding of ceftiofur to plasma proteins was determined by employing the equilibrium dialysis technique (Gupta *et al.*, 2006).

There were no adverse effects after s.c. administration of ceftiofur in buffalo calves. The logarithm of plasma ceftiofur concentration versus time curves (Mean±SE) was best fitted into a two-compartment open model (Fig 1).

The various pharmacokinetic parameters for ceftiofur and its metabolite DFC were calculated and presented in table 1. The calculated % T e" MIC values for ceftiofur using pharmacokinetic parameters obtained after s.c. dose for 8, 12, 18 and 24 h dosing intervals and MIC for 0.1, 0.125, 0.2, 0.25, 0.4 and 0. 5 ìg ml^{-1} are presented in Table 2.

DISCUSSION

Absorption half life ($t_{½ka}$) in the present study was (0.13 h) very short indicating faster absorption of the drug after s.c. injection compared to previous studies which might be due to difference in species, routes of administration and formulations of the ceftiofur used (Brown *et al.*, 1996). Distribution half life of 3.15 h was observed in the present study indicated persistence of drug in the tissue and extracellular fluid.

The value of area under curve (AUC) was 25.2 $μg.h.ml^{-1}$ which showed longer plasma exposure in buffalo calves. However, it was less than that observed in cows following s.c. administration of ceftiofur hydrochloride (Okker *et al.*, 2002). In comparison to the present study, AUC seen in cow calves was three times (77.3 $μg.h.ml^{-1}$) higher (Brown *et al.*, 1996). Possible reason behind difference in values of AUCs in cattle might be due to variation in the route injection, age and species of the experimental animals (Brown *et al.*, 2000).

Volume of distribution ($Vd_{area)}$ of 1.9 $L.kg^{-1}$ in the present study indicated better distribution and penetration of the antimicrobial to various body fluids and tissues. The mean resident time (MRT) of 24.5 h was observed in the present study which was 2.5 times higher than that seen in cattle (10.9 h) after s.c. administration of ceftiofur sodium at the dose rate 2.2 $CFAE.kg^{-1}$ (Brown *et al.*, 2000). The longer MRT of the drug confirms the longer persistent of the drug in the animal body as shown by better distribution half life. This was further confirmed by slower drug elimination as indicated by lower Cl_B (0.08 $L.kg^{-1}.h^{-1}$). The low value of protein binding of ceftiofur (40.9%) indicates that more drug is available in free form to produce the therapeutic effect. Low value of protein binding normally does not give rise to unusual kinetic behaviour of the drug.

The minimum inhibitory concentration (MIC_{90}) of ceftiofur against common pathogenic microorganisms has been reported to be in the range 0.125–0.5 $ìg.ml^{-1}$ (Witte *et al.*, 2011). As per the study conducted in our laboratory, MIC of 0.1 $μg.ml^{-1}$ against *Pasteurella multocida* has been reported (Singh, 2014). The important pharmacokinetic-pharmacodynamic index correlating the *in vivo* efficacy of cephalosporins is the time length that the unbound concentration of the antibiotics remains above MIC as percent of dosing interval (Andes and Craig, 2002). Ceftiofur has time-dependent bacterial killing (Plumb, 2008) therefore the length of duration during which concentration of ceftiofur remains above the MIC_{90} value is an important consideration (Turnidge, 1998). For cephalosporin antibiotics, a T e" MIC of 35-40% of the interdose interval has been recommended as optimal for bacteriostatic action while a T e" MIC of 60-70% is obligatory for a bactericidal effect (Toutain *et al.*, 2002). In the present study, ceftiofur and DFC concentration above MIC was seen up to 24 h post administration (% T e" MIC is 73.8) for the range of MICs (0.125 – 0.5 $ìg.ml^{-1}$) reported. This indicates the selected dose can be repeated at 24 h interval for the effective treatment of the diseases caused by the pathogens having MIC d" 0.5 $ìg.ml^{-1}$, which is five times the reported MIC of *Pasteurella spp*.

A single subcutaneous injection of ceftiofur sodium at the dose of 2 $mg.kg^{-1}$ body weight in healthy buffalo calves produced plasma concentrations of ceftiofur that exceed the reported range of MICs. The dosage regimen is hence suggested for potential clinical testing of ceftiofur in buffalo species against susceptible micro-organisms. The present pharmacokinetic data reported can be useful to conduct therapeutic drug monitoring which can subsequently be used to justify the optimum therapeutic regimen.

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Research Article

EFFECT OF *MORINDA CITRIFOLIA* ON HEMATO-BIOCHEMICAL PROFILE ON DIMETHOATE TOXICITY IN RATS

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ABSTRACT

In present study, thirty six adult male wistar albino rats were randomly divided into six groups, of six rats in each. Group I served as negative control and treated with distilled water. Group II was administered with 1/10th of LD_{50} of dimethoate (31mg/kg). Group III served as standard amelioration group received dimethoate (31mg/kg) along with Vitamin E (150mg/kg). While group IV, V and VI received dimethoate in same dose (31mg/kg) along with hydroethanolic extract of fruit of *Morinda citrifolia* @ 75 mg/kg, 150 mg/kg and 300 mg/kg body wt respectively for 28 days. Study revealed significant increased levels of ALT, AST, ALP, and Uric acid in group II where as Group IV, V and VI showed decreasing trend in the levels of all parameters with significant ($P<0.05$) result in group III (dimethoate + Vit E) and VI. However, it did not produce any significant change in the level of LDH. Decreased level of total protein and hemoglobin (Hb) was observed in dimethoate treated group which was found to change significantly ($P<0.05$) in different treatment groups. Total erythrocyte count (TEC) and Total leukocyte count (TLC) showed non significant changes when compared with Group II. It is concluded from this study that the fruit extract of *Morinda citrifolia* possessed a potential protective effect against dimethoate induced toxicity.

Key words: Dimethoate, , *Morinda citrifolia,* Vitamin E, hydro- ethanolic extract

INTRODUCTION

Organophosphate (OP) compounds are widely used pesticides in agriculture and public health programs. Indiscriminate use of these compounds cause environmental pollution. One of the most important pesticides, commonly used in the agriculture and sanitary hygiene, is dimethoate – an organophosphorus insecticide with a wide spectrum of activity. It is a "Class II, moderately hazardous" organophosphorus systemic pesticide (WHO, 2002) with anticholinesterase activity. It is widely used in agriculture on fruit trees, olive trees, vegetables, ornamental plants, tobacco, cotton, and other crops. Majority of the population get exposed to lower doses of dimethoate through contaminated water, food or by wide application of household insecticides. In several studies it is found that dimethoate intoxication can cause oxidative stress in mice and rats (Sivapiriya *et al*., 2006).

Plants are the potential source of natural antioxidants. Plant extract with antioxidant activity are traditionally used in various diseases. *Morinda citrifolia* is commonly known as Noni, which belongs to family Rubiaceae and is a bush found wild and cultivated nearly all over the India (Nadkarni, 1976). It has been used for more than 2000 years in traditional healing practices of culture throughout the world. Antitumorogenic, Anti-inflammatory, Analgesic, Immunomodulatory properties of Morinda citrifolia are well documented by Nayak and Shettigar (2010). It has been demonstrated that Noni juice contain some antioxidant or anti-inflammatory ingredients (Kamiya *et al.,* 2004). However, information regarding effect of noni on pesticide induced toxicity is meager. Therefore, present study was planned to observe the ameliorative effect of Morinda *citrifolia* fruits extract against Dimethoate induced toxicity with specific reference to changes in serum biochemical and hematological profiles.

MATERIALS AND METHODS

Chemicals and plant material

Dimethoate technical grade was kindly provided by Rallis (Mumbai) India. All the other chemicals used in this study were of analytical grade. The fruits of *Morinda citrifolia* were procured from Buldhana district, Maharashtra, India and were identified by Department of Botany, Institute of Science, Nagpur University, Nagpur Maharashtra.

Preparation of plant extract

The unripe fruits were chopped in small pieces and dried at 40-50°C and powdered. The powder was stored in tightly screwed glass bottle in a cool dry place, away from direct sunlight and used for preparation of hydro-ethanolic extract. The Hydro-ethanolic extract (40% distilled water + 60% ethanol) was prepared as per the method of Barua et al. *(2009).The extract was stored in airtight screw cap vials and kept in the desiccators until further used in this study.*

Experimental design

The present study was carried out on thirty six male rats of albino Wistar strain, weighing around 150 ± 20g, procured from Laboratory Animal Breeding Centre, were divided equally and randomly into six groups. Group I served as negative control and given distilled water. Group II was administered with 1/10th of LD_{50} of dimethoate (31mg/kg). Group III served as standard amelioration group received dimethoate (31mg/kg) along with Vitamin E

(150mg/kg). While group IV, V and VI received dimethoate in same dose (31mg/kg) along with hydroethanolic extract of fruit of *Morinda citrifolia* @ 75 mg/kg, 150 mg/kg and 300 mg/kg b wt, respectively for 28 days. Rats were acclimatized for a period of 7 days before the conduct of the experiment. They were given *ad lib* pellet feed and wholesome drinking water throughout the period of experiment**.** The protocol used in this study was approved by Animal Ethical Committee.

Estimation of hematobiochemical parameters

Blood was collected from retro-orbital sinus under ether anesthesia on 28th day of the experiment in both EDTA added and non-EDTA added tubes .Whole blood was utilized for estimating different hematological parameters (Benzamin, 2001) like hemoglobin, PCV, TEC and TLC. Serum was used for the analysis of different biochemical parameters like AST, ALT, ALP, LDH, total protein and uric acid using commercially available kit from span diagnostics. The data generated were analyzed statistically by standard statistical procedure by applying One- way analysis of variance (ANOVA) (Snecdecor and Cochron, 1994).

RESULTS AND DISCUSSION

The results of hematological profile and serum biochemical are depicted in Table 1.Repeated oral administration of dimethoate produced decreased concentration of hemoglobin and TEC values as compared to control animals however; changes in TEC values noted were of non-significant level. Khogali *et al*. (2005) also reported the similar type of decreasing trend in the values of TEC in mice treated with different concentration of dimethoate and noticed non-significant changes in these values. PCV values were showing increasing trend in treatment groups as compared to control animals however changes observed were of significant level only in highest treatment group. Khogali *et al*. (2005) studied different concentration of dimethoate and reported decreased levels of PCV values. In present study decreased values of PCV by dimethoate was found to increase by 300 mg of *M. citrifolia* which is a positive sign of being antioxidant in nature. Increasing trend in TLC values were noticed in different groups of animals as compare to control group but these changes were of non-significant level. Similar finding was previously reported by Khogali *et al*. (2005).

In serum biochemical values AST, ALT, alkaline phosphatase and Lactate dehydrogenase were studied. Damaged hepatocytes release AST and ALT enzymes following exogenous toxin exposure. Therefore, these elevated serum enzymes levels are good indicators of liver damage. Dimethoate impaired the levels of transferases, alkaline phosphatase and lactate dehydrogenase. Biochemical parameters on experimental rat serum where they observed increase in the level of ALT, AST and alkaline phosphatase was also reported earlier by Attia and Nasr (2009) and Yahya *et al.* (2012). In present study these levels have been significantly decreased by *M. ctrifolia* is an indication of antioxidant activity of *M. citrifolia* on dimethoate induced toxicity. Similar type of findings were also reported by Saafi *et al.* (2011) where all values were counteracted by date palm fruit extract (*Phoenix dactylifera* L.). Wang *et al.* (2008) reported the protective effect of *M. citrifolia* juice against acute CCl_4 induced toxicity in rats. Observations reported in present study for plant are in agreement to Wang *et al.* (2008).Kidney function was evaluated by studying biochemical marker serum uric acid. Various chemical could affect plasma and or serum uric acid levels by influencing the net reabsorption of uric acid in the proximal tubule of the nephron (Reyes, 2003). The effect of hydroethanolic extract of *M. citrifolia* was studied on serum protein and uric acid. Dimethoate induced the oxidative damage to all vital organs and perhaps due to which values of total protein was found to decrease. Attia and Nasr (2009) also reported the similar type of observation after dimethoate stress. Rao and Subramanian (2009)

Table 1:
Effect of Hydroethanolic extract of Morinda *citrifolia* on haemato-biochemical parameters (Mean ± SE)in different groups (n=6).

Parameters	Group I	Group II	Group III	Group IV	Group V	Group VI
Hb(g/dl)	14.96^{a} ±0.64	9.63^{c}±0.37	12.06^{b}±1.29	9.63^{c} ±0.28	8.2^{c}±0.24	12.0^{b}±1.02
TEC(x10^{6}/cmm)	8.03±0.41	7.59±1.04	7.69±0.57	8.23±1.19	6.23±1.06	7.89±0.78
TLC(x10^{3}/cmm)	9.95±1.38	12.7±1.44	11.26±0.90	11.55±1.12 N	9.76±1.08 NS	8.13±0.66
PCV (%)	26.16^{c}±2.93	26.16^{c}±4.49	34.0^{ab}±0.81	27.83^{bc}±2.07	32.16^{abc}±1.66	36.16^{a}±1.13
ALT (IU/L)	36.77^{d}±1.92	52.11^{a}±1.51	42.54^{c}±2.49	50.57^{ab}±1.20	45.43^{bc}±2.12	47.17^{abc}±1.73
AST (IU/L)	62.22^{e}±3.98	107.18^{a}±4.02	73.83^{d}±1.58	99.50^{ab}±4.87	91.01^{bc}±3.37	85.74^{c}±3.33
ALP (IU/L)	63.25^{e}±1.45	88.56^{a}±1.51	71.11^{b}±1.68	85.15^{ab}±1.11	82.60^{b}±1.50	76.62^{c}±1.39
LDH (IU/L)	631.66±18.43	688.5±31.11	675.16±31.56	684.5±30.79	685.16±31.81	680.5±34.75
TP (g/dl)	5.62^{a}±0.37	3.91^{c}±0.11	4.81^{b}±0.24	4.17^{bc}±0.17	4.30^{bc}±0.17	4.50^{bc}±0.17
Uricacid(mg/dl)	3.24^{d}±0.12	4.19^{a}±0.12	3.62^{c}±0.12	4.13^{ab}±0.11	4.13^{ab}±0.11	3.81^{bc}±0.11

Values not sharing a common superscript in a row differ significantly (P<0.05); NS: Non significant; Values not sharing a common superscript in a row differ significantly (P<0.05) and not bearing any superscript do not differ significantly when compared in a row ; Hb:Haemoglobin; TEC: Total Erythrocyte Count; TLC: Total Leukocyte Count; PCV: Packed Cell Volume, Alanine transaminase (ALT), Aspartate transaminase (AST), Alkaline Phosphatase (ALP), Lactate dehydrogenase (LDH), Total Protein (TP) and Uric acid.

studied changes in biochemical parameters in hypoglycemic rats and studied antioxidative effect of *M. citrifolia* fruit extract where they have reported decreased levels of total protein which was increased by fruit extract of *M. citrifolia*. In present observation also decreased level of total protein was found to be elevated by *M. citrifolia* extract. Thus the present findings are in agreement to Rao and Subramanian (2009). Further, uric acid in blood is the most important antioxidant (Ames *et al.,* 1981). Uric acid is the end product of purine catabolism and reduces oxidative stress by scavenging various ROS (Samet *et al.,* 2008). Significant result has also been observed in uric acid parameter. The oxidative damage caused by dimethoate is also reported by Salih (2010) as in present study. Dimethoate intoxication resulted in a significant decrease in total protein and increased in activity of AST and ALT this could be attributed in part to the damaging effect of dimethoate on liver cells. This oxidative damage is also overcome significantly by *M. citrifolia* fruit extract.

The present study highlights the protective role of *Morinda citrifolia* fruit extract against dimethoate induced toxicity. Protective effect might be attributed due to presence of several antioxidants in *Morinda citrifolia* fruits as reported in several studies. But mechanism involved is still unclear. So further work is needed to clarify how this plant extracts work exactly.

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Research Article

HEMATO-BIOCHEMICAL AND HISTOPATHOLOGICAL EVALUATION FOLLOWING REPEATED ORAL ADMINISTRATION OF ATORVASTATIN IN HYPERLIPIDEMIC RATS

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ABSTRACT

The study was carried out to evaluate the toxic effects of atorvastatin in hyperlipidemic rats. Hyperlipidemia was induced by poloxamer-407 @ 500 mg/kg, i. p., at every 3 days interval in rats. A total of 30 rats were divided randomly and equally into 5 groups. Groups I and II served as vehicle and hyperlipidemic control and groups III, IV and V were given atorvastatin at 0.5, 2.5 and 5.0 mg/kg b wt, respectively for 28 days. At the end of study, haemo-biochemical and histopathological examinations of vital organs were performed. An increase in levels of AST, ALT, ALP and total bilirubin was observed. Histopathological lesions indicated hepatocellular damage suggesting atorvastatin induced hepatotoxicity in rats @ 2.5 and 5.0 mg/kg. Additionally myotoxicity was confirmed by observing increased level of creatinine, CK, LDH and AST along with histopathological lesions in heart and skeletal muscles in group V. Sections of kidney of rats of group V showed tubular degeneration and necrosis along with rise in creatinine suggesting nephrotoxicity. On repeated exposure, atorvastatin has been found to produce antihyperlipidemic effects and dose dependent damage to the liver, skeletal muscles, heart muscles and kidney in poloxamer-407 induced hyperlipidemic rats.

Key Words: Atorvastatin, blood chemistry, histopathology, hyperlipidemia, rats.

INTRODUCTION

Atorvastatin (AT) is an inhibitor of conversion of HMG-CoA to mevalonate, the major rate-limiting step of the sterol pathway responsible for cholesterol biosynthesis (Goldstein and Brown, 1990) and thereby significantly reduces TC, LDL-C and plasma TG in clinical studies (Bakker-Arkema *et al.,* 1996). Atorvastatin myopathy in 1–5% of patients (Thompson *et al.,* 2003). There is 10-fold increase in myopathy in patients taking a high dose of AT (80 mg/day) compared to patients on a lower dose (Silva *et al.,* 2007). The cellular mechanism of AT myopathy is not clearly understood; however, it may progress into a potentially fatal rhabdomyolysis (Graham *et al.,* 2004). As there is lack of literature for data regarding toxic effect of AT in hyperlipidemic rats, the present study was planned with objective to explore toxic effects of AT in poloxamer-407 induced hyperlipidemic rats.

MATERIALS AND METHODS

Experimental animals

Thirty healthy male Wistar rats of 8-12 weeks of age, procured from Zydus Research Centre (ZRC), Ahmedabad, India, were housed in standard polypropylene cages (three rats per cage) and maintained under controlled room temperature (22 ± 2^{0}C) and humidity (55 ± 5%) with 12h light and12h dark cycle. All the rats were maintained on *ad libitum* commercially available pellet diet and deionized water throughout the course of the experiment and acclimatized for 5 days prior to grouping. The study was conducted after approval of IAEC.

Drugs and chemicals

Atorvastatin, obtained from ZRC (Ahmedabad, India), was dissolved in 0.5 % W/V sodium bicarbonate in distilled water. Poloxamer-407 was purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). 5% W/V trisodium ethylene diamine tetraacetate (K_3EDTA) solution was purchased from Beacon Diagnostics Pvt. Ltd., Gujarat. Reagents used for serum biochemical analysis were purchased from Merck Specialities Pvt. Ltd. India.

Induction of hyperlipidemia

To produce and maintain high level of cholesterol in rats, poloxamer-407, prepared by mixing in normal saline followed by overnight refrigeration to facilitate dissolution by the cold method (Schmolka, 1991), was given @ 500 mg/kg, i.p., on one day prior to start of study and subsequently at every third day throughout study period (Joo *et al.,* 2010).

Experimental design

Thirty rats were randomly divided in five groups. Rats of group I served as vehicle control and received 0.5 % W/V sodium bicarbonate in distilled water while rats of group II served as hyperlipidemic control and received treatment only for induction of hyperlipidemia. Rats of group III, IV and V were treated with AT @ 0.5, 2.5 and 5.0 mg/kg b wt orally daily for 28 days and received treatment for induction of hyperlipidemia.

Collection of blood and tissues

At the end of study period, on 29th 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 collected at 3000 rpm for 10 minutes at 10 °C (Eppendorf 5804 R, Germany) and stored at -35 °C. After collection of blood samples, all the rats were sacrificed humanely for gross and microscopic changes in cerebrum, cerebellum, lung, liver, kidney, heart, spleen and muscle. Aorta was collected and preserved in neutral buffered formalin for histopathological examination.

Estimation of hematological and serum biochemical parameters

Blood samples collected in test tubes with K_3EDTA were subjected to estimation of various hematological parameters by auto hematology analyzer (BC-2800 Vet, China). On the day of blood collection, Hb, PCV, TEC, TLC, DLC, MCV, MCH and MCHC were estimated. Serum biochemical parameters were estimated in clinical serum biochemistry analyzer (Junior Selectra, Vital Scientific, NV) including triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), serum creatine kinase (CK), lactate dehydogenase (LDH), serum aspartate aminotransferase (AST)/ serum glutamic- oxaloacetic transaminase (SGOT), serum alanine aminotransferase (ALT)/ serum glutamic- pyruvic transaminase (SGPT), serum alkaline phosphatase (ALP), total bilirubin and serum creatinine.

Histopathology

Sections from all the tissues were cut at 5-6 microns thickness with automatic section cutting machine (Leica Automatic Microtome Machine) and were stained with Haematoxylin and Eosin (H & E) stains (Luna, 1968). Sections from aorta were also cut at 4-7 microns thickness with frozen section cutting machine (Minotome) and were stained with oil-red-o stain (Paigen *et al.,* 1987).

Statistical analysis

All the data have been presented as mean ± SE and compared by student 't' test for significant difference at 1% and 5% level of significance.

RESULTS

Effects of sub-acute oral exposure of Atorvastain for 28 days in hyperlipidemic rats on various hematological parameters 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 reduction in platelet counts in hyperlipidemic rats of group V.

In rats of hyperlipidemic control group, serum concentration of TG, TC and LDL-C were increased by 15.1, 5.2 and 8.7 fold respectively; serum concentration of HDL-C was decreased by1.5 fold as compared to rats of vehicle control group. Rats of group IV and V showed significant reduction in TG and LDL-C as compared to hyperlipidemic control group. Rats of group IV and V exhibited significant rise HDL-C, AST, ALT, ALP and total bilirubin level as compared to hyperlipidemic control group. Rats of group V revealed significant reduced level of TC as compared to hyperlipidemic control group. Rats of group V also revealed significant raised level of creatinine, CK and LDH as compared to hyperlipidemic control group.

Detailed post-mortem examination of all the animals of different groups was performed on 29th day. Liver collected from rats of group II to V showed slight paleness as compared to vehicle control group. Thigh muscles collected from group II, III and IV did not reveal gross changes. Thigh muscles collected from rats of group V showed tenderness and wasting condition (Figure-1). No appreciable gross changes were observed in cerebrum, cerebellum, aorta, heart, lungs, kidney and spleen of any experimental animal.

Organs like cerebrum, cerebellum, aorta, lungs and spleen collected from group II, III, IV and V did not show any marked histopathological changes. Vehicle control animals were found physiologically normal and no microscopic changes were observed in any of the organ examined. All organs collected showed normal microscopic structure. H & E stained sections and oil-red-o stained frozen sections of aorta collected from rats of group II did not reveal atherosclerotic lesions or atheroma formation.

Histopathological examination of liver of rats of different groups revealed dose dependant varying degree of degenerative as well as necrotic changes. The lesions were mild to severe in nature. Sections of liver from rats of group II showed fatty changes (Figure-2). Liver of rats of groups III did not reveal any noticeable changes in histological structure. Sections of liver of rats of group IV showed necrosis of hepatocytes (Figure-3) with mild fatty changes. Sections of liver of rats of group V showed hepatocellular necrosis.

On histopathological examination, sections of skeletal muscles, heart and kidney of rats of groups II, III and IV did not reveal any noticeable changes in histological structure. Sections of skeletal muscles from rats of group V showed loss of striations in myofibers (Figure-4), whereas sections of heart of rats of group V showed mild to moderate degeneration of myocardium (Figure-5). Additionally sections of kidney of rats of group V showed renal tubular degeneration and necrosis (Figure-6).

DISCUSSION

The aim of this study was establish a reliable and reproducible hyperlipidemic rat model, suitable for

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

Parameter	Group I	Group II	Group III	Group IV	Group V
TEC (10v /μL)	8.27 ± 0.49	8.23 ± 0.63	8.55 ± 0.90	8.23 ± 0.61	9.47 ± 0.52
Hb(g/dl)	16.80 ± 0.48	17.28 ± 1.11	17.60 ± 1.58	16.95 ± 1.04	19.73 ± 1.26
PCV(%)	43.88 ± 1.33	42.35 ± 1.72	43.88 ± 3.41	42.68 ± 2.35	49.38 ± 3.32
MCV (fL)	55.03 ± 1.15	53.23 ± 1.61	53.50 ± 1.12	52.98 ± 0.99	52.35 ± 0.95
MCH (pg)	20.68 ± 0.84	21.35 ± 1.33	21.40 ± 0.78	21.67 ± 0.69	20.50 ± 1.10
MCHC (g/dl)	38.75 ± 1.24	40.53 ± 1.49	40.48 ± 1.42	39.68 ± 0.59	40.18 ± 0.84
TLC(10^3/μl)	9.96 ± 0.73	7.95 ± 0.79	7.70 ± 0.51	9.30 ± 0.25	8.10 ± 0.60
Platelets(10^5/μl)	5.56 ± 0.18	6.09 ± 0.26	5.37 ± 0.40	5.53 ± 0.44	4.58 ± 0.37*
Neutrophils (%)	16.99 ± 1.89	13.28 ± 0.74	15.86 ± 1.61	12.44 ± 1.39	13.21 ± 1.65
Lymphocytes (%)	75.20 ± 1.92	79.48 ± 0.95	77.00 ± 1.55	80.50 ± 1.59	80.08 ± 1.96
Monocytes(%)	3.60 ± 0.29	3.13 ± 0.22	3.13 ± 0.24	2.98 ± 0.13	2.78 ± 0.23
Eosinophils (%)	2.28 ± 0.06	2.26 ± 0.07	2.20 ± 0.08	2.21 ± 0.08	2.14 ± 0.09
Basophils (%)	1.85 ± 0.05	1.87 ± 0.04	1.82 ± 0.05	1.87 ± 0.08	1.80 ± 0.07

*significant at $p < 0.05$. TEC: Total erythrocyte count; Hb: Haemoglobin; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; TLC: Total leukocyte count.

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

Parameter	Group I	Group II	Group III	Group IV	Group V
TC (mg/dl)	63.47 ± 1.68	327.85 ± 15.26**	317.37 ± 10.12	297.87 ± 11.92	206.15 ± 5.39**
TG (mg/dl)	83.93 ± 1.06	1270.43 ± 21.64**	1246.12 ± 27.11	1068.62 ± 32.78**	1017.15 ± 27.13**
LDL-C (mg/dl)	14.13 ± 0.68	122.80 ± 1.94**	120.78 ± 1.39	109.78 ± 4.77*	87.48 ± 2.06**
HDL-C(mg/dl)	32.54 ± 0.55	22.37 ± 1.97**	24.97 ± 1.30	27.78 ± 1.10*	30.32 ± 1.14**
AST (U/L)	87.12 ± 1.69	90.43 ± 1.55	91.24 ± 2.43	97.32 ± 1.95*	110.43 ± 3.63**
ALT (U/L)	35.48 ± 1.42	36.75 ± 1.57	38.20 ± 2.01	50.28 ± 2.79**	64.48 ± 3.44**
ALP (U/L)	115.49 ± 2.30	116.62 ± 1.82	118.82 ± 2.48	123.72 ± 2.08*	127.23 ± 1.66**
Total bilirubin (mg/dl)	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.03	0.33 ± 0.02*	0.44 ± 0.04**
Creatinine (mg/dl)	0.42 ± 0.01	0.42 ± 0.04	0.44 ± 0.04	0.45 ± 0.04	1.13 ± 0.09**
CK (U/L)	82.85 ± 3.13	84.54 ± 2.24	88.45 ± 2.05	90.02 ± 2.89	114.49 ± 5.68**
LDH (U/L)	209.77 ± 5.55	218.17 ± 7.72	219.18 ± 6.12	244.83 ± 10.51	384.30 ± 12.20**

*significant at $p < 0.05$, **highly significant at $p < 0.01$. TC: Total cholesterol; TG: Triglyceride; LDL-C: Low density lipoprotein cholesterol; HDL- C: High density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; CK: Creatine kinase; LDH: Lactate dehydogenase

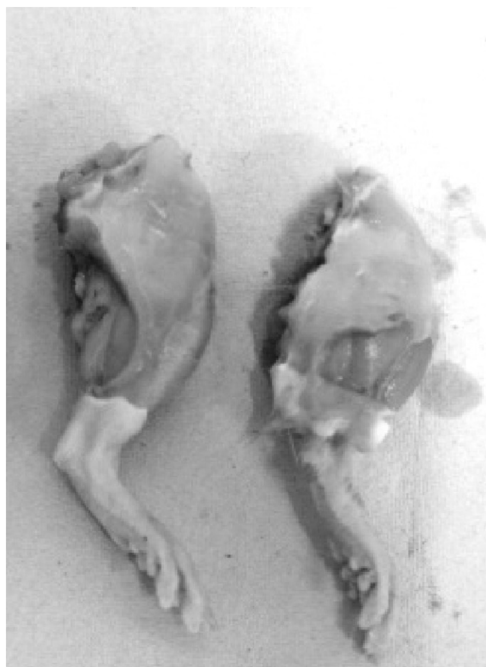

Fig. 1:
Tenderness and wasting condition in muscles of rats of group V

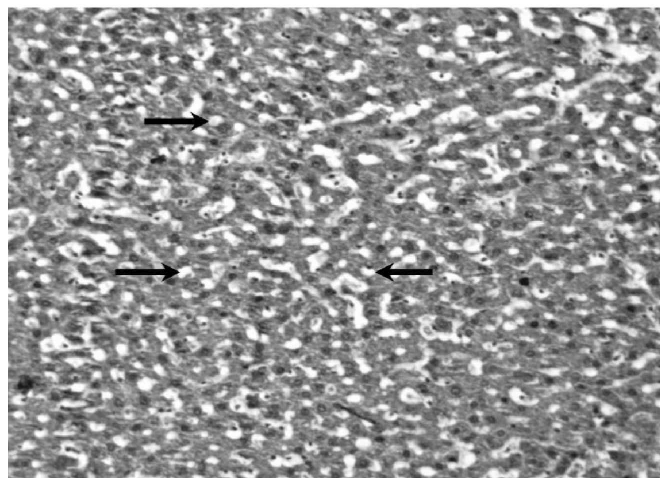

Fig. 2:
Fatty changes liver from rat of group II.

evaluating toxic effects of atorvastatin. Treatment given for induction of hyperlipidemia has lead to increased serum concentration of TG (15.1 fold), TC (5.2 fold) and LDL-C (8.7 fold) in rats of hyperlipidemic control group as compared to rats of vehicle control group. Conversely

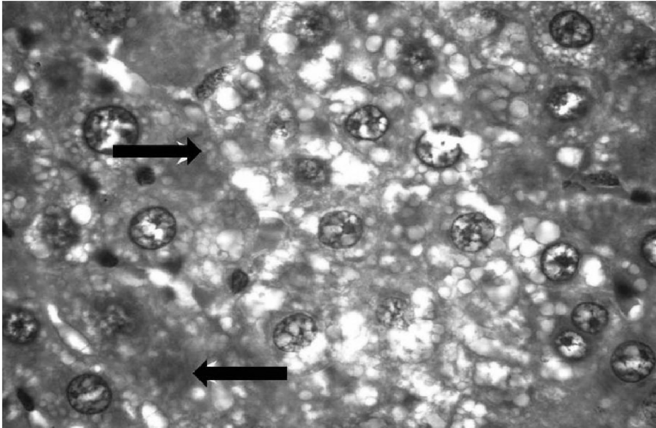

Fig. 3:
Necrosis of hepatocytes of rat of group IV

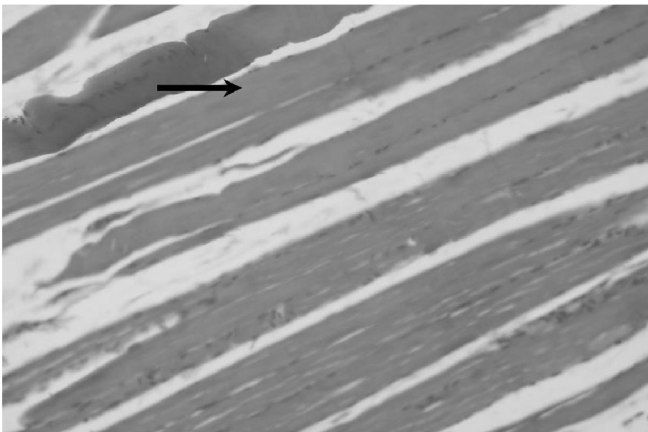

Fig. 4:
Loss of striations in myofibers skeletal muscles from rat of group V

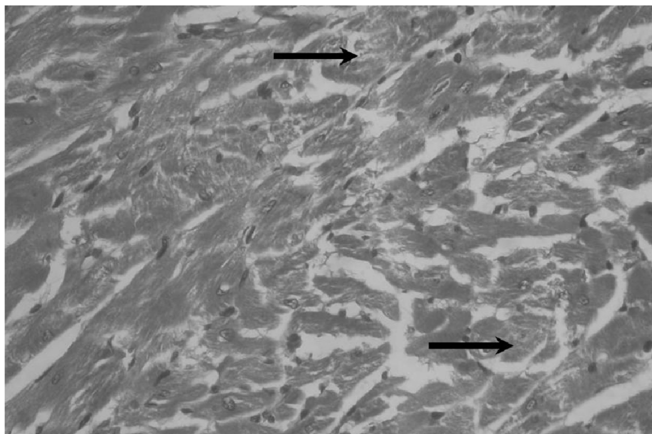

Fig. 5:
Moderate degeneration of myocardium heart of rat of group V

striking reduction has been observed in level of HDL-C in rats of hyperlipidemic control group as compared to rats of vehicle control group. The results obtained with the poloxamer-407 model are comparable to those in the literature (Hor *et al.,* 2011, Wasan *et al.,* 2003) , where i.p.

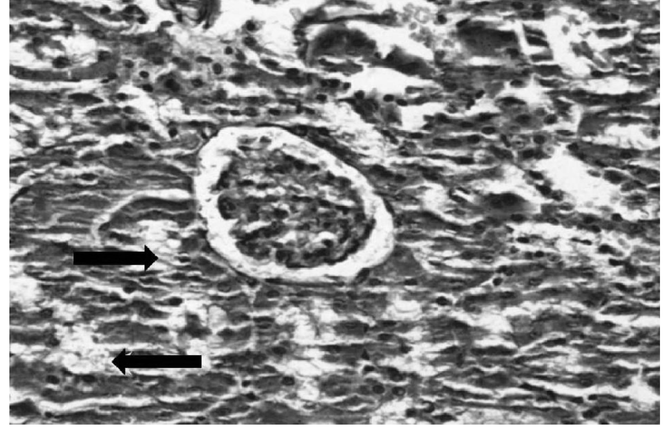

Fig. 6:
Renal tubular degeneration and necrosis kidney of rat of group V

administration of poloxamer-407 has been shown to cause significant increase in TC, TG and LDL-C level as compared to the vehicle control rats suggesting establishment of successful hyperlipidemic rat model in the present study.

Significant thrombocytopenia has been observed in hyperlipidemic rats of group V. This finding is well supported by observation of significant reduction in platelets counts following administration of simvastatin at 10 mg/kg for six weeks in human (Ames, 2008).

In the present study, results of lipid profile of rats of group III, IV and V demonstrated dose dependent antihyperlipidemic effects of atorvastatin. In accordance to the finding of present findings, there are similar reports on reduction in TC, TG and LDL-C levels along with rise in HDL-C level upon administration of atorvastatin in hyperlipidemic rat model. (Shah *et al.,* 2010; Bhandari *et al.,* 2011).

Now a day, there are increasing numbers of reports on the atorvastatin associated complications. The elimination half-life of atorvastatin is longer as compared to other statins. Following once and twice daily administration, atorvastatin accumulation has been reported to be 1.6-fold and 3.3-fold on the 22nd day, respectively suggesting chances of tissue accumulation of AT on repeated administration (Cilla *et al.,* 1996). Upon daily administration of atorvastatin at 2.5 and 5.0 mg/kg in hyperlipidemic rats, significant rise in AST, ALT, ALP and total bilirubin level are supported by variable extent of histopathological lesions observed in sections of liver of the respective study group. Liver was an expected target organ of toxicity, as it is the primary site of cholesterol synthesis in the body. Hepatotoxicity has also been reported with statins administered to rodents and humans (Davidson *et al.,* 2002).

In fact, there is no specific biomarker available to date for statin induced myotoxicity. Elevation of creatinine and CK is generally used to establish myotoxicity of statin

with elevation of other parameters like LDH and AST (Hodel, 2002). In the present study, myotoxicity is confirmed by rise in creatinine, CK, LDH and AST levels along with histopathological lesions in heart and skeletal muscles, which could be due to hydrophobic (lipophilic) nature of AT, which allows more permeation of AT in plasma membrane. It is well established that the adverse effects of AT are dose dependent.

On histopathological examination, sections of kidneys of hyperlipidemic rats of group V showed renal tubular degeneration and necrosis which is in accordance with serological rise in creatinine in respective study group. In literature, AT administration at 0.5 and 1.0 mg/kg in rats has been found to produce no effect on histological structure of kidney (Aktas *et al.,* 2011). Which again substantiate that repeated oral administration of AT results in accumulation and exposure of AT to extra-hepatic tissues leading to adverse effects in the kidney.It is concluded from this study that 28 days repeated oral administration of AT produced antihyperlipidemic effects and dose dependent damage to the liver, skeletal muscles, heart muscles and kidney in poloxamer-407 induced hyperlipidemic rats.

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IN-VITRO ANTIBACTERIAL ACTIVITY OF ALKALOIDS ISOLATED FROM CHLOROFORM EXTRACT OF *PROSOPIS JULIFLORA* LEAVES

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ABSTRACT

Extracts (aqueous, alcoholic, n-butanol, chloroform and n-hexane) of *Prosopis juliflora* leaves and fractions of alkaloids isolated from chloroform extract of *P. juliflora* were evaluated for their antibacterial activity against gram-positive and gram-negative bacteria using the disk diffusion method. Phytochemical and elemental analysis of extracts of *P. juliflora* along with heat stability study of alkaloid fraction were also carried out. Although presence of alkaloids in alcoholic, aqueous, chloroform and n-butanol extracts of the plants, chloroform extract showed highest activity against all tested bacteria compared to other extracts. Upon chromatographic separation of the chloroform extract, four alkaloids were observed with Rf values of 0.24, 0.44, 0.48, 0.55 cm. On elemental analysis, very low amount of metal have been found in isolated fraction of alkaloids compared to leaf powder of the plant. *In vitro* antibacterial activity of the alkaloid was excellent against various bacteria. Autoclaving of solution of alkaloids revealed persistent antibacterial activity. Thus, fraction of alkaloids from chloroform extract of *P. juliflora* showed antibacterial potential may further be investigated to develop as novel a formulation for bacterial infection in human and animals.

Key words: Chromatography, antibacterial , *in-vitro,* alkaloids, *Prosopis juliflora,*

INTRODUCTION

Medicinal plants are good source of natural novel antimicrobial compounds (Preethi, *et al.,* 2010). The genus *Prosopis* of family Leguminosae has been found to have many active principles with pharmacological activities.. *P. juliflora,* commonly found in Gujarat, is considered fast growing, hardy and drought resistant evergreen spiny tree or shrub with drooping branches which is suitable for forestation of arid and semiarid lands (Gurumurti *et al.,* 1984). It has been reported that the leaves of *P. juliflora* having antimicrobial activities against various pathogens which might be due to presence of alkaloids (Pfoze *et al.,* 2011).

As the presence of active principles in medicinal plants may vary according to nature of soil and climate, it is an essential to evaluate the medicinal properties of plants collected from different geopgraphical locations. Information on the phytochemical constituents of *P. juliflora* of Indian origin and its antimicrobial activity is not available in literature. Thus, present study was carried out to undertake phytochemical analysis, *in vitro* antimicrobial activity, elemental analysis and heat stability of alkaloids isolated from *P. juliflora.*

MATERIALS AND METHODS

Plant materials

Fresh leaves of *P. juliflora* were collected from Anand region, Gujarat, India and were authenticated by botanist at Department of Botany, Anand Agricultural University, Anand, India. Specimen of the leaves of plant was deposited in the Herbarium of the Department of Pharmacology and Toxicology, College of Veterinary Science and A.H., Anand Agricultural University, Anand, India. The leaves were washed thoroughly with clean water, dried in shade, powdered and stored in an air-tight container in refrigerator for further use.

Preparation of extracts

Powder of leaves of *P. juliflora* (150 g) was subjected to serial extraction in soxhlet apparatus using water, methanol, n-butanol, chloroform and n-hexane (Chhabra *et al.,* 1992) which were of analytical grade obtained from SD Fine Chem. Mumbai, India. The mixture was then filtered and the filtrate was reduced using a rotatory vacuum evaporator and stored at -20 °C for further use.

Phytochemical analysis

Presence of various phytochemical constituents in different solvent extracts have been determined by various tests (Harbone, 1984; Evans, 1996).

Test microorganisms

Typed cultures of *Escherichia coli* (ATCC25922), *Pseudomonas aeruginosa* (ATCC27853), *Bacillus subtilis* (ATCC6633), *Staphylococcus aureas* (ATCC 25923), and *Streptococcus agalactiae* (NCBI 8778) were procured from National Chemical Laboratory (NCL), Pune to evaluate the antimicrobial activity of extracts of *P. Juliflora.*

Identification and extraction of alkaloids

Based on preliminary antimicrobial activity of

different extracts of *P. Juliflora,* the chloroform extract of the plant selected for further study and was reconstituted in chloroform for chromatographic separation using HPTLC apparatus (DESAGA AS30, SARSTEDT, Germany). The mobile phase was mixture of chloroform: methanol: ammonia (80:20:3). Chromatographic separation of the extract was done on TLC plate (Silica gel 60 F 254 Merck, Germany). Tank saturation was followed for 30 min with filter. The plates were developed for 7 cm. and identification of alkaloids was done by spraying Wagner's reagent on developed TLC plate.

For bulk isolation of alkaloids from the plant extract, the chloroform extract was heated with water at 50 °C approximately for 30 min. The resultant water soluble fraction was filtered and the residue was discarded. The filtrate was evaporated to obtain residue. The residue obtained was brownish red in colour and having semi-solid consistency. Thin Layer Chromatographic separation of this residue showed bands which were identified as alkaloids after spraying Wagner's reagent.

Antimicrobial activity of different extracts and alkaloids

Dried extracts were sterilized by exposing to UV light for 24 h. Sterility was checked by streaking the extract on nutrient agar plate and incubated at 37C for 24-48 h. Extracts were reconstituted in dimethyl sulphoxide (DMSO) at concentration of 500 mg/ml. Solution of extracts (50 µl) were dispensed on blank sterile discs (Himedia Ltd., Mumbai) and sterilized under exposure to UV light for 24 h. Antibacterial activity of different extracts was evaluated using Muller Hinton agar (MHA) plate by disc diffusion assay (Murray *et al.,* 1999). Pure DMSO was used as negative control.

Standard antibacterial discs of cefepime, enrofloxacin, tetracycline, gentamicin, and amoxicillin were used to compare the zone of inhibition (mm) of plant extracts against test bacteria. The assay was carried out in triplicates and the result thus obtained is taken as the mean of the three readings. Based on preliminary screening of different extracts, it has been found that chloroform extract of plants has highest activity against almost all test organisms. Thus, the chloroform extract was selected for further extraction of active principles (alkaloids) and evaluation of antimicrobial activities against test microorganisms.

Heat stability of alkaloids

A solution of alkaloids at 10 mg/ml concentration was prepared in distilled water and autoclaved at 121-122°C and 15 psi pressure for 30 min. Solution of alkaloids (50 µl) were dispensed on blank sterile discs and sterilized under exposure to UV light for 24 h. Evaluation of antibacterial activity of autoclaved and non-autoclaved solution of alkaloids was done using Muller Hinton agar plates by disc diffusion assay (Murray *et al.,* 1999) as described earlier. The zones of inhibition (mm) against test bacteria were measured.

Elemental analysis

Alkaloid solution (20 mg/ml) was prepared in distilled water (Solution A). Ash of the leaf powder of the plant was prepared by method 7300 as per NIOSH manual of analytical methods with slight modification. In brief, 15 ml acid mixture was prepared by mixing perchloric acid and concentrated nitric acid in ratio 1:4. One gram of leaf powder was taken in a flask and 5 ml of acid mixture was added to it and carefully evaporated till fumes ceased to evolve. The process was repeated twice. Finally, the sample in the flask was evaporated to near dryness and diluted quantitatively with distilled water to 100 ml (Solution B). The solutions A and B were subjected to elemental analysis using Atomic Absorption Spectrophotometer (Perkin-Elmer, AAS 3110) at Department of Micronutrient, Anand Agricultural University, Anand. A blank solution was prepared by evaporating 15 mL of acid mixture to dryness and diluted to 100 ml with distilled water.

RESULTS

Extractability and phytochemical constituents in aqueous, alcoholic, butanol and chloroform extracts of *P. juliflora* is shown in Table 1. During preliminary screening for *in-vitro* antibacterial activity, excellent zone of inhibition were observed with chloroform extract against typed bacterial culture whereas other extracts of the plant did not show activity against all bacteria. The range of zone of inhibition with chloroform extract was from 21.40 to 28.90 mm. The values of zone of inhibition of different extracts of the plant are depicted in Table 2.

Considering the excellent *in vitro* antibacterial activity, chloroform extracts of the plant was used for identification and separation of bioactive principles through thin layer chromatography. Upon chromatographic separation of the chloroform extract on TLC plate, four alkaloid bands were identified with Rf values of 0.24, 0.44, 0.48, 0.55 cm. Per cent extractability of alkaloids from chloroform extract with solvent extraction method was calculated to be 4.30. *In vitro* antibacterial activity of the alkaloid solution was found excellent against typed culture. The values of mean zone of inhibition of fraction of alkaloids and different antibacterials against various bacteria are depicted in Table 3. The values of zone of inhibition against test bacteria with alkaloids are comparable to those observed with standard antibacterial drugs of different groups. It has also been observed that the fraction of alkaloids extracted was heat stable and antibacterial activity was not altered after sterilization by autoclaving. *In vitro* antibacterial activity (zone of inhibition) of autoclaved and non- autoclaved solutions of alkaloids is shown in Table 4.

On elemental analysis, very low amount of metal

Table 1:
Extractability (percent) and phytochemical constituents of the different extracts of *P. juliflora.*

Parameters	Extract				
	Aqueous	Methanol	n-Butanol	Chloroform	Hexane
Extractability	21.65 %	18.65 %	16.25 %	14.28 %	13.35 %
	Phytochemical constituents				
Steroids (Salkovaski test)	+	-	-	-	-
Alkaloids (Wagner's test)	+	+	+	+	-
Tannins (Ferric chloride test)	+	-	-	-	-
Flavonoids (Lead acetate test)	-	+	-	-	-
Glycosides (Fehling's test)	+	-	+	-	-
Saponins (Foam test)	+	-	-	+	-
Triterpenes (Lieberman Burchardt test)	+	+	-	+	-
Diterpenes (Copper acetate test)	-	+	-	-	+

(+): presence; (-): absence

Table 2:
Mean Zone of inhibition (mm) of different extracts of *P. Juliflora* against various bacteria.

Microorganisms	Extracts				
	Aqueous	Methanol	n-Butanol	Chloroform	Hexane
	Zone of inhibition (mm)				
E. coli (ATCC 25922)	13.76	27.50	24.82	21.40	———
P. aeruginosa (ATCC 27853)	15.36	17.25	———	23.16	———
B. subtilis (ATCC 6633)	———	15.24	———	22.64	———
S. aureus (ATCC 25923)	———	17.04	———	23.89	———
S. agalactiae (NCBI 8778)	13.76	29.12	15.66	28.90	———

Table 3:
Mean zone of inhibition (mm) of alkaloid fraction of chloroform extract of *P. juliflora* and different antibacterials drugs against bacteria.

Organisms	Alk	Cef	Tet	Enx	Gen	Amx
E. coli (ATCC 25922)	28.12	29.72	17.60	13.70	20.72	27.70
P. aeruginosa (ATCC 27853)	30.94	24.80	24.30	25.48	21.92	—-
B. subtilis (ATCC 6633)	39.01	29.00	40.00	43.60	14.00	31.00
S. aureus (ATCC 25923)	32.61	22.36	15.66	26.14	25.14	—-
S. agalactiae (NCBI 8778)	36.42	22.70	14.88	27.94	20.00	—-

Alk: Alkaloids; Cef: Cefepime; Enx: Enrofloxacin; Tet: Tetracycline; Gen: Gentamicin; Amx: Amoxicillin; N.M.: Not measured

Table 5:
Concentration of metals (ppm) in leaf powder and alkaloid fraction collected from *P. Juliflora.*

Material	Concentration of Metals in ppm w.r.t. dry weight								
	Fe	Mn	Zn	Cu	Cd	Co	Cr	Ni	Pb
Leaf Powder	622.00	86.00	42.00	83.00	3.00	3.00	11.00	6.00	10.00
Alkaloid fraction	19.50	74.50	0.00	37.00	1.00	3.00	1.00	7.00	1.00

Table 4:
***In vitro* antibacterial activity of autoclaved and non-autoclaved solutions of alkaloids.**

Organism	Mean zone of Inhibition (mm)	
	Autoclaved Solution	Non-Autoclaved Solution
E. coli (ATCC 25922)	20.88	21.00
P. aeruginosa (ATCC 27853)	—-	—-
B. subtilis (ATCC 6633)	14.70	14.72
S. aureus (ATCC 25923)	15.30	15.56
S. agalactiae (NCBI 8778)	15.34	15.66

have been found in isolated fraction of alkaloids compared to leaf powder of the plant. The values of level of metals (ppm) in leaf powder and fraction of alkaloids of *P. juliflora* are shown in Table 5.

DISCUSSION

Antimicrobial activity of alkaloids and alcoholic extract of the plant were evaluated by various scientists (Lakshmi *et al.,* 2010; Shachi Singh *et al.,* 2011). However, the studies on antibacterial activity of chloroform, n-butanol and hexane extract were not reported elsewhere. Alcoholic extract showed good activity against Gram-positive and Gram-negative bacteria. Similar activity of ethanolic leaf extract of *P. Juliflora* was reported by Shachi Singh *et al.* (2011). However, the highest antibacterial activity was found with chloroform extract in the present experiment during the preliminary screening for antimicrobial activity which may be due to presence of all different types of

alkaloids in chloroform extract. The chloroform extract showed larger zone of inhibition against all tested bacteria compared to other extracts.

Elemental analysis indicates the presence of iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), cadmium (Cd), cobalt (Co), chromium (Cr), Nickel (Ni) and lead (Pb) in the plant samples however the amount of it found to be less in fraction of alkaloids. The concentrations of the elements appear to be lower which is within safety limit according to WHO (1996).

Antimicrobial activity of fraction of alkaloids was remarkable as compared to chloroform extract with promising values of zone of inhibition. Shachi Singh *et al.* (2011) also reported good antibacterial activity of alkaloids isolated from ethanolic extract of the *P. Juliflora* leaves. The activity of extract from stem, pod, flower and root of the plant would not be promising as compared to those observed with extract from leaves of the plant (Shachi Singh *et al.,* 2011). The heat stability experiment showed that the alkaloids from the plant remains stable even at high temperature thus the formulation of alkaloids would be with retained activity even after sterilization. The values of zone of inhibition were lower due to lower concentration of the alkaloids used in that experiment. Due to lower concentration of alkaloids, the activity against *Pseudomonas* spp. was not found. In conclusion, fraction of alkaloids from chloroform extract of *P. juliflora* may suitable for the development of novel formulation for bacterial infection in human and animals.

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Research Article

COMPARISON OF PESTICIDE RESIDES VARIOUS DISTRICTS OF KUMAON REGION OF UTTARAKHAND

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ABSTRACT

The present investigation was carried out for comparison of pesticide residues of chlorpyrifos and endosulfan (alfa, beta and sulfate) in samples of poultry meat (muscle, liver, fat and kidney) and poultry feed and water collected from different districts and respective sub-centers of Kumaon region of Uttarakhand by using reverse phase HPLC. Mean difference of residual concentrations of chlorpyrifos irrespective of the sample type of district US Nagar vary significantly ($P<0.05$) with the districts of Almora, Champawat, Bageshwar and Pithoragarh and non significantly with district Nainital. Mean difference of residual concentrations of endosulfan irrespective of the sample type of district US Nagar varied significantly ($P<0.05$) only with the districts Bageshwar and Pithoragarh

Key words: Chlorpyrifos, endosufans, HPLC, residue, poultry meat.

INTRODUCTION

Endosulfan, an organochlorine insecticide (OCI) and chlorpyrifos, an organophosphate insecticide (OPI), have been used in the public health sector for disease vector control and in agriculture to control crop pests for the past several decades in India. They are characterized by low water solubility and high lipid solubility, leading to their bioaccumulation in fatty tissues. Therefore, they can accumulate in human body fats and the environment posing problems to human health (Ejobi *et al.,* 1996). Out of various pesticide residues likely to be encountered in meat industry, OCI and OPI and their metabolites are more prevalent. Endosulfan and chlorpyriphos are widely used for the control of pests, mites, flies and lice affecting the livestock and poultry (Loomi *et al.,* 1972) and detected in poultry egg, meat and cow milk and milk products (Rawat *et al.,* 2003). In view of this fact, this study was undertaken for detection and comparison of pesticide residual concentrations among various districts of Kumaon region of Uttarakhand.

MATERIALS AND METHODS

Samples were collected from different places of Kumaon region of Uttarakhand state. For sample collection, Kumaon region was divided into six centers (districts) and different samples were collected by multistage cluster sampling technique. The centers were selected on the basis of use of pesticides in various agricultural operations, public health programmes and animal husbandry practices. From Kumaon region, identified six centers were U.S Nagar, Nainital, Almora, Champawat, Bageshwar and Pithoragarh. For collection of samples, from each identified selected center (district) and in turn certain places were selected as sub centers. Twenty locally produced poultry farms were selected randomly from each districts sub-center. A total of 140 samples from each district were collected and it comprised of poultry meat (muscle, liver, kidney and fat) egg, feed and water were collected for each specimen from respective sub centers and analyzed within 3 days of collection by HPLC.

In the present study, reverse phase HPLC was used to detect and quantify the chlorpyrifos and endosulfan residues in different tissues of poultry birds. Acetonitrile (ACN) was used as a mobile phase (ACN: water, 65: 35). A wavelength of 220 nm was used for detection of endosulfan and chlorpyrifos.

Extraction from poultry tissue sample and water

The extraction of pesticide residues from different tissues (liver, kidney, muscle and fat) of poultry birds was done as per the method described by Loerger and Smith (1993) with slight modifications. Ten ml of HPLC grade acetonitrile was mixed with 5 gm of tissue and homogenized in a mortar. The homogenate was sonicated at 10 amplitude microns for 30secs, with a pause of 5 seconds (a total of 15 cycles) by using ultrasonic tissue disintegrator. The sonicated tissue was centrifuged at 12000 rpm for 15 min and supernatant collected in a petridish and dried overnight at 37^0C. The residue was reconstituted in 2ml acetonitrile and subjected to clean-up procedure. Clean up process was done as per the technique described by Telling and Sissons (1977) with slight modifications using solid- phase extraction C_{18} cartridges. The dried eluate was reconstituted in 2ml of acetonitrile and loaded onto the conditioned C18 cartridges (conditioning was done first with water and then by acetonitrile) and allowed to pass through vacuum (20mmHg). The cartridges were then washed with 2ml of acetonitrile.The eluate which was obtained after loading of cartridges then filtered through 0.22 µm filter paper. 20 µl of the sample thus obtained was injected into HPLC system for detection and quantification of pesticide residues in tissues of poultry birds.

Extraction of residues

Extraction of yolk

One gm of yolk was put in 10ml test tube, mixed with 2.9ml distilled water. To this mixture 500µl of acetonitrile (ACN) and 2 ml of ethyl acetate was added. After vortexing 1.2g Nacl + 100µl of 1N HCl +500µl of n-hexane was added. The vortexed mixture was centrifuged at 1400g for 5 min. 1.5 ml of supernatant was put in a petri dish and allowed to evaporate. Residue was reconstituted with 700 µl of acetonitrile, vortexed for 2 min and then centrifuged at 23100g for 5 min. 500µl of supernatant was used for HPLC analysis.

Extraction of albumin

One gm of albumin was put in a 5ml test tube mixed with 0.5ml of distilled water. The mixture was vortexed. 1.2g Nacl + 2ml of ethyl acetate was added to the mixture and centrifuged at 4100g for 5 min. 2ml of supernatant was collected and evaporated and residue was reconstituted with 700 µl of acetonitrile, vortexed for 2 min and then centrifuged at 4100g for 5 min. 500ul of supernatant was taken for HPLC analysis.

Extraction from poultry feed samples

Ten gram of chopped fodder sample was mixed with 10ml acetone crushed in a mortar with pestle, then blended in high speed blender for 15 mint to make fine paste. To this paste, 30ml acetone was added and blended again for 2 min. The sample was homogenized for 15 min at 16000 rpm. The homogenate was filtered with suction through Buchner funnel with glass septum. Jar and residue were washed with 10ml acetone and washing were filtered through Buchner funnel. All the filtrate was transferred to separators funnel and 90ml sodium sulfate solution (4%), 20ml dichloromethane, 20ml acetone and 1.4g sodium chloride were added to it. The separatory funnel was shaken vigorously for 2-3 mints to dissolve most of the sodium chloride and follow to stand for separation. Further steps were followed similarly as described for tissue analysis.

RESULTS AND DISCUSSION

Mean difference of the residual concentration of pesticides (Chlorpyrifos and Endosulfan) in different districts of Kumaon region of Uttarakhand are shown in the Table

Table 1:
Comparison of mean difference residual concentrations of endosulfan and chlorpyrifos irrespective of the sample types within the districts of Kumaon region of Uttarakhand.

District (I)	Districts (J)	Mean difference conc. I-J (µg/g)	Std. Error	Mean difference conc. I-J (µg/g)	Std. Error
		Endosulfan		Clorpyrifos	
US Nagar	Nainital	0.00216	0.007907	0.014454	0.0064728
	Almora	0.009579	0.007907	0.0186747*	0.0064728
	Champawat	0.017465	0.007907	0.0222547*	0.0064728
	Bageshwar	.0249643*	0.007907	0.0293547*	0.0064728
	Pithoragarh	.0288914*	0.007907	0.0342254*	0.0064728
Nainital	US Nagar	-0.00216	0.007907	0.014454	0.0064728
	Almora	0.007419	0.007907	0.0042207	0.0064612
	Champawat	0.015305	0.007907	0.0078007	0.0064612
	Bageshwar	0.0228043*	0.007907	0.0149007	0.0064612
	Pithoragarh	0.0267314*	0.007907	0.0197714*	0.0064612
Almora	US Nagar	-0.00958	0.007907	0.0186747*	0.0064728
	Nainital	-0.00742	0.007907	0.0042207	0.0064612
	Champawat	0.007886	0.007907	0.00358	0.0064612
	Bageshwar	0.015385	0.007907	0.01068	0.0064612
	Pithoragarh	0.019312	0.007907	0.0155507	0.0064612
Champawat	US Nagar	-0.01747	0.007907	0.0222547*	0.0064728
	Nainital	-0.01531	0.007907	0.0078007	0.0064612
	Almora	-0.00789	0.007907	0.00358	0.0064612
	Bageshwar	0.007499	0.007907	0.0071	0.0064612
	Pithoragarh	0.011426	0.007907	0.0119707	0.0064612
Bageshwar	US Nagar	-.0249643*	0.007907	0.0293547*	0.0064728
	Nainital	-.0228043*	0.007907	0.0149007	0.0064612
	Almora	-0.01539	0.007907	0.01068	0.0064612
	Champawat	-0.0075	0.007907	0.0071	0.0064612
	Pithoragarh	0.003927	0.007907	0.0048707	0.0064612
Pithoragarh	USNagar	-.0288914*	0.007907	0.0342254*	0.0064728
	Nainital	-.0267314*	0.007907	0.0197714*	0.0064612
	Almora	-0.01931	0.007907	0.0155507	0.0064612
	Champawat	-0.01143	0.007907	0.0119707	0.0064612
	Bageshwar	-0.00393	0.007907	0.0048707	0.0064612

* Mean difference concentrations differ significantly ($p<0.05$).

1. More the mean difference between the districts less will be the residual concentrations detection difference with the comparing district and vice versa.

Mean difference of residual concentrations of chlorpyrifos irrespective of the sample type of district US Nagar with the districts Almora, Champawat, Bageshwar and Pithoragarh vary significantly at $P<0.05$. The mean difference concentrations of chlorpyrifos vary with districts Nainital non-significantly. Mean difference of residual concentrations of chlorpyrifos irrespective of the sample type of district Pithoragarh with the districts US Nagar and Nainital varied significantly ($P<0.05$) whereas non-significantly with districts Almora, Champawat and Bageshwar.

Mean difference of residual concentrations of endosulfan irrespective of the sample type of district US Nagar with the districts Bageshwar and Pithoragarh vary significantly ($P<0.05$). Contrast to that, mean difference concentrations of endosulfan vary non-significantly with districts Almora, Nainital and Champawat. Mean difference of residual concentrations of endosulfan, irrespective of the sample type of district Pithoragarh with the districts US Nagar and Nainital varied significantly ($P<0.05$). Contrast to that, mean difference concentrations of endosulfan varied non-significantly with districts Almora, Champawat and Bageshwar.

In Kumaon region mean residual concentration of chlorpyrifos and endosulfan irrespective of the sample type were detected maximum in district U.S nagar and minimum in district Pithoragarh.Levels of chlorpyrifos and endosulfan residues were present in poultry meat eggs, feed and water samples. These findings are in conformity with the presence of pesticides in samples of feed grain of this region. The contamination of poultry meat, eggs and water with pesticide residues could be due to their accumulation in food grain used in preparation of poultry feed (Noble,1990). Pesticide residue were also reported in samples of poultry meat collected from Tarai, Kumaon region and adjoining plains (Taneja, 2000; Mishra, 2001). Various other several reports from India (Tripathi *et al.,* 1973; Singh and Chawla, 1988) and USA and Japan (Chen *et al.*,1997) also revealed accumulation of pesticide residues in tissues of poultry and other samples of ground and surface water in India (Flavio *et al.*, 1999; Mishra, 2001**)**.

It is concluded from the residual study of the pesticide in this investigation that mean residual concentrations of chlorpyrifos and endosulfan and its isomers were found more in US Nagar and least in Pithoragarh. As we move from plains to high altitude of Kumaon region less frequent use of pesticides has been exercised, this may be one the reasons that in plain areas more concentrations of pesticides has been detected than in high altitude areas.

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EVALUATION OF DETOXIFIED JATROPHA CURCAS SEED CAKE AS FEED SUPPLEMENT ON IMMUNOLOGICAL PARAMETERS IN WHITE LEGHORN COCKERELS

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ABSTRACT

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

Key words : Humoral and cellular immunity, *Jatropha curcas* seed cake, white leghorn cockerels.

INTRODUCTION

Jatropha curcas, (family Euphorbiaceae), commonly called ratanjyot is a drought-resistant succulent shrub/ tree, and is recognized as biodiesel plant. All parts of the *Jatropha* plant have been used traditionally as folk medicines, for both human and veterinary ailments, from a long time for having potential anticancerous, hepatoprotective, antiulcer, antifungal, tocolytic, antidiabetic, anti-inflammatory, antioxidant, antimicrobial, wound healing and anthelmintic activity. The present study was conducted to evaluate the effect of detoxified seed cake on immunological response following feeding of normal and detoxified *Jatropha curcas* seed cake as 5% and 10% supplement in normal feed for 90 days in white leghorn cockerels..

MATERIALS AND METHODS

Detoxification of *Jatropha curcas* seed cake

The present study was designed for evaluation of the detoxified *Jatropha curcas* seed cake as 5% and 10% supplement in normal feed for 90 days in white leg horn cockerels. The seed cake was detoxified by boiling at 121°C, 15 lbs pressure for 20 minutes, followed by soaking with 0.2N potassium hydroxide solution for 30 minutes and subsequently washing with water to remove potassium hydroxide. Treated seed cake meal was dried at 60ºC in oven for overnight. Dried meal was milled to reduce the particle size for the proper mixing in the normal poultry ration.

Experimental design

The study was conducted in 35 white leghorn cockerels for 90 days. Thirty five (6 weeks old) white leghorn (WLH) cockerels were procured from Instructional Poultry Farm of the college and maintained in experimental poultry shed in battery cage system under standard conditions. All the birds were fed with starter ration for initial 2 weeks followed by grower ration till the end of the study. The birds were divided randomly and equally into five groups with seven birds in each group. Group I served as control, groups II and III were fed untreated *Jatropha* seed cake at 5% and 10%, respectively, and groups IV and V were fed detoxified *Jatropha* seed cake at 5% and 10%, respectively.

Cell mediated immunity

The cell-mediated immune response of the birds was estimated using lymphocyte stimulation test and delayed type hypersensitivity.

Delayed Type Hypersensitivity (DTH)

Delayed type hypersensitivity reaction to dinitrochlorobenzene (DNCB) 14 days post exposure was carried out by the procedure given by **Phanuphak *et al.* (1974)** at the end of the study.

For sensitization, a 10 cm^2 featherless area on either side of the keel bone was selected and prepared aseptically. 0.25 ml acetone was applied over the skin as control on one side and 0.25 ml of DNCB solution (10 mg/ ml) in acetone was applied on other side as test. The day of application was taken as day 0. Sensitized birds were challenged on 14th day by applying 0.25 ml of DNCB solution (1 mg/ml) in acetone on test site & 0.25 ml of acetone as control on other site and the site being the same as of day 0. The response to DNCB was assessed by measuring the thickness of the skin at the site of application with the help of Vernier calipers at 0, 12, 24, 48 and 72 hours post challenge in each group. The response was also recorded as erythema, induration,

ulceration and scab formation.

Lymphocyte stimulation test (LST)

The evaluation of the activity of T lymphocytes was carried out at 60th and 90th day of study. For the stimulation of T cell blastogenesis, phytohaemaglutin (Con-A) was used as the mitogen. Con-A was dissolved in RPMI-1640 cell culture media in a concentration of 5 μg/ml for stimulation of cells.

Separation of lymphocytes

Three ml of blood was collected in a sterilized heparinised (125 IU/ml) syringe and diluted with equal volume of RPMI-1640 media. This mixture was then layered carefully over 3 ml of histopaque (Sigma) so as to avoid mixing of the blood and histopaque followed by centrifugation in a swing type centrifuge at 400 g for 30 minutes. The middle buffy layer of lymphocytes was carefully aspirated by sterilized Pasteur pipette. This layer was further washed with RPMI-1640 twice and cell viability was examined using 0.5% trypan blue dye exclusion test. Live cells did not take any stain but dead were stained black**.** The final concentration of the cells was adjusted to be 1 X 10^7 cells per ml of RPMI-1640 medium. To the final cell suspension 10 % foetal calf serum was added.

Test procedure

For the estimation of lymphocyte blastogenesis, 96 well, flat bottom tissue culture plates (Cellstar, Grenier bio-one) were used. Each sample was used in triplicate whose average was used as the final reading for the given sample. Each sample had its own control, also in triplicate for comparison. For the triplicate wells, 100 μl of cell suspension, 50 μl of media and 50 μl of CON-A (5μg/ml)/ LPS (4μg/ml) as mitogen were added. For blank 100 μl of media alone was used.

After loading, the plates were sealed with parafilm (Pechiney plastic packaging) and were incubated at 37^0C for 72 hours in CO_2 incubator (Forma Scientific) with 5% CO_2 pressure. Four hours before the completion of the incubation period, 50 μl of MTT (4 mg/ml, Sigma) was added to each of the well (Rai-el-Balhaa *et al.,* 1985). It was again incubated for four hours. After the completion of 72 hours of incubation, 100 μl of isopropyl alcohol was added to each well to stop the reaction of cells with MTT. The plate was then subjected to ELISA plate reader to read the optical density (OD) at 570 nm. Triplicate sample wells were averaged to get the final OD of a sample wells. Average of the control wells was taken as the final OD of control wells. The difference of the sample and control wells was expressed as the final reading in terms of "OD.

Humoral immunity

Immunoglobulin estimation

Total serum immunoglobulin was estimated by using zinc sulphate turbidity test **(McEven *et al*., 1969)** at 60 and 90 days of study. 6ml of 0.025% $ZnSO_4$ solution was added to control and test sample. Shaked well and kept at room temperature for 60 minutes. O.D was read at 545 nm by spectrophotometer and calculated by using the following formula:

Zinc sulphate (ZnSO4) turbidity (ZST units) = Test – Control X 10

Total immunoglobulin (g dL^{-1}) = 0.04 + 0.98 ZST units.

Statistical analysis

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

RESULTS AND DISCUSSION

There was a significant (P<0.05) decrease in the values of Ä OD in group II and III at 60 day feeding interval and II, III and V group as compared to control after 90 day of trial. Group IV & V revealed significantly (P < 0.05) higher Ä OD values than group III at 60 day interval and group IV showed significantly (P < 0.05) higher Ä OD as compared to group III at 90 day feeding interval in WLH cockerels. At the end of the trial I, II and IV groups showed significant (P<0.05) increase in the values of Ä OD as compared to that of 60 days feeding interval (Table 1). A significant (P<0.05) decrease in the delta OD in all Con-A treated groups at the end of the trial as compared to control. Decrease in the lymphocyte proliferation with Con-A is an indication of suppression of T-cell blastogenesis which are essential for mounting of both cell mediated and humoral immune response

There was a significant (P<0.05) decrease in skin thickness in II, III, IV and V groups as compared to control after 0, 12, 24, 48 and 72 hr of DNCB challenge. The decrease in skin thickness in 5% and 10% detoxified groups was significantly (P < 0.05) lower in 5% and 10% normal seed cake fed groups after 0, 12, 24, 48 and 72 hr of DNCB challenge (Table 2). The decreased DTH reaction observed in the present study indicates suppression of cell mediated immunity in birds. Reduction in DTH reaction was more in the groups fed with normal seed cake. As also evident by LST along with their functional activity

Table 1:

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

Groups	Feed supplement	OD	
		60 day	90 day
I	Normal feed	0.759±.004	0.855±.003[a]
II	5% normal JSC	0.652±.003	0.716±.004[a, w]
III	10% normal JSC	0.581±.003[w]	0.608±.004[wx]
IV	5% detoxified JSC	0.716±.004[y]	0.798±.004[a, y]
V	10% detoxified JSC	0.689±.004[y]	0.667±.003[wz]

a= Value differ significantly with 1st coloumn in a row.w= value differs significantly with control group

.x= value differs significantly with II group.y=value differs significantly with III group.z=value differs significantly with IV group.

Table 2:
Effect on DTH by measuring skin thickness (cm) following 90 days feeding of normal and detoxified *Jatropha curcas* seed cake as 5% and 10% supplement in normal ration in WLH cockerels (Mean±SE, n=7).

Hrs/Groups	0 hr	12 hr	24 hr	48 hr	72 hr
I	1.86±0.039	2.31±0.030^{a}	2.45±0.047^{ab}	2.65±0.027^{abc}	2.12±0.019^{abcd}
II	1.52±0.032^{w}	1.59±0.034$^{a, w}$	1.66 ±0.030$^{ab, w}$	1.51±0.025$^{c, w}$	1.34 ±0.037$^{abcd, w}$
III	1.39±0.016^{wx}	1.47±0.05$^{a, wx}$	1.58 ±0.012$^{ab, wx}$	1.37 ±0.02^{bc,wx}	1.27±0.014$^{abcd, wx}$
IV	1.75±0.05^{xy}	1.85±0.033$^{a, wxy}$	1.92±0.029$^{ab, wxy}$	1.74±0.041^{bc,wxy}	1.64±0.024^{abcd,wxy}
V	1.65±0.022^{wxy}	1.73±0.031$^{a, wxyz}$	1.82±0.033$^{ab, wxy}$	1.66±0.041^{bc,wxyz}	1.46±0.046^{abcd,wxyz}

a= Value differ significantly with 1st coloumn in a row.b= Value differ significantly with 2nd coloumn in a row w= value differs significantly with control group. x= value differs significantly with II group.y=value differs significantly with III group.z=value differs significantly with IV group.

Table 3:
Effect on total immunoglobulins (g/l) following 90 days feeding of normal and detoxified *Jatropha curcas* seed cake as 5% and 10% supplement in normal ration in WLH cockerels (Mean±SE, n=7)

Groups	Feed supplement	Total immunoglobulins	
		60 day	90 day
I	Normal feed	2.75 ±.034	2.72 ±.03
II	5% normal JSC	2.06 ±0.027^{w}	1.76 ±.044^{w}
III	10% normal JSC	1.82 ±0.059^{wx}	1.38 ±.019^{wx}
IV	5% detoxified JSC	2.69 ±0.44^{xy}	2.02 ±.032$^{a, wxy}$
V	10% detoxified JSC	2.62 ±.031^{wxy}	1.89 ±.049$^{a, wxyz}$

a= Value differ significantly with 1st column in a row.w= value differs significantly with control group.x= value differs significantly with II group. y=value differs significantly with III group.z=value differs significantly with IV group.

which is also a contributory factor in the reduction of DTH response. The decrease in DTH response may be due to decreased T-cell response.

Total immunoglobulins were significantly ($P < 0.05$) reduced in groups II and III at 60 day interval and in II, III, IV and V groups at 90 day feeding interval as compared to control group. Total immunoglobulins were significantly ($P < 0.05$) high in IV and V groups as compared to both the normal JSC supplement fed groups (Table 3). Immunotoxic potential of *J. curcas* is also evident by decreased values of total immunoglobulins in 10% normal and detoxified JSC fed groups at both 60 and 90 day feeding intervals. . Reduction in DTH reaction was more in the groups fed with normal seed cake. The decreased DTH reaction observed in the present study indicates suppression of cell mediated immunity in birds Similar results were found in lambs fed on cotton seed meal as they showed lower humoral and DTH response (Nagalakshmi *et al.*, 2001).

As also evident by LST along with their functional activity which is also a contributory factor in the reduction of DTH response. The decrease in DTH response may be due to decreased T-cell response. Immunotoxic potential of *J. curcas* is also evident by decreased values of total immunoglobulins in 10% normal and detoxified JSC fed groups at both 60 and 90 day feeding intervals. It is concluded from the 90 days study in white leghorn cockerels that feeding of detoxified *Jatropha* seed cake as 5% supplement produced suppression in immunological response mild toxic effects only after 60 days. Thus, feeding of detoxified *Jatropha* seed as 5% supplement in feed for 60 days did not affect immune response in WLH cockerels.

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Research Article

EVALUATION OF PROTECTIVE EFFECT OF *ERYTHRINA VARIEGATA* AND *SPIRULINA PLATENSIS* IN COCKERELS INTOXICATED WITH IMIDACLOPRID

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ABSTRACT

The study was carried out for evaluation of ameliorative potential of *Erythrina variegata* and *Spirulina platensis* on haemato-biochemical parameters in cockerels simultaneously intoxicated with imidacloprid. The study was conducted in 42 white leghorn cockerels for 60 days. The birds were divided randomly and equally into six groups with seven birds in each group. Group I served as control and other groups were administered as SP @ 0.2% in II, IMI @ 50 PPM in III, SP @ 0.2% plus IMI @ 50 PPM in group IV, EVLP (1%) in group V and EVLP (1%) plus IMI @ 50 PPM in VI, respectively, for 60 days and parameters were recorded at 0, 30 and 60 days interval. A significant ($P<0.05$) reduction in Hb, PCV, TEC, TLC was observed in group III and a significant ($P<0.05$) increase in Hb, PCV, TEC, TLC was observed in group II as compared to control after 60 days. Groups IV and VI showed a significant ($P<0.05$) improvement in Hb, PCV, TEC, TLC as compared to group III. A significant decline in total serum protein, albumin and globulin was reported in imidacloprid treated cockerels of group III as compared to control. Groups IV and VI showed significant ($P<0.05$) increase in total serum protein, albumin and globulin as compared to group III showing ameliorative effect of SP and EVLP medication. A significant ($P<0.05$) increase in triglycerides, cholesterol, total bilirubin, indirect bilirubin, creatinine, AST, ALT, ALP was observed in cockerels of imidacloprid treated Group III, whereas, a significant ($P<0.05$) decline in value of these parameters was observed in groups IV and VI supplemented with SP and EVLP, respectively. It is concluded from the present study that the supplementation of feed with 1% EVLP and 0.2% SP, simultaneously given with imidacloprid for 60 days have a protective effect on body weight gain and haemato-biochemical parameters.

Key words: *Erythrina variegata, Spirulina platensis*, Imidacloprid.

INTRODUCTION

Imidacloprid, 1((6-chloro-3-pyridinyl) methyl)-N-nitro-2-imidazolidinimine, a chloronicotyl is a globally used insecticide for crop protection in the world wide from the last decade due to its low soil persistence and high insecticidal activity at low application rate (Chao and Casida, 1997). It is fastest growing in sales as insecticide because of its high selectivity for insects and apparent safety for humans (Matsuda *et al*., 2001). Its selective toxicity results from its high affinity to insects nicotinic acetylcholine receptors compared to mammals (Zhang *et al*., 2000). Recently imidacloprid has raised concern as it causes egg shell thinning, reduce egg production and hatching time, possibly due to endocrine disturbances and honey bee colony collapse disorder (Matsuda *et al*., 2001).

Erythrina variegata also called *Erythrina indica* is a thorny deciduous tree growing to 60 feet tall. A wide range of chemical compounds have been isolated, mainly alkaloids, flavonoids, triterpenoids, and lectin. Different parts of the plant have been used in traditional medicine as nervine sedative, collyrium in ophthalmic, antiasthmatic, antiepileptic, anthelmintic, antiseptic, and as an astringent. The alkaloids extracted from the leaves of *Erythrina variegata* are reported to have anti-inflammatory and analgesic activity. Isoflavonoids isolated from *E. variegata* having antibacterial and activity. *E. variegata* shows several other characteristic pharmacological effects like neuromuscular blockade, smooth muscle relaxant, CNS depressant, and hydrocholeretic. The plant has been reported for various medicinal uses in the indigenous system of medicine (Kumar *et al*., 2010).

The recent trend in the feed business is currently directed toward the use of natural ingredients as alternatives to antibiotics, synthetic colors, and other chemicals. Spirulina (blue-green alga) is one of the high quality natural feed additives that can be used in animal and poultry nutrition. There are two different species of Spirulina: *Spirulina maxima* and *Spirulina platensis*, with varying distribution throughout the world. *Spirulina platensis* is more widely distributed and found mainly in Africa, Asia and South America (Vonshak, 2002). The blue-green algae (*Spirulina platensis*) have been used for hundreds of years as a food source for humans and animals due to the excellent nutritional profile and high carotenoid content. Spirulina is relatively high in protein with values ranged between 55-65% and includes all of the essential amino

acids (Anusuya *et al*., 1981).

MATERIALS AND METHODS

Plant materials and chemicals

Erythrina variegata leaves used in the study were collected from GBPUAT Pantnagar campus and taxonomically identified vide letter no. *CHN/64/2012/Tech-II dated 2.10.2012*, Office of Scientist 'F', Central National Herbarium, Botanical Survey of India, Botanical garden, Howrah 711-103 as *Erythrina variegata* (L.) and *Spirulina platensis* (Spirulina DXN) manufactured by twenty first century pharmaceuticals, Chennai were used in the study.Imidacloprid (Midas, Imidacloprid 17.8 % SL) was purchased from local market of Pantnagar.

Experimental design

Forty two male white leghorn cockerels weighing between 250-275 gm of 4-6 months age were selected and procured from Instructional Poultry Farm, Pantnagar for this study and were kept in deep litter system under standard managemental conditions in Instructional Poultry Farm, Pantnagar. The animals were maintained on the standard poultry feed and water *ad libitum*. Experimental chicks were maintained according to the suggested ethical guidelines for the care of laboratory animals.

After acclimatizing for 15 days, Forty two male white leghorn chicks were divided equally and randomly into six groups viz. I, II, III, IV, V and VI. Group I served as control. Group II was given *Spirulina platensis* @ 0.2 % mixed in feed. Group III was given imidacloprid @ 50 ppm mixed in feed. Group IV was given *Spirulina platensis 0.2%* and Imidacloprid @ 50 ppm mixed in feed. Group V birds were given *Erythrina variegata @ 1 %* mixed in feed. Group VI were given *Erythrina variegata 1 %* and Imidacloprid @ 50 ppm mixed homogenously in feed. The study was carried out for 60 days and body weight was recorded at 15 days interval whereas haemato-biochemical and antioxidative parameters were recorded at 30th and 60th day of the study. During the study period, the birds were observed for any toxic signs and symptoms. After 60 days, all the birds were sacrificed humanely and tissue samples of liver, kidney, brain and testes were collected for gross and histopathological examination. After the completion of experiment, all the birds were buried scientifically. The research project was approved by Institutional animal ethics committee for conducting this investigation.

Haemato-biochemical examination

Haematological parameters such as total erythrocyte count (TEC) and total leucocyte count (TLC) were determined according to the method of Natt and Herrick (1952) using poultry blood diluting fluid. Packed cell volume (PCV) and haemoglobin were estimated using the method of Jain (1986). All the haematological parameters were done immediately after the collection of blood. The serum collected from blood samples was used for the estimation of biochemical parameters viz. glucose, total proteins, albumin, cholesterol, total bilirubin, direct bilirubin, triglyceride, and creatinine using ERBA diagnostic kits. Serum enzymes viz. aspartate aminotransferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) were estimated by using Autospan diagnostic kits. The absorbance for different parameters is recorded on UV-VIS spectrophotometer (BIORAD Smart Spec™ Plus). All the parameters were determined immediately after collection of serum within 24 hour of blood collection.

RESULTS AND DISCUSSION

No apparent clinical signs or behavioral changes were seen in birds during study period. There was a significant (P<0.05) decline in body weight gain in imidacloprid treated cockerels. Whereas, SP and EVLP treatments alone and in combination with imidacloprid revealed an improvement in body weight gain indicating their ameliorative effect against imidacloprid (Table 1). A significant (P<0.05) increase in body weight was seen in SP supplemented group as compared to control.

A significant (P<0.05) reduction in Hb, PCV, TEC, TLC is seen in group III, and a significant (P<0.05) increase in Hb, PCV, TEC, TLC was observed in group II. Group IV and VI showed a significant (P<0.05) increase in Hb, PCV, TEC, TLC as compared to group III. A significant (P<0.05) improvement in hematological parameters was induced by *Spirulina platensis* and *Erythrina variegata* in 0.2% and 1% groups respectively (Table 2).

The increment in the blood indices in group II supplemented with *Spirulina platensis* may be related to the rich mineral content in *Spirulina platensis* of Fe, Cu, and zinc (Babadzhanov, *et al*., 2004). It is well known that iron plays an important role in hemoglobin and red blood cells biosynthesis to prevent anemia and is essential for metabolic enzymes biosynthesis such as cytochromes, superoxide dismutase and glutathione reductase. The increment in white blood cells count may be attributed to increased thymus gland weight in the present study and/or increased zinc concentration in *Spirulina platensis*. A significant decrease in all hematological parameters is seen in group supplemented with imidacloprid. Difference in the results may be due to increased dose and duration. Imidacloprid is a ring structured compound and can act as hapten that combines with the protein constituent of leucocytes to form an antigen to which the animal develops antibodies that are toxic to leucocytes, causing either lysis or agglutination (Benjamin, 1978). Decrease in Hb, PCV, TEC in our study might have been due to decreased appetite and feed intake. No change in hematological parameters in groups simultaneously supplemented with *Spirulina platensis* and *Erythrina*

Table 1:
Effect of feeding of 0.2% *Spirulina platensis* (SP) and 1% *Erythrina variegata* leaf powder (EVLP) daily as feed supplement for 60 days on B Wt (grams) in imidacloprid intoxicated WLH cockerels. (Mean±SE, n=7)

Groups	Feed supplement	Time intervals				
		0th day	15th day	30th day	45th day	60th day
I	**Normal feed**	267.14±5.21	328.29±3.13^{B}	457.43±6.68^{C}	612±3.52^{C}	937.43±3.03^{C}
II	**SP @ 0.2 %**	265.14±5.43	380.14±2.72^{A}	549.29±4.50^{A}	657.29±1.26^{A}	1018.40±10.99^{A}
III	**IMI @ 50 PPM**	257.43±3.89	322.23±2.57^{B}	440.00±3.80^{D}	606.57±3.23^{C}	916.14±2.10^{D}
IV	**SP @ 0.2 % + IMI @ 50 PPM**	259.43±5.67	330.57±6.69^{B}	458.29±3.31^{C}	617.14±2.30^{C}	936.71±5.25^{C}
V	**EVLP @ 1 %**	261.57±3.35	333.29±4.44^{B}	482.14±6.93^{B}	642.43±9.71^{B}	965.14±6.54^{B}
VI	**EVLP @ 1 % + IMI @ 50 PPM**	262.29±3.77	322.43±3.35^{B}	456.86±2.51^{C}	619.86±2.41^{C}	935.57±2.85^{C}

Means bearing different superscripts i.e., a, b and c differ significantly (P<0.05) when compared horizontally in a row.
Means bearing different superscripts i.e., A, B, C and D differ significantly (P<0.05) when compared vertically in a column.

variegata, along with imidacloprid depicts their protective effect.

Group III showed a significant (P<0.05) reduction in glucose, group II showed a significant increase in glucose. Group IV showed a significant (P<0.05) increase in glucose as compared to group III (Table 4). Thus, *Spirulina platensis* revealed a protective effect by elevating the glucose level. Hypoglycemia induced by imidacloprid occurs due to sensitization of thyroid by imidacloprid leading to enhanced BMR which further declined the blood glucose levels. Ameliorative efficacy shown by *Spirulina platensis* in imidacloprid treated cockerels as well as increment in glucose levels may be attributed to high carbohydrate and carotenoid content present in *Spirulina platensis*. Hypoglycemia observed in EVLP treated chicks may be due to its hypoglycemic activity as reported by Kumar *et al.* (2011).

A significant (P<0.05) decrease in total serum protein, albumin and globulin was noticed in group III. A significant (P<0.05) increase in total serum protein, albumin and globulin was seen in group II. Group IV and VI showed significant (P<0.05) increase in total serum protein, albumin and globulin as compared to group III showing the ameliorative effect of SP and EVLP supplementation (Table 3). Group III showed a significant (P<0.05) rise in A:G ratio both after 30 and 60 days. Group II, IV and VI showed a significant (P<0.05) decline in A:G ratio as compared to group III.

The significant (P<0.05) decrease in serum total proteins, albumin and globulin in imidacloprid treated cockerels might be due to damage of filtration system in kidney leading to leakage and passage of proteins in urine. Damage in kidney glomerulus and tubules is also confirmed by histopathology examination in this study. Ameliorative effect shown by *Spirulina platensis* in imidacloprid treated cockerels may be attributed to its high level and good quality proteins contents in *Spirulina* (55-65 %) and presence of phycocyanins which might have reversed the degenerative changes induced by imidacloprid in kidney. An absolute increase is also seen in total protein and albumin levels in *Spirulina platensis* supplemented group. control. Protective effect of EVLP might be attributed to presence of flavonoids and proteins in its leaves. Flavonoids acts as antioxidant and scavenges reactive oxygen species produced by oxidative damage, thereby diminishing the oxidative radicals induced cytotoxic effect in liver, kidney and other organs.

Group III showed a significant (P<0.05) increase in triglycerides and cholesterol. Group IV and VI had shown a significant (P<0.05) decrease in triglycerides and cholesterol as compared to group III. Group II showed a reduction in triglyceride and cholesterol as compared to control (Table 4). The significant reduction in serum cholesterol caused by *Spirulina platensis* supplemented feed may be attributed to reduction of absorption and/ or enhanced synthesis of cholesterol in the gastro intestinal tract after increased *Lactobacillus* population (Mariey *et al.,* 2014). Lactobacillus have been found to have a high bile salt hydrolytic activity and ability to reduce the cholesterol in the blood by deconjugating bile salts in the intestine (Sunoro, 2003), thereby preventing them from acting as precursors in cholesterol synthesis. Similar results are reported by Fong *et al.* (2000) in rats fed *Spirulina platensis* supplemented diets. Ameliorative effect shown by EVLP may be attributed to its flavonoids content, which has protective effect on oxidative damage in imidacloprid intoxicated cockerels.

A significant (P<0.05) increase in total and indirect bilirubin was observed in group III. However, group IV and VI showed significant (P<0.05) decrease in total and indirect bilirubin as compared to group III. A significant (P<0.05) increase in creatinine was reported in group III. Group IV and VI showed significant (P<0.05) fall in creatinine as compared to group IIII (Table 5). These findings on bilirubin and creatinine further suggest the ameliorative action of *Spirulina platensis* and *Erythrina variegata.*

The significant increase in creatinine level in imidacloprid treated cockerels in the present study may be due to degenerative changes produced by imidacloprid toxicity in kidney, as evident from histopathological examination. *Spirulina platensis* along with imidacloprid treated chicks was able to restore the creatinine levels to

Table 2:
Effect of feeding of 0.2% *Spirulina plantesis* (SP) and 1% *Erythrina variegata* leaf powder (EVLP) daily as feed supplement for 60 days on HB Level (gm/100 ml), PCV (%),TEC ($\times10^6$/µl) and TLC ($\times10^3$/µl) in imidacloprid intoxicated WLH cockerels. (Mean±SE, n=7)

Groups	HB			PCV			TEC			TLC		
	0th day	30th day	60th day	0th day	30th day	60th day	0th day	30th day	60th day	0th day	30th day	60th day
I	10.93±0.14	11.36±0.21^{C}	11.57±0.32^{B}	31.57±0.53	31.71±0.47^{B}	31.85±0.74^{AB}	3.33±0.03	3.32±0.06^{B}	3.35±0.08^{B}	10.19±0.08	10.37±0.12^{A}	10.50±0.17^{A}
II	11.22±0.18	12.54±0.09^{aA}	12.60±0.19^{aA}	30.71±0.61^{b}	33.71±0.56^{aA}	33.85±0.55^{aA}	3.28±0.06^{b}	3.48±0.05^{aA}	3.53±0.02^{Aa}	10.60±0.186	10.57±0.12^{A}	10.46±0.08^{A}
III	11.87±0.19^{a}	10.88±0.19^{bC}	10.45±0.16^{bC}	30.14±0.91^{a}	29.71±0.68^{abB}	27.42±0.92^{bC}	3.36±0.03^{a}	3.04±0.02^{bC}	2.80±0.04^{Cc}	10.57±0.16^{a}	9.75±0.05^{bB}	8.92±0.07^{cC}
IV	11.20±0.22^{b}	11.92±0.16^{aB}	11.96±0.31^{aAB}	30.14±1.05	30.14±0.51^{B}	30.85±0.46^{B}	3.41±0.05^{a}	3.29±0.04^{abB}	3.27±0.04^{Bb}	10.50±0.09	10.54±0.11^{A}	10.39±0.08^{A}
V	11.17±0.28	11.32±0.26^{C}	11.89±0.30^{AB}	29.85±0.99	30.28±0.56^{B}	32.29±1.20^{AB}	3.41±0.06	3.33±0.04^{B}	3.40±0.02^{AB}	10.58±0.16	10.48±0.07^{A}	10.35±0..56^{A}
VI	11.29±0.26	11.17±0.18^{C}	11.27±0.19^{B}	30.00±0.53	29.71±0.92^{B}	30.14±0.99^{B}	3.39±0.04	3.28±0.03^{B}	3.36±0.07^{bB}	10.57±0.12^{a}	10.34±0.04^{aA}	9.74±0.07^{bB}

Means bearing different superscripts i.e., a, b and c differ significantly (P<0.05) when compared horizontally in a row.
Means bearing different superscripts i.e., A and B differ significantly (P<0.05) when compared vertically in a column.

Table 3:
Effect of feeding of 0.2% *Spirulina plantesis* (SP) and 1% *Erythrina variegata* leaf powder (EVLP) daily as feed supplement for 60 days on Total protein (g/dl), Albumin (g/dl), Globulin (g/dl) and A:G Ratio in imidacloprid intoxicated WLH cockerels. (Mean±SE, n=7)

Groups	TOTAL PROTEIN			ALBUMIN			GLOBULIN			A:G		
	0th day	30th day	60th day	0th day	30th day	60th day	0th day	30th day	60th day	0th day	30th day	60th day
I	3.79±0.09	3.84±0.10^{B}	3.87±0.04^{B}	1.50±0.01	1.50±0.02^{C}	1.43±0.07^{C}	2.26±0.05	2.33±0.11^{A}	2.44±0.09^{A}	0.67±0.02	0.66±0.04^{C}	0.60±0.05^{C}
II	3.90±0.05^{b}	4.83±0.14^{aA}	5.06±0.09^{aA}	1.48±0.02^{b}	2.78±0.15^{aA}	2.55±0.14^{aA}	2.25±0.05	2.05±0.24^{A}	2.51±0.17^{A}	0.66±0.02^{b}	1.51±0.22^{aB}	1.07±0.15^{abB}
III	3.94±0.06^{a}	3.34±0.13^{bC}	3.10±0.15^{bC}	1.49±0.01^{c}	1.11±0.14^{aB}	1.29±0.12^{bB}	2.28±0.05^{a}	1.11±0.14^{bB}	1.29±0.12^{bC}	0.65±0.01^{b}	2.37±0.49^{aA}	1.49±0.18^{abA}
IV	3.87±0.07	3.69±0.07^{BC}	3.74±0.07^{B}	1.50±0.01	1.46±0.06^{C}	1.43±0.09^{C}	2.25±0.05	2.22±0.11^{A}	2.30±0.08^{AB}	0.67±0.02	0.68±0.07^{C}	0.63±0.06^{C}
V	3.89±0.04	3.73±0.14^{BC}	3.58±0.12^{B}	1.49±0.01	1.55±0.11^{C}	1.65±0.09^{BC}	2.32±0.05	2.18±0.19^{A}	1.93±0.12^{B}	0.65±0.02	0.78±0.13^{C}	0.89±0.09^{BC}
VI	3.89±0.06	3.65±0.14^{BC}	3.54±0.14^{B}	1.50±0.01	1.58±0.08^{C}	1.62±0.08^{C}	2.38±0.04	2.07±0.09^{aAB}	1.92±0.18^{bB}	0.63±0.01	0.77±0.05^{C}	0.94±0.17^{BC}

Means bearing different superscripts i.e., a, b and c differ significantly (P<0.05) when compared horizontally in a row.
Means bearing different superscripts i.e., A, B, C and D differ significantly (P<0.05) when compared vertically in a column.

Table 4:
Effect of feeding of 0.2% *Spirulina plantesis* (SP) and 1% *Erythrina variegata* leaf powder (EVLP) daily as feed supplement for 60 days on Glucose (mg/dl), cholesterol (mg/dl) and triglycerides (mg/dl) in imidacloprid intoxicated WLH cockerels. (Mean±SE, n=7)

Groups	GLUCOSE			CHOLESTEROL			TRIGLYCERIDES		
	0th day	30th day	60th day	0th day	30th day	60th day	0th day	30th day	60th day
I	300.58±5.22	305.54±7.86^{AB}	286.08±9.55^{BC}	135.96±3.63	132.05±1.47^{A}	133.65±2.5^{B}	82.75±1.34	81.12±1.37^{AB}	80.96±1.22^{BC}
II	298.49±1.29^{c}	318.77±8.16^{bA}	346.44±5.14^{aA}	134.14±2.52^{a}	120.44±0.84^{bB}	111.93±1.57^{cC}	81.27±1.65^{a}	70.68±1.21^{bC}	68.35±1.71^{bD}
III	300.25±1.63^{a}	239.69±8.45^{bC}	216.13±7.51^{cD}	133.33±2.47^{b}	134.14±1.91^{bA}	148.31±4.90^{aA}	81.43±1.41^{b}	85.24±1.23^{abA}	90.22±2.25^{aA}
IV	296.11±1.78	289.51±7.59^{B}	301.42±8.93^{B}	132.85±1.69	133.07±0.07^{A}	132.53±2.27^{B}	81.12±1.35	81.51±1.71^{AB}	80.80±2.46^{BC}
V	296.10±1.21^{a}	261.68±5.42^{bC}	262.89±9.37^{bC}	134.51±2.4	134.51±1.91^{A}	134.35±2.34^{B}	81.97±0.74^{a}	79.79±1.75^{abB}	75.98±2.28^{Bc}
VI	297.65±1.68^{a}	207.85±11.02^{bD}	214.13±9.56^{D}	132.96±1.65	133.98±1.96^{A}	135.38±1.65^{AB}	80.10±1.08	82.60±2.30^{AB}	84.07±2.98^{AB}

Means bearing different superscripts i.e., a, b and c differ significantly (P<0.05) when compared horizontally in a row.
Means bearing different superscripts i.e., A, B, C and D differ significantly (P<0.05) when compared vertically in a column.

Table 5:
Effect of feeding of 0.2% *Spirulina plantesis* (SP) and 1% *Erythrina variegata* leaf powder (EVLP) daily as feed supplement for 60 days on total bilirubin, direct bilirubin, indirect bilirubin and creatinine in imidacloprid intoxicated WLH cockerels. (Mean±SE, n=7)

Groups	TOTAL BILIRUBIN			DIRECT BILIRUBIN			INDIRECT BILIRUBIN			CREATININE		
	0th day	30th day	60th day	0th day	30th day	60th day	0th day	30th day	60th day	0th day	30th day	60th day
I	1.02±0.01	1.00±0.04	1.04 ± 0.03^{B}	0.23±0.01	0.23±0.01	0.23 ± 0.01^{A}	0.79±0.01	0.77±0.04	0.81 ± 0.04^{B}	0.36±0.004	0.34 ± 0.005^{C}	0.35 ± 0.006^{C}
II	1.01±0.04	0.99±0.08	1.02 ± 0.04^{B}	0.23±0.01	0.23±0.01	0.23 ± 0.01^{A}	0.78±0.02	0.76±0.03	0.79 ± 0.02^{B}	0.33±0.005	0.34 ± 0.01^{C}	0.33 ± 0.01^{C}
III	1.04 ± 0.01^{b}	1.10 ± 0.05^{b}	1.30 ± 0.09^{aA}	0.23 ± 0.01^{a}	0.23 ± 0.01^{a}	0.18 ± 0.01^{bB}	0.80 ± 0.02^{b}	0.87 ± 0.05^{b}	1.12 ± 0.10^{aA}	0.33 ± 0.01^{c}	0.50 ± 0.02^{b}	0.77 ± 0.10^{aA}
IV	1.05±0.02	1.04±0.03	1.09 ± 0.02^{B}	0.23±0.01	0.23±0.01	0.23 ± 0.01^{A}	0.81±0.01	0.78±0.03	0.77 ± 0.02^{B}	0.33±0.01	0.34 ± 0.01^{C}	0.33 ± 0.02^{C}
V	1.04±0.06	1.02±0.01	1.00 ± 0.03^{B}	0.23±0.01	0.23±0.01	0.23 ± 0.01^{A}	0.80±0.06	0.78±0.01	0.77 ± 0.02^{B}	0.33±0.01	0.34 ± 0.01^{C}	0.33 ± 0.02^{C}
VI	1.03±0.02	1.11±0.05	1.16 ± 0.06^{AB}	0.23±0.01	0.23±0.01	0.23 ± 0.01^{A}	0.78±0.02	0.87±0.05	0.93 ± 0.05^{B}	0.33±0.01	0.42 ± 0.01^{B}	0.42 ± 0.05^{B}

Means bearing different superscripts i.e., a, b and c differ significantly (P<0.05) when compared horizontally in a row.
Means bearing different superscripts i.e., A, B, C and D differ significantly (P<0.05) when compared vertically in a column.

Table 6:
Effect of feeding of 0.2% *Spirulina plantesis* (SP) and 1% *Erythrina variegata* leaf powder (EVLP) daily as feed supplement for 60 days on AST, ALT and ALP (IU/L) in imidacloprid intoxicated WLH cockerels. (Mean±SE, n=7)

Groups	AST			ALT			ALP		
	0th day	30th day	60th day	0th day	30th day	60th day	0th day	30th day	60th day
I	108.04±1.00	106.36 ± 1.34^{CD}	107.00 ± 2.21^{B}	21.81±0.91	22.72 ± 1.37^{C}	22.97 ± 1.94^{C}	191.11±1.97	187.17 ± 3.24^{B}	184.49 ± 3.33^{C}
II	104.89±0.89	104.70 ± 2.17^{D}	103.92 ± 2.51^{B}	20.91±0.84	21.89 ± 2.56^{C}	22.15 ± 1.16^{C}	191.22±1.43	189.14 ± 5.03^{B}	183.16 ± 2.28^{C}
III	105.42±1.99	134.68 ± 2.07^{bA}	144.77 ± 5.07^{aA}	21.01±1.99	28.25 ± 1.74^{bB}	$43.45\pm1.51^{a,A}$	189.24 ± 2.2^{C}	204.87 ± 7.31^{bA}	$226.53\pm3.01^{a,A}$
IV	103.26±2.34	113.54 ± 2.64^{BC}	105.93 ± 5.96^{B}	20.65 ± 1.01^{c}	32.05 ± 1.64^{aAB}	22.82 ± 2.04^{bC}	186.06±4.2	201.62 ± 8.95^{AB}	190.83 ± 5.09^{C}
V	104.32±1.96	107.60 ± 3.04^{CD}	105.89 ± 3.02^{B}	21.19±0.86	$22.47\pm.74^{C}$	21.7 ± 1.61^{C}	193.58±3.29	195.32 ± 3.87^{AB}	186.28 ± 1.37^{C}
VI	105.81±3.66	118.08 ± 2.78^{B}	115.79 ± 6.71^{B}	19.97 ± 0.85^{b}	33.52 ± 1.46^{aA}	30.00 ± 2.03^{aB}	194.97±2.83	211.63 ± 8.98^{A}	200.78 ± 3.35^{B}

Means bearing different superscripts i.e., a, b and c differ significantly (P<0.05) when compared horizontally in a row.
Means bearing different superscripts i.e., A, B, C and D differ significantly (P<0.05) when compared vertically in a column.

normal, showing its protective effect in restoring the glomerular filtration. Protective action of *Spirulina platensis* @ 1000 mg/kg b wt against deltamethrin toxicity in rats were also reported by Abdel-Daim *et al*. (2013) and protective action of *Spirulina fusiformis* @ 400 mg/kg b wt against bromobenzene induced toxicity in rats were also reported by Mahima *et al.* (2014). *Spirulina platensis* treatment might have prevented the oxidative damage caused by imidacloprid owing to presence of phycocyanins in *Spirulina platensis.*

A significant (P<0.05) increase in AST, ALT and ALP was seen in imidacloprid treated birds of group III. Group IV showed significant (P<0.05) reduction in AST, ALT and ALP in comparison to group III after 30 and 60 days, significant (P<0.05) reduction in AST, ALT and ALP in comparison to group III is also shown by group VI in comparison to group III after 60 days (Table 6). The increase in the levels of ALP, ALT and AST in this study might have resulted due to prolonged duration of exposure of imidacloprid that the level of enzymes has increased for detoxification of imidacloprid, which may possibly be based on mutation of genes responsible for the synthesis of these enzymes. Similar results were also reported in other species by Toor *et al.* (2013) following oral administration of imidacloprid @ 9 and 45 mg/kg b wt for 4 weeks in female albino rats. The protective effect shown by *Spirulina platensis* may be due to presence of phycocyanins, carotenoids, minerals, vitamins, proteins, lipids and carbohydrates in it (Upasani and Balaraman, 2003). Ameliorative action of *Spirulina platensis* were also reported by Abdel-Daim *et al*. (2013) @ 1000 mg/kg b wt against deltamethrin toxicity in rats. The protective effect shown by *Erythrina variegata* might be due to presence of flavonoids, which might have shown antioxidative activity by direct scavenging of free radicals, because of the high activity of hydroxyl group of the flavonoids, free radicals are made inactive (Korkina and Afanasev, 1997).

It is concluded from the present study that the supplementation of feed with *Spirulina platensis* and *Erythrina variegata* have protective effect on simultaneously induced imidacloprid toxicity and *Spirulina platensis* may be used as an excellent growth promoter and as an alternative to antibiotics and other toxic chemicals currently used as growth promoters.

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Research Article

MOLECULAR CHARACTERIZATION OF *SALMONELLA* (RARE SEROVARS) ISOLATED FROM GANGETIC WATER BASED ON ERIC-PCR

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ABSTRACT

Considering zoonotic importance of Salmonella Molecular Typing of 20 isolates belonging to 7 different serovars (Abuja, Lagos, Chinkual, Zwickau, Pontypridd, Goldenberg and Oritamerin) was carried out by ERIC-PCR using two primers ERIC-C1 and ERIC-C2. In all 20 isolates amplification occurred. Total 10 profiles were observed in 20 isolates. The present study reveals that ERIC-PCR can be used to differentiate strains of same serovars. ERIC-PCR used in combination with other molecular typing methods will provide further vital information in serovars differentiation. The discrimination index was moderate to high (D value 0.92) calculated by Simpson"s index of diversity.

Keywords: ERIC-PCR, Salmonella, Serovars, Discriminative Index, Dendrogram.

INTRODUCTION

Salmonella infections may be severe, especially in very young, older or immunodepressed people, with a possible infecting dose for healthy person of 10 CFU. Serotypes *S. Typhimurium* and *Enteritidis* are two most widespread serotypes that cause human salmonellosis (CDC, 2005). *Salmonella* has more than 2500 serotypes based on the Kaufmann and White scheme in which somatic and flagellar (H) antigens are identified and occasionally capsular (Vi) antigens may be detected.

Conventional methods like ribotyping, biotyping, OMP profiling and Phage typing used for characterization and identification of strains But these conventional methods are time consuming and less specificity. Genotyping has versatility over traditional methods because genomic analysis not only facilitates identification and fast detection of an organism but also determines it's taxonomic position in the evolutionary hierarchy. Pulsed-field gel electrophoreses (PFGE) and ERIC-PCR (Saxena *et al.,* 2002) have been found useful target for molecular typing of *Salmonella* serovars. PFGE is considered the gold standard, but it requires elaborate set-up and costly equipment, whereas ERIC-PCR is simple to perform.

MATERIALS AND METHODS

Isolation of *Salmonella* from Ganga water

Total 500 water samples were collectedfor isolation of *Salmonella* from Ganga 10 locations Gangotri, Uttarkashi, Rishikesh, Haridwar, Hastinapur, Garh mukteshwar, Narora, Kanpur, Allahabad, and Varanasi in autoclaved bottles. Approximately 200 ml of water was collected. Fifty samples were collected from each location. All samples were brought to the laboratory on ice as soon as possible.

Samples were then slowly filtered through 0.45 µm syringe filter. The filter membrane was inoculated in 5ml LB broth test tube and incubated for 18 hr in incubator shaker at 37^0C for pre-enrichment of the bacteria in the media.From each LB tube, 1 ml of culture was inoculated in 9 ml of tetrathionate broth for selective enrichment of *Salmonella* and incubated at 37^0C for 18 hr.After 18 hr of incubation, the enriched LB media was streaked on Brilliant Green Agar (BGA) plates containing 100 µl of Novobiocin (50 mg/ml) and incubated for 18 hr at 37^0C.Then colonies showing characteristic pink colour were selected. The single colony was used for the preparation of lysate of individual sample. For identification "*his"* Gene by PCR.

Isolated colony suspected as *Salmonella* was reinocculated in 5 ml LB media and incubated for 24 hr at 37^0C. Then 1.5 ml culture was centrifuged in a micro centrifuge tube at 10000 rpm for 10 minutes and pellet was collected .The pellet was resuspended in 100 µl autoclaved distilled water and boiled at 100^0C for 10 minutes. Immediately after that the samples were ice cooled for 10 minutes. Then 5 µl of supernatant was collected in fresh PCR tube. This lysate was used for confirmation of *Salmonella* by 'his' gene PCR.

Samples showing positive results in '*his*' gene PCR was subjected to a panel of biochemical tests in *Salmonella* identification kit. These tests 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 (Table1).

Isolates showing biochemical profile of *Salmonella* were sent for serotyping at National *Salmonella* Research Centre, IVRI, Izatnagar, U.P.

ERIC-PCR analysis

For amplification of ERIC sequence genomic DNA was isolated as per C-tab method (Wilson *et al.*, 1987) with some modifications. DNA was quantified and 40ng of template DNA was taken for 25 µl of PCR reaction mixture containing 100µM/L of dNTPs, 5pmol of each primer 1 (ERIC C-1 5' CAG CCA TGA ACA ACT GGT GGC G 3') and primer 2(ERIC C- 2 5' TGC TTT GCG CAG GGA AGA TTC 3') and 1.5 Unit of Taq DNA polymerase in 1X PCR buffer (1.5Mm Mgcl2). PCR was performed with an initial denaturation step (94ºC for 5 min) followed by 30 cycles of denaturation (94ºC for 1 min.), annealing (55ºC for 1 min.) and extension (72ºC for 5 min.) with final extension at 72ºC for 10 minutes REP-PCR repeated thrice to check reproducibility. Amplified products wre resolved by electrophoresis in 2% agarose gel containin ethidium bromide 0.5µg/ml for1.5 hr in 1X TAE buffer. As size marker 100-300 bp DNA low range rular (Banglore Genei) was used. The NTSYSpc (version-2.11V) software was used to construct phylogenetic tree (Table 3 & Fig 3) and analysis of bands.

RESULTS AND DISCUSSION

In ERIC PCR, amplicons were observed in all the 20 isolates (Table-2). No Amplicon was seen in negative control (Fig1& Fig2). It that ERIC sequence is present in all the isolates. Out of 20 isolates, 10 different profiles designated as E1 to E10 were observed. Profiles E1 and E3, E7 and E10 were found to be very rare as these were observed in only two isolates. Profile E6 was the most common profile and observed in four isolates. No serovar specific profile could be observed in this study. The band size varied from 138 bp to 4286 bp. No common band was observed in the serovars but the bands of 508 bp, 192 bp, 138 bp and 292 bp were observed in most of the isolates. A band of 278 bp was observed in one isolate G-2(*S.*Abuja) only. This may be serovar *S.* Zwikau specific band. The efficiency of the differentiation calculated by Simson's Index of Diversity (D) was 0.92.

ERIC-PCR (target ERIC sequence) (Saxena *et al.,* 2002) used for the molecular typing of 24 isolates of *Salmonella* serovars, namely *Abortus equi*, Choleraesuis, Barielly and Dublin. In ERIC-PCR, seven molecular types were observed with ERIC-C1 primer, and nine molecular types with ERIC-C2 primer. When the results of both the ERIC-PCR were combined for molecular typing, 21 molecular types were observed, which indicated a high degree of discrimination. Both the ERIC primers are designed from the ERIC consensus sequence, yet they gave different profiles, indicating that they supplement each other ERIC sequences were found to be useful target for molecular typing. The different profiles observed appear to be due to differences in ERIC sequences and differences in inter ERIC distance.

The first time use of ERIC-PCR (Millemann *et al.,* 1996) for molecular epidemiological studies of *Salmonella*. Discrimination of 70 *Salmonella* strains previously studied by ribotyping was done by RAPD and ERIC-PCR analysis. RAPD results of 56 *S.* Typhimurium isolates did not closely match those of ribotyping with ERIC-PCR, two fingerprints only were obtained. For the 14 *S.* Enteritidis strains, a helpful discrimination was obtained with RAPD analysis, while ERIC-PCR resulted in a single fingerprint. ERIC-PCR fingerprinting (Chmielewski *et al.,* 2002) used for analysis of *Salmonella* Enteritidis strains of poultry by which they divided these isolates into three distinct genomic groups

In the present study, all 20 rare isolates of *Salmonella*

Table 1:
Biochemical characterization of Samples

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

Table2.
Band Profile of rare serovars of *Salmonella* obtained from Primer (ERIC-C1 and ERIC-C2).

Sl. No.	Strain No.	Serovars	Band size (bp)	Profile
1.	G-2	*S.* Abuja	292, 192, 138	E1
2.	G-4	*S.* Abuja	508, 192, 138	E2
3.	G-6	*S.* Abuja	1165, 192, 138	E3
4.	G-7	*S.* Pontypridd	508, 192, 138	E2
5.	G-9	*S.* Abuja	1165, 508, 292, 192, 138	E4
6.	G-12	*S.* Lagos	784, 562, 486, 417, 352, 271, 211, 153, 112	E5
7.	G-13	*S.* Lagos	784, 562, 486, 417, 352, 271, 211, 153, 112	E5
8.	G-15	*S.* Lagos	508, 292, 192, 138	E6
9.	G-17	*S.* Chinkual	192, 138	E7
10.	G-19	*S.* Abuja	1165, 508, 138	E8
11.	G-22	*S.* Abuja	508, 292, 192, 138	E6
12.	G-24	*S.* Chinkual	508, 292, 192, 138	E6
13.	G-25	*S.* Zwickau	780, 508, 192, 138	E8
14.	G-26	*S.* Goldenberg	4286, 508, 292, 192, 138	E9
15.	G-27	*S.* Lagos	784, 562, 486, 417, 352, 271, 211, 153, 112	E5
16.	G-30	*S.* Pontypridd	4286, 292	E10
17.	G-31	*S.* Abuja	4286, 508, 292, 192, 138	E9
18.	G-32	*S.* Abuja	1165, 508, 292, 192, 138	E4
19.	G-39	*S.* Oritamerin	1165, 508, 292, 192, 138	E4
20.	G-43	*S.* Oritamerin	508, 292, 192, 138	E6

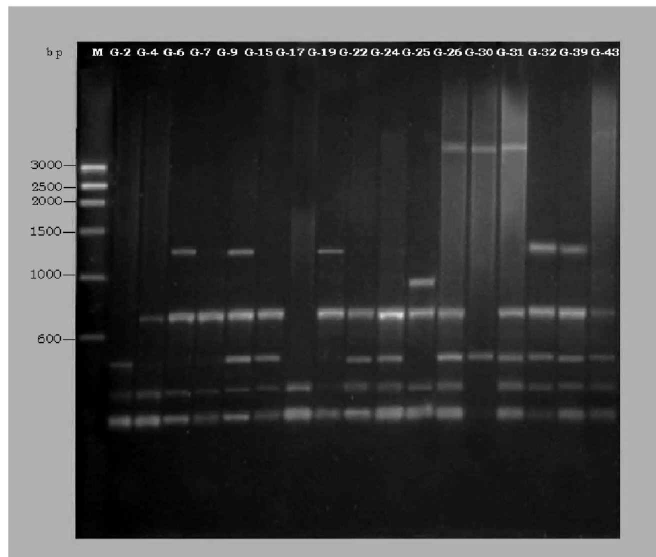

Fig.1:
Band Profile of Salmonella Rare isolates in ERIC-PCR. Lane M : DNA Marker (100-3000 bp) (DNA low range DNA Rular, Banglore Genei); Lane 2-18: *Salmonella* isolates G-2, G-4, G-6 G-7, G-9, G-15 G-17, G-19, G-22, G-24, G-25, G-26, G-30, G-31, G-32, G-39, G-43

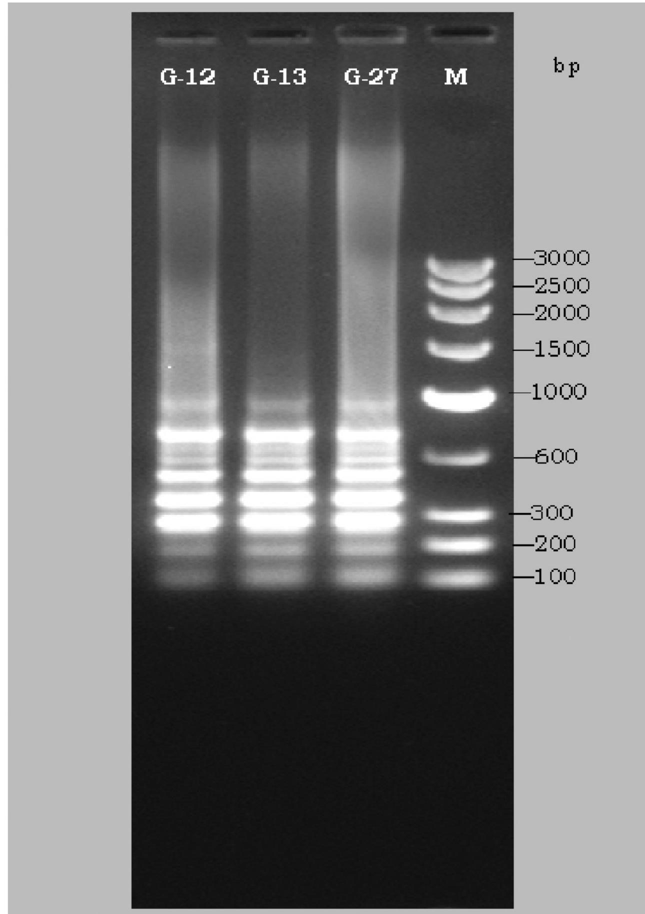

Fig.2:
Band profile of Salmonella Rare isolates in ERIC-PCR. Lane M: DNA Marker (100-3000 bp); (DNA low range DNA Rular, Banglore Genei), Lane1-3: *Salmonella* isolates G-12, G-13, G-27.

showed ten different band profiles. As isolates G-2 showed E1 band profile while isolates G-4 and G-7 showed E2 band profile and G-6 showed E3 band profile. G-9, G-32 and G-39 are having E4 profile. The G-12, G-13, G-27 showed E5 band profile,G-15, G-22, G-24 and G-43 make the profile E6, Isolate G-17 is of band profile E7 and isolates G-19, G-25 have the band profile E8. The G-26 and G-31 have the profile E9, and the isolate G-30 showed the band profile E10. 508, 192,138 and 292 bp bands were common in most of the isolates while 780 bp bands was found only in isolate G-25 and this band may be *S.* Zwickau specific. Common band profiles observed in maximum number of isolates concluded that

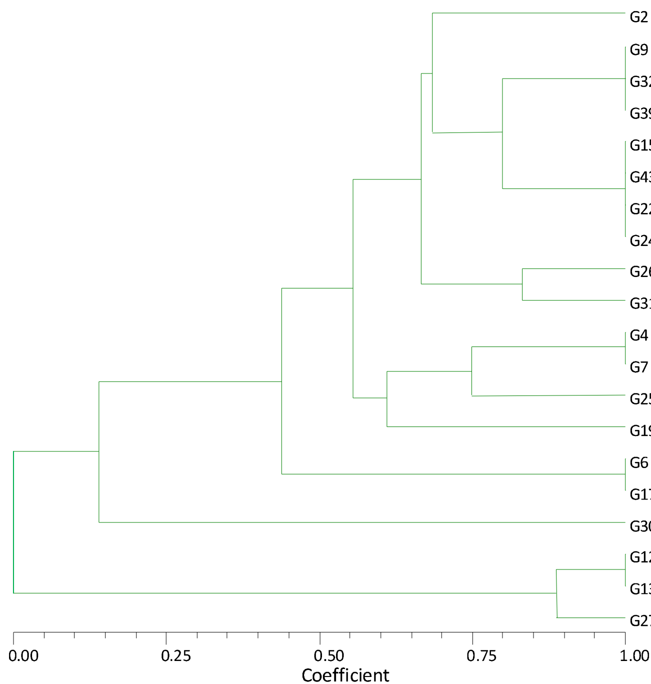

Fig. 3:
Phylogenatic tree of *Salmonella* isolates (rare serovars) based on ERIC-PCR profiles

ERIC PCR can be used for strain differentiation of *Salmonella* Rare isolates.

These observations were different from other researchers like (Lupski and Weinstock, 1992; Millemann *et al., 1996*). The preliminary study reveals that ERIC PCR can be used for strain differentiation within a serovars. ERIC sequence on one strand of DNA is not related to ERIC sequence of other strand of DNA. The present study reveals that ERIC PCR has a limitation that it can produce the similar profiles among different serovars as E2 profile (508, 192, 138 bp) was showen by *S*.Abuja (G-4) and *S*.Pontypridd (G-7) and profile E4 (1165, 508, 292, 192 bp) was exhibited by *S*.Abuja(G-9,G-32) and *S*.Oritamerin(G-39) and similarly profiles E5, E6, E8 and E9 were showed by different serovars**.**

The D value of ERIC-PCR was found to be 0.92; no serovars specific bands were obtained. Similar results were reported by several other workers. The present study has revealed that ERIC-PCR could be used for strain differentiation within a serovar. As the D value of ERIC-PCR was found to be 0.92 or 92%, so we suggest that this technique should be used with some other typing methods like REP-PCR, RFLP, and AFLP.

Thus, ERIC is the repetitive elements present in the family enteriobactericae. In the present study ERIC-PCR (target ERIC sequence) was used for the molecular typing of 20 isolates of *Salmonella* serovars, namely Abuja, Lagos, Chinkual, Pontypridd, Zwickau, Goldenburg, Oritamerin. Total 20 serovars of *Salmonella* were discriminated into 10 molecular types. The discrimination value (D value) of ERIC-PCR was found to be 0.92. The different profiles observed to be due to differences into ERIC distances. The study indicates that ERIC-PCR can form an efficient tool for molecular typing of *Salmonella* species and its efficiency can be further improved by combined molecular typing using ERIC-PCR with other typing methods.

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HAEMATOLOGY AND PLASMA BIOCHEMISTRY INFLUENCED BY ADMINISTRATION OF MARBOFLOXACIN IN SHEEP: SAFETY IMPACT

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ABSTRACT

A safety evaluation of marbofloxacin was carried out to assess clinical impact of intravenous administration of marbofloxacin on various haematological and biochemical parameters in sheep to establish safety profile of marbofloxacin. Six healthy male sheep were treated with single intravenous dose of marbofloxacin @ 2.0 mg/kg body weight. The blood samples before and after intravenous drug administration at different time interval were collected and plasma were separated. The haematological and plasma biochemical analysis were done. The intravenous administration of marbofloxacin @ 2.0 mg/kg in sheep causes statistically significant alteration in the values of neutrophils, lymphocytes, total erythrocytes and haemoglobin, but within normal clinical range. The values of alanine aminotransferase and blood glucose showed statistically difference before and after drug administration. Remaining biochemical parameters remains unaffected. Urine examination did not show any relevant alterations. Clinical examination of sheep during and after drug administration didn't reveal any clinical signs and symptoms of adverse drug reaction or toxicity. All the animals were clinically normal with respect to body temperature, respiration rate and heart rate. Form the findings of present study, it is concluded that single intravenous dose of marbofloxacin @ 2.0 mg/kg in sheep does not produce any clinically relevant adverse effects or toxicity.

INTRODUCTION

The increased incidence of infectious diseases and resistance to classical antimicrobials, are global challenge for veterinary clinicians in twenty first century (DaSilva *et al.,* 2013). The only way to counteract these challenges is to introduce more effective and safer newer antimicrobials in veterinary practice. Marbofloxacin is a synthetic third-generation fluoroquinolone, exclusively developed for veterinary treatment (Schneider *et al.,* 1996; Aliabadi and Lees, 2002; Meunier *et al.,* 2004). Marbofloxacin achieves rapid bactericidal activity by inhibiting bacterial DNA gyrase, an enzyme responsible for packaging DNA within cells (Gellert, 1981; Shen *et al.,* 1989). The drug is approved for use in veterinary species *viz.,* cattle, pigs, dogs, horses and cats for respiratory, urinary tract, soft tissue and dermatological infections (Cotard *et al.,* 1995, Thomas *et al.,* 1997, 1998[a], 1998[b] and 2001). The studies on pharmacokinetics of marbofloxacin in veterinary species including sheep (Sidhu, *et al.,* 2010) have been reported to propose suitable dosage regimens for clinical application. But there are scanty reports on clinical impact of marbofloxacin on haematology and plasma biochemistry in sheep or in other veterinary species. Therefore present investigation on clinical impact of single intravenous administration of marbofloxacin on haematological, biochemical and urine parameters in sheep was carried out to exclude possibilities of adverse reactions associated with use of marbofloxacin in sheep and to recommend its safe use among field veterinarians for treating infectious diseases. The results of the present study would help to establish the safety profile of marbofloxacin in sheep. It will also open new avenue for clinical use of novel drug (marbofloxacin) in sheep suffering from infectious diseases which are not responding clinically to treatment by conventional or older antimicrobials.

MATERIALS AND METHODS

Experimental Animals

Six healthy male sheep of 'Patanwadi' breed having body weight between 30 to 38 kg and 1 to 1.5 years of age were selected for study. The research protocol was approved by IAEC of the institution. The animals were subjected to acclimatization period of 15 days during which all the animals were subjected to daily clinical examination in order to exclude possibility of any diseases condition, which otherwise may interfere with results of experiment. All the animals were kept in individual pan of experimental sheep shed at Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar. The latitude and longitude of experimental location are; N 24.324309 and E 72.296369, respectively. All the animals were offered combination of green and dry fodder along with concentrates as per the standard schedule of Livestock Research Station. Fresh potable water was made available *ad libitum* to all animals during experimental period.

Drug administration and collection of samples

The drug formulation for intravenous administration was prepared from certified samples of marbofloxacin

procured from Naxia Enterprise, Mumbai, India. The purity assay of marbofloxacin reference sample used was between 99.0 to 101 % (percent w/w on dried substance) and compliance to all standards as described in European Pharmacopoeia 7.0. The 0.01 M acetic acid and water for injection was used to prepare the marbofloxacin injection having final drug concentration of 10 mg/ml. Single dose of the freshly prepared drug formulation was injected intravenously into left jugular vein of sheep at the dose rate of 2.0 mg/kg.

Approximately 3 ml of blood samples were collected from right jugular vein of sheep before administration of drugs (0 day) and were considered as control in this study. Similarly, remaining blood samples were collected into K_2EDTA vacutainer tubes and sterile heparinized test tubes at 6 h, 12 h, 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day for haematological and plasma biochemical analysis, respectively. Plasma was separated after refrigerated centrifugation of blood samples at 1600 revolutions per minute (rpm) for 10 minutes. The plasma samples were transferred to cryo-vials and then stored at 2-8° C until assayed for enzyme estimation. Approximately 15 ml of urine sample were collected from animals using urinary catheter (Sterile disposable AI Sheath) through urethral opening before drug administration and at 6 h, 12 h, 1, 2, 3, 4, 5, 6 and 7th day after drug administration.

Haematological, Biochemical and Urine analysis

Haematological parameters were analyzed with Automatic Haematology Analyzer, MEK-6450 Celltac á (Nihon Khoden, Japan) immediately after collection. The biochemical parameters were estimated with analytical kits (Sigma diagnostics Pvt. Ltd., Vadodara) using Automatic Biochemistry Analyzer, Pictus 400 (Diatron, Germany). Chemical, physical and microscopical examination of urine samples were conducted immediately after collection of samples using Uriscan® urine strips (U41, YD Diagnostics, Korea) for urine analysis.

RESULTS

Average values of the haematological parameters including total leukocyte count (TLC), differential leukocyte counts (DLC) (lymphocytes, monocytes, neutrophils, eosinophils and basophils), total erythrocyte count (TEC), haemoglobin (Hb) concentration, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) count were estimated before (0 day) and after single dose intravenous administration of marbofloxacin at the dose rate of 2.0 mg/kg on 6 h, 12 h, 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day in present study and their values are given in Table 1.

The average value of blood biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), acid phosphatase (ACP), alkaline phosphatase (ALP), creatine kinase (CK), lactose dehydrogenase (LDH), creatinine (CR), total bilirubin (T-BIL), direct bilirubin, blood glucose, blood urea nitrogen (BUN) and total protein estimated before (0 day) and after single dose intravenous administration of marbofloxacin at the dose rate of 2.0 mg/kg on 6 h, 12h, 1st, 2nd, 3rd, 4th, 5th, 6th and 7th day in present study are presented in Table 2. The average values of specific gravity and pH of urine samples were presented in table 3. The values of all parameters observed at 0 day (before drug administration or pre-dose) were compared with values observed on remaining days (after drug administration or post dose).

DISCUSSION

The statistically significant differences were observed in the values of neutrophils and lymphocytes at 6 h, 1st, 2nd, 3rd and 6th day, when compared to values of 0 day (p d" 0.05). The increased values of neutrophils were observed at these time interval, while decreased values were noticed for lymphocytes. The values of TEC at 4th, 5th and 6th day interval showed decreased values as compared to values on 0 day. On 7th day, the value of TEC returned to normal range and did not show any significant difference (p d" 0.05). The value of haemoglobin was found to be significantly lower at 6 h at 1% significant level. Same was significantly lower on 3rd and 6th day at 5.0 % significant level. Significant differences were observed in the value of PCV on 3rd day ($p<0.01$). The values of MCH and MCHC obtained on 6 h; and neutrophil at 6th day were found to be significantly different (p d" 0.01). The normal values of haematological parameters in sheep *viz*., haemoglobin (8 - 16 g/dL), RBC count (9 -15 x 10^6/µL), TLC/Total WBC (4-12 x 103/µL), neutrophils (20-42 %), lymphocytes (40-70 %), monocytes (0-6 %), eosinophils (0-10 %), basophils (0-3 %), PCV (37-48 %), MCV (23-48 fl), MCH (8-12 Pg), MCHC (31-38 g/dL) were reported by RAR (2014) and Radostits *et al.,* (2006). Comparing these normal values of haematological parameters and observed values in present experiment, it is clear that though alteration were statistically significant, but were within normal clinical ranges of reference values reported for sheep species. Additionally no sheep treated with marbofloxacin exhibited any clinical sign or symptoms of adverse drug reaction or toxicity.

The statistically significant difference was evident in the values of ALT (44.48 ± 1.64 U/L) and blood glucose (55.55 ± 2.58 mg/dL) obtained at 12 h post dosing when compared with values obtained on 0 day *i.e*., pre-dosing or before drug administration. These transient alterations in the values of ALT and blood glucose did not persist for longer period and the values of both parameters became normal with subsequent sampling. The normal values of ALT (22-38 U/L) and blood glucose (42-76 mg/dL) as reported by RAR (2014) justifies that the alteration in ALT

Table 1:
Haematological parameters (mean ± SE) before and after single dose intravenous administration of marbofloxacin (2.0 mg/kg body weight) in sheep

Parameter	Unit	Values of parameters (Mean ± SE) Time of Collection									
		0 day	6 h	12 h	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
TLC	x10³ /µL	9.72± 1.13	10.18 ± 0.87	10.87 ± 0.81	9.33 ± 1.05	9.72 ± 0.87	9.32 ± 0.73	10.12 ± 0.40	8.72 ± 0.58	9.37 ± 0.39	7.32 ± 0.48 [a]
Neutrophils	%	28.17 ± 2.27	38.83 ± 2.61[a]	35.17 ± 2.77	43.83 ± 3.19 [a]	35.67 ± 1.23[a]	37.00 ± 2.92[a]	32.83 ± 1.35	31.83 ± 3.45	36.33 ± 2.43[b]	32.5 ± 1.76
Basophils	%	0.17 ± 0.17	0.00 ± 0.00	0.17 ± 0.17	0.33 ± 0.21	0.00 ± 0.00	0.17 ± 0.17	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.17	0.17 ± 0.17
Eosinophil	%	3.67 ± 0.56	2.67 ± 0.56	4.00 ± 0.36	1.83 ±0.40	4.00 ± 0.58	5.50 ± 1.50	4.17 ± 0.31	1.83 ±0.70	3.00 ± 0.26	2.83 ±0.70
Lymphocytes	%	65.83 ± 2.48	56.67 ± 2.32[a]	58.83 ± 2.64	51.67 ± 3.07[a]	56.17 ± 1.00[a]	54.50 ± 2.72[a]	60.17 ± 1.58	63.67 ± 3.71	58.17 ± 2.97[a]	62.50 ± 2.29
Monocytes	%	2.33 ± 0.56	1.83 ± 0.31	1.83 ± 0.31	2.33 ± 0.56	4.17 ± 0.98	2.83 ± 0.31	2.83 ± 0.31	2.67 ± 0.33	2.33 ± 0.42	2.00 ± 0.26
TEC	x10⁶ /µL	10.66 ± 0.22	10.49 ± 0.19	10.52 ± 0.28	10.45 ± 0.24	10.01 ± 0.15	09.90 ± 0.13[b]	9.88 ± 0.12[a]	9.75 ± 0.17[a]	10.00 ± 0.14[a]	9.34 ± 0.13
Hb	g / dL	10.50 ± 0.25	9.43 ± 0.31[b]	10.47 ± 0.29	10.30 ± 0.11	10.23 ± 0.09	10.10 ± 0.18[a]	10.05 ± 0.17	9.78 ± 0.23	9.80 ± 0.22[a]	9.53 ± 0.11
PCV	%	30.43 ± 0.71	29.98 ± 0.79	30.26 ± 0.67	27.45 ± 2.78	29.67 ± 0.30	29.02 ± 0.51[b]	28.87 ± 0.41	28.58 ± 0.57	28.50 ± 0.58	27.52 ± 0.38
MCV	fL	28.62 ± 0.49	28.60 ± 0.50	28.83 ± 0.69	29.05 ± 0.62	29.67 ± 0.44	29.33 ± 0.27	29.27 ± 0.56	29.32 ± 0.29	28.53 ± 0.72	29.45 ± 0.41
MCH	Pg	9.90 ± 0.21	9.00 ± 0.27[b]	9.98 ± 0.29	9.87 ± 0.24	10.23 ± 0.23	10.20 ± 0.15	10.18 ± 0.26	10.03 ± 0.13	9.80 ± 0.26	10.22 ± 0.16
MCHC	g/L	34.50 ± 0.34	31.45 ± 0.55[b]	34.55 ± 0.28	33.98 ± 0.17	34.50 ± 0.42	34.82 ± 0.36	34.82 ± 0.39	34.23 ± 0.33	34.37 ± 0.32	34.68 ± 0.26

Values having [a] and [b] superscript in same row indicate significant difference at pd" 0.05 and d" 0.01 respectively.

Table 2:
Biochemical parameters (mean ± SE) before and after single dose intravenous administration of marbofloxacin (2.0 mg kg^{-1} body weight) in sheep

Parameter	Unit	Values of parameters (mean ± SE) Time of Collection									
		0 day	6 h	12 h	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
ALT	U/L	46.13±1.37	46.08±1.89	44.48±1.64[a]	44.56±1.41	46.16±0.87	40.84 ±3.2	43.41±2.43	46.77±2.44	46.06±2.93	45.96±2.06
AST	U/L	57.32±1.13	57.98±1.26	57.42±1.82	60.36±0.71	53.91±2.94	56.73±1.58	54.69±1.94	57.68±3.52	57.84±2.67	57.08±1.70
Acid Phosphate	U/L	224.39±11.47	221.45±4.45	224.6 ±3.32	232.78±17.49	219.45 ±6.74	227.37±13.4	231.92±12.64	218.37 ±5.56	219.39±3.31	222.26 ±1.18
Alkaline Phosphatase	U/L	100.27±19.13	135.64±18.94	131.60 ±16.1	129.00±13.77	122.71±13.97	109.25±22.88	126.45±13.34	148.20±19.82	139.52±12.87	111.41±10.77
Creatine kinase	IU/L	153.39±7.99	156.22±9.44	129.79±1.28	145.43±11.50	147.04±9.43	140.52±7.37	388.68±249.56	144.90±8.78	156.88±8.27	161.81±15.49
LDH	IU/L	705.46±5.09	670.67±34.74	665.69 ±33.48	666.26±28.55	643.13±36.69	657.30±28.38	659.93±27.20	627.68±26.93	644.55±22.56	637.28±27.46
Creatinine	mg/dL	0.82 ±0.01	0.86 ±Q0.02	0.84 ±0.01	0.85 ±0.02	0.85 ±0.03	0.79 ±0.05	0.80 ±0.03	0.84 ±0.02	0.85 ±0.02	0.85±0.02
Total bilirubin	mg/dL	0.35 ±0.03	0.36 ±0.03	0.33 ±0.02	0.32 ±0.01	0.27 ±0.04	0.33±0.02	0.34±0.02	0.35 ±0.04	0.31 ±0.08	0.32±0.01
Direct bilirubin	mg/dL	0.13 ±0.01	0.13 ±0.02	0.14 ±0.03	0.17 ±0.04	0.16 ±0.02	0.14 ±0.02	0.10 ±s0.03	0.11 ±0.04	0.25 ±0.15	0.14±0.03
Blood glucose	mg/dL	49.60 ±1.91	55.20±3.51	55.55 ±2.58[a]	52.21±2.44	51.30 ±1.76	49.92±2.01	50.89±1.90	51.33±2.09	51.79±1.96	50.98±3.58
BUN	mg/dL	18.19±0.39	18.21±0.57	18.23±0.71	18.90±0.71	18.69±0.44	18.84±0.53	19.04±0.58	18.74±0.66	18.71±0.98	19.15±1.13
Total Protein	g/dL	8.08 ±0.57	8.17 ±0.43	7.95 ±0.45	8.16 ±0.28	8.33±0.33	7.96 ±0.46	7.98±0.38	8.39 ±0.41	7.79 ±0.25	8.44 ±0.20

Values having [a] superscript in same row indicate significant difference at pd" 0.05

Table 3:
Urine specific gravity and pH (mean ± SE) before and after single dose intravenous administration of marbofloxacin (2.0 mg kg^{-1} body weight) in sheep

Time of collection	Specific gravity	Urine pH
0 day	1.02 ± 0.003	7.83 ± 0.167
6 h	1.02 ± 0.004	8.33 ± 0.211
12 h	1.02 ± 0.004	7.83 ± 0.167
1 day	1.02 ± 0.003	8.00 ± 0.258
2 day	1.02 ± 0.002	7.83 ± 0.167
3 day	1.02 ± 0.003	8.00 ± 0.00
4 day	1.02 ± 0.003	7.83 ± 0.167
5 day	1.01 ± 0.002	7.83 ± 0.307
6 day	1.02 ± 0.004	8.17 ± 0.167
7 day	1.01 ± 0.002	8.17 ± 0.167

and blood glucose level in treated sheep are clinically irrelevant. The increases in blood glucose level following treatment may be due to most variable nature of this parameter or stress of repeated blood sampling on experimental animals. Stress results in release of epinephrine which promotes glycogen breakdown and inhibits glycogen synthesis. This ultimately directs all available glucose residues and precursors into production of free blood glucose (Lehninger, 1978).

No alterations were observed in the colour, odour, transparency, turbidity, specific gravity and pH of urine before and after single dose intravenous administration of marbofloxacin @ 2.0 mg/kg in sheep. There was no presence of any abnormal chemical constituents in urine of treated sheep. No microscopic abnormality was detected in urine sediments.

In present experiment, the behaviour, appetite, defaecation, urination and body temperature were observed to be normal in all the treated sheep. None of animals showed any signs related to urinary discomfort; and urine frequency was found to be normal in all treated animals before and after drug administration. The similar results were also reported in safety study of multiple doses of marbofloxacin in sheep, which revealed no obvious adverse effects and no haematological or histological changes (Robert, 2013). Similarly in cats, no clinically meaningful haematological and biochemical alterations were observed in cats administrated with high dose (ten times therapeutic dose) of marbofloxacin (FDA, 1999).

In contrary to present findings for marbofloxacin, another fluoroquinolones trovafloxacin caused elevated enzyme level of ALT, AST and LDH after multiple injection with clinically significant signs of neutrophilia and liver damage in sheep (Roberts, 2013). When high doses of marbofloxacin were administered during safety testing, the varieties of toxic reactions were being reported to occur in dogs and cats (USP monograph, 2007). But these effects are produced only at high doses, which were not serious and life threatening.

Clinical use of fluoroquinolones has been regarded as safe and tolerable in most of animal species. The safety of fluoroquinolones have been reported in many species *viz*., moxifloxacin in sheep (Modi *et al.,* 2013[a,b]) and lactating goats (Chaudhari, 2012). Gatifloxacin and ciprofloxacin, widely used fluoroquinolones in human medicines when administered repeatedly for two weeks to sheep (growing lambs) resulted into no significant signs of chrondotoxicity and other toxicity (Janson *et al.,* 2009). The safety profile of marbofloxacin in sheep suggests that the drug is safe and well tolerated without any side effects or toxicity and does not cause any clinically apparent sign and symptoms of discomfort. Other fluoroquinolones like ciprofloxacin when given by repeated doses in calves *via* intramuscular dose in calves did not cause any significant alteration in the values of biochemical parameters (Bhavsar *et al.,* 2004). The same findings were also reported for enrofloxacin in yak (Khargharia, 2007) and levofloxacin in layer birds (Patel *et al*., 2009). In human also, fluoroquinolones as a class are generally well tolerated; most adverse effects are mild in severity, self-limiting and rarely result in treatment discontinuation (Ball *et al.,* 1999; Mandell and Tillotson, 2002). The findings in present study are in agreement with other reports of fluoroquinolones in other veterinary species also. It can be concluded that the single intravenous administration of marbofloxacin in sheep at the dose rate of 2.0 mg/kg is safe and well tolerated.

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Research Article

ANTHELMINTIC ACTIVITY OF *CLERODENDRON INFORTUNATUM* LEAVES AGAINST COMMON G.I. NEMATODES IN CALVES

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ABSTRACT

Eighteen naturally infested calves with common G.I. nematodes were treated with albendazole (5 mg/kg b wt, po) and *C. infortunatum* (100mg/kg, orally) for 3 consecutive days. The post treatment EPG observation and efficacy percentage of drug were taken before and after treatment on day 3, 7, 10, 14 and 21, respectively. Albendazole in the study showed 100 % efficacy against common G.I. nematodes of calves on day 14 post treatment EPG count where as *C. infortunatum* leaves powder showed 93.94 % efficacy on day 21 post-treatment. For assessment of safety of *C. infortunatum* leaves powder, the monitoring of the haematological parameters i.e. Hb, TEC, PCV, MCV, MCH, MCHC, TLC and DLC were carried out. The values of Hb, TEC, PCV, MCV, MCH, MCHC were found to be significantly improved in albendazole and *C. infortunatum* leaves powder treated calves on day 21 post-treatment as compared to untreated control group. The TLC values showed significant decrease in treated group as compared to untreated control. It was concluded that the leaf powder of *C. infortunatum* may be used safely at the dose of 100 mg/kg b wt against the common G.I. nematodes infections in calves.

Key words: Albendazole, *C. infortunatum,* haematology, calves

INTRODUCTION

Clerodendron infortunatum (Hindi name- bhant) is a shrub distributed in Jharkhand region and other parts of India, America, Australia, Philippines, Fiji and South Africa. The leaves are bitter acrid, thermogenic, laxative, cholagogue, demulcent, anti-inflammatory, vermifuge, expectorant, antipyretic, anticonvulstant and tonic (Nadkarni, 1954). The phytochemical studies reveals that it contains clerodin, clerodolon, clerodone, clerodol, clerosterol, â-sitosterol, flavonoid like apigenin and acacetin (Banerjee, 1936). A variety of parasites external as well as internal and infest them and affect their health adversely. Among common parasites affecting calf are blood sucking species of family *Ancylostomedae, Strongylidae* and *Trichostrongylidae* etc. Therefore, the present work aims at the identification of the efficacy of leaves powder of *Clerodendron infortunatum* as an anthelmintic in calves.

MATERIALS AND METHODS

Experimental animals and drugs

Eighteen calves (100 kg to 150 kg b wt) having natural gastro-intestinal nematodes infestation were used. These calves were divided into 3 groups and were housed under proper hygienic conditions in college dairy farm, maintained on balanced diet and sufficient amount of drinking water. The standard anthelmintic drug albendazole (5mg/kg b wt orally) and test compound as leave powder of *C. infortunatum* (100 mg/kg b wt orally) for 3 consecutive days were given in the experiment.

Examination of faecal content

The faecal samples, collected from rectum of the each calf and transferred to small polythene bags were subjected to examination for positive or negative following the concentration method and the samples found negative during the direct smear test were also subjected to sedimentation and centrifugation floatation method of examination (Soulsby, 1982).

Cultivation of common GIT nematodes

Applying the direct faecal culture method (Sahai, 1965), positive faecal samples kept in small petri dishes were further transferred to bigger size petri dishes containing water. The outer dish covered with another dish of equal size to avoid any contamination with dirt particles. The culture was always kept moist for the development of larvae. It took 6 to 10 days to develop the larvae. The infective larvae were collected in water from the faecal culture, care was taken to prevent the contamination of cultured materials. The ova were identified based on the characters described by Soulsby (1982).

Identification of infective larvae

The larvae cultured and collected were examined for their identification on the basis of characters mentioned by Whitlock (1960), Sahai (1965) and Soulsby (1982). Pre and post-treatment nematodes egg per gram (EPG) count of calf faeces and hematological examination were undertaken on day 0, 3, 7, 10, 14 and 21.

Estimation of egg per gram (EPG)

The estimation of pre and post-treatment EPG of faeces was carried out as per standard method (Stoll,

1923). The per cent efficacies of the drugs were calculated by the formula as follows:

$$\text{Per cent efficacy} = \frac{\text{Pre-treatment mean EPG} - \text{Post-treatment mean EPG}}{\text{Pre-treatment mean EPG}} \times 100$$

Haematological examination

The haematological estimation of haemoglobin (Hb, gm/dl), packed cell volume (PCV %), total erythrocyte count (TEC x 10^6/µl), mean corpuscular volume (MCV fL), mean corpuscular haemoglobin (MCH pg), mean corpuscular haemoglobin concentration (MCHC gm/dl) and total leucocyte count (TLC x10^3/µl) and differential leucocyte count (DLC %) of pre and post-treatment blood samples were determined by cell counter.

Data obtained in this study was analyzed by one way ANOVA at 5% level of significance.

RESULTS

In albendazole treated group the EPG count recorded on the day 3rd indicated 61.80 per cent efficacy whereas 86.23 and 96.87 per cent efficacy was observed on the day 7th and 10th respectively. The 100 percent of recovery from the infestation was observed on day 14 and 21 post-treatment. (Table 1). The percent efficacy in *C. infortunatum* treatment was 42.45, 58.67, 70.34, 85.78 and 93.94 on day 3, 7, 10, 14 and 21, respectively. The infested untreated control animals continued to suffer till the end of observation. (Fig 1).

Haematological data is given in Table 2. Hb was significantly higher in both treated group as compared to control on 3rd, 7th and 14th days post treatment. On day 21st the Hb value of group I was significantly lower than the rest of the two groups. Whereas on day 0, the Hb value was not significantly different from control (Table 2). TEC of Group I were significantly lower than the other two treated groups on day 14 and 21, respectively (Table 2). PCV was significantly higher in both treated group as compared to control throughout the observation period. There was improvement of MCV value of other two treated groups from day 0 to day 21. There were reasonable improvement in the MCH value of treated group II and III, respectively, during the observation period whereas there was decrease in the MCH value in of group I during the same observation period and it was significantly lower than rest two groups.

The TLC values were found to be significant on day 14 and highly significant on day 21. The TLC value of group I consistently increased up to day 21. The TLC value of group II and III were significantly lower than Group 1 on day 14 and day 21 (Table 2). As shown in Table 3, on DLC, lymphocyte count revealed a significant difference between groups I, II and III on day 14 while difference was highly significant on day 21.

The difference with respect to neutrophil between the groups on day 21 was found to be highly significant. The difference eosinophil count among groups I, II and III was highly significant on day 7, 14 and 21, respectively.

DISCUSSION

The maximum pathogenicity of gastro-intestinal parasitic infestation in calves is due to the indiscriminate grazing practices. The EPG count revealed that albendazole showed 100 per cent efficacy against common G.I. nematodes of calves (*Haemonchus, Strongyloides, Trichostrongylus, Oesophagostomum, Ostertagia* spp.) on day 14 post treatment. The above finding was in agreement with the observations recorded by Kumari (2004) and Sinha (2006).

In comparison to albendazole, *C. infortunatum* leaves powder showed 93.94 per cent efficacy on day 21 post-treatment as observed by the EPG count. It may be interpreted here that the anthelmintic effect of the leaves powder of plant might be due to the presence of an active principle 'Clerodin' in the leaves of *C. infortunatum* (Sharma, 2001). Earlier reports also mentioned that the fresh leaves juice of *C. infortunatum* when introduced into the rectum remove ascarides worm burden from the host (Bakshi *et al*., 1999).

The haematological observations such as Hb, TEC and PCV in treated groups indicated significant improvement on day 21 post-treatment to that of control group. Whereas naturally infested untreated control calves

Table 1:
EPG of faeces Mean± S.E. in naturally infested calves on G.I. nematodes* after albendazole and *C. infoturnatum* leaves powered treatment.

Groups	Treatment	Dose and route	Av.Pre-treatment EPG Day 0	Post treatment EPG of faeces Day 3	Day 7	Day 10	Day 14	Day 21
I	-	-	1370± 48.46	1485± 35.67	1675.6± 55.67	1799.93± 34.46	2075.7± 38.63	2377.67± 47.63
I	Albenda-zole	5mg/kg b wt ,po, for 3 days	1533.3± 45.91	585.57± 48.37 (61.80)	211.14± 43.34 (86.23)	47.99± 39.78 (96.87)	0 (100)	0 (100)
III	*C.infortunatum* leaves' powder	100 mg/kg b wt, po, for 3 days	1480± 41.05	851.74± 45.46 (42.45)	611.68± 65.67 (58.67)	338.97± 64.5 (70.34)	210.45± 35.43 (85.78)	89.69± 45.43 (93.94)

Data in parenthesis show % efficacy;**Common G.I. nematodes : Haemonchus, Strongyloides, Oesophagostomum, Ostertagia, Trichostrongylus*

Table 2:
Hematological profile (Mean±S.E, n=6)) of calves in each group of naturally infested with G.I. nematodes* pre and post albendazole and *C. infortunatum* leaves' powder treated groups.

Parameters	Groups	Pre-treatment hematological observation	Post-treatment hematological observation			
		Day 0	Day 3	Day 7	Day 14	Day 21
Hb (g/dl)	I	9.28±0.19^{ab}	9.16±0.19^{ab}	8.84±0.18^{a}	8.32±0.24^{a}	7.81±0.24^{a}
	II	9.69±0.21^{b}	9.70±0.21^{b}	10.03±0.20^{b}	10.2±0.20^{b}	11.61±1.44^{b}
	III	8.64±0.30^{a}	8.64±0.32^{a}	9.03±0.30^{ab}	9.61±0.27^{ab}	9.84±0.28^{ab}
TEC(x10^{6}µL)	I	7.29±0.27^{a}	7.13±0.25^{a}	6.83±0.25^{a}	6.56±0.24^{a}	6.34±0.26^{a}
	II	7.85±0.19^{a}	7.89±0.21^{a}	8.39±0.21^{a}	8.71±0.21^{b}	8.99±0.19^{b}
	III	7.60±0.75^{a}	7.63±0.75^{a}	8.11±0.75^{a}	8.41±0.77^{ab}	8.64±0.74^{b}
PCV (%)	I	27.86±0.59^{ab}	27.50±0.57^{ab}	26.54±0.54^{a}	24.96±0.73^{a}	23.65±0.74^{a}
	II	29.06±0.63^{b}	29.24±0.60^{b}	30.25±0.51^{b}	30.62±0.61^{b}	30.84±0.62^{b}
	III	25.92±0.91^{a}	25.94±0.90^{a}	27.09±0.90^{ab}	27.59±0.88^{ab}	29.54±0.86^{b}
MCV(fL)	I	43.61±0.77^{a}	43.18±0.78^{a}	42.59±0.79^{a}	42.24±0.80^{a}	44.59±0.76^{a}
	II	40.56±1.24^{a}	41.18±1.22^{a}	41.83±1.25^{a}	42.43±1.25^{a}	42.92±1.24^{a}
	III	41.72±1.08^{a}	42.17±1.07^{a}	42.68±1.06^{a}	43.22±1.05^{a}	43.50±1.16^{a}
MCH(pg)	I	12.40±0.29^{a}	12.51±0.29^{a}	12.68±0.29^{a}	12.52±0.30^{a}	12.39±0.28^{a}
	II	12.14±0.27^{a}	12.26±0.27^{a}	12.53±0.27^{a}	12.70±0.28^{a}	12.86±0.28^{a}
	III	12.23±0.18^{a}	12.35±0.17^{a}	12.77±0.18^{a}	12.92±0.17^{a}	13.04±0.16^{a}
MCHC(g/dl)	I	30.35±1.25^{ab}	30.16±1.25^{ab}	29.71±1.25^{ab}	30.02±1.22^{ab}	29.39±1.25^{ab}
	II	34.62±1.23^{b}	34.49±1.24^{b}	34.77±1.19^{b}	34.63±1.22^{b}	34.03±1.25^{b}
	III	27.24±0.87^{a}	27.44±0.86^{a}	27.81±0.84^{a}	27.72±0.85^{a}	27.58±0.87^{a}

*Common G.I. nematodes : Haemonchus, Strongyloides, Oesophagostomum, Ostertagia, Trichostrongylus
Means with different alphabet as superscript as differ significantly (P<0.05) by one way ANOVA.

Table 3:
TLC and DLC of naturally infested calves with common G.I. nematodes.

Parameters	Groups	Pre-treatment hematological observation	Post-treatment hematological observation			
		Day 0	Day 3	Day 7	Day 14	Day 21
TLC(10^{3}/µL)	I	9.52±0.35^{a}	9.66±0.33^{a}	9.85±0.31^{a}	10.45±0.27^{b}	10.71±0.23^{b}
	II	9.66±0.13^{a}	9.49±0.16^{a}	9.35±0.18^{a}	9.16±0.12^{a}	9.02±0.19^{a}
	III	9.68±0.21^{a}	9.59±0.24^{a}	9.44±0.27^{a}	9.27±0.16^{a}	8.92±0.20^{a}
Lymphocyte (%)	I	44.79±1.66^{a}	44.12±0.95^{a}	42.95±0.78^{a}	40.98±.51^{a}	39.46±0.47^{a}
	II	43.19±0.51^{a}	44.26±0.57^{a}	45.52±0.53^{a}	47.19±0.59^{b}	48.60±0.65^{b}
	III	44.04±0.54^{a}	44.25±0.66^{a}	45.10±0.77^{a}	46.11±0.96^{b}	47.48±1.22^{b}
Neutrophil (%)	I	44.09±0.97^{a}	44.83±0.89^{a}	45.77±0.81^{a}	46.97±0.82^{b}	47.96±1.07^{b}
	II	47.91±0.91^{b}	47.45±0.89^{b}	46.15±0.87^{a}	43.30±0.86^{a}	42.27±1.13^{a}
	III	47.36±0.66^{b}	46.99±0.64^{b}	45.87±0.76^{a}	44.18±0.93^{ab}	43.93±1.1^{a}
Eosinophil (%)	I	5.53±0.41^{a}	5.77±0.42^{a}	6.37±0.44^{b}	7.23±0.41^{b}	8.24±0.49^{b}
	II	5.72±0.21^{a}	5.35±0.18^{a}	4.83±0.15^{a}	3.42±0.20^{a}	1.86±0.23^{a}
	III	5.52±0.21^{a}	5.25±0.20^{a}	4.50±0.20^{a}	3.40±0.21^{a}	2.22±0.17^{a}
Monocyte(%)	I	2.41±0.13	2.48±0.12	2.63±0.136	2.65±0.13	2.73±0.14
	Gr.II	2.32±0.07	2.29±0.06	2.30±0.06	2.46±0.06	2.58±0.0.74
	Gr.III	2.34±0.072	2.37±0.074	2.39±0.076	2.44±0.075	2.51±0.076
Basophil (%)				ABSENT		

Means with different alphabet as superscript as differ significantly (P<0.05) one way ANOVA.

continued to have reduced haematological values on day 21 of observations. The above finding was in close agreement with the observations of other workers viz. Ghulam *et al*. (1995) and Kumari (2004). The reduction of Hb, TEC and PCV value during G.I. nematodes infestation in calves observed in this study might have been due to suppressive effect of the toxic material secreted by the parasite *in vivo* in the host affecting their haemopoietic system (Das, 2001).

The calves infested with intestinal parasites had decreased Hb, TEC and PCV value which might be due to decrease in total number of cells surviving and increase in the ratio of fluid to cell particles as well as probable blood sucking nature of parasite. It suggests why the treated animals regained their normal haematological values following the treatment indicating stoppage in release of toxic material and other factors after elimination of parasites. Thus, the parasite controlling drugs and herbal preparations like extracts of *C. infortunatum* were found to aid the suffering animals in recovery from the damage caused by parasites.

Similarly, the haematological parameters with

respect to MCV, MCH and MCHC indicated improvement when comparison was made between treated group with albendazole and leaves powder to that of untreated control group on 21 day post treatment showed significant improvement whereas, the untreated control showed almost no change during the period . The MCH and MCHC values varied significantly on 21day. The haematological parameters with respect to TLC and DLC indicated significant improvement in groups II and III when compared to the control untreated group. The TLC values showed significant decrease in treated group as compared to untreated control which showed increased TLC value. These findings were in close agreement with the findings of Kumari (2004). In the DLC observation , lymphocyte value of treated groups were found to be normal in both treated group but untreated control group continued to decrease (Table 3).

. The neutrophil and eosinophil counts were significantly increased in infested calves but after treatment they approached towards normal on day 21 post treatment. These findings were in close agreement with the findings of Pal *et al*. (2001) and Kumari (2004). Increased eosinophil count indicated helminth infestation (Soulsby, 1982). Eosinophilia was observed in pre treatment and there was significant difference in pre and post treatment values of eosinophil which was in close resemblance with the findings of Kumari (2004) indicating increased eosinophil counts in the parasitic infestation. Eosinophilia is a well known characteristic feature of helminth infection and act as killer cells for some larval parasite as a sequel to the development of immune response of the host against the parasite (Butterworth *et al*., 1977).

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EFFECT OF PRETREATED TRIKATU ON THE PHRMACOKINETICS OF LEVOFLOXACIN IN CROSSBRED COW CALVES

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ABSTRACT

Pharmacokinetic study of levofloxacin without and with pretreated trikatu was carried out in six crossbred cow calves of 9-12 months of age weighing 70-90 kg. Levofloxacin was given intravenously at the dose rate of 4 mg/kg b.wt alone in combination with trikatu (0.2 g/kg b.wt., orally for seven days) in each of six crossbred cow calves. Estimation of concentration of levofloxacin in plasma was carried out by microbiological assay technique (Agar gel diffusion technique) by using *E. coli* (ATCC 25922) as test organism. Various kinetic parameters were calculated by using two compartment open models. Attempts were made to calculate the rational dosage regimens of levofloxacin on the basis of kinetic data and maintenance of therapeutic concentrations in plasma. Following intravenous administration of levofloxacin, significantly higher plasma concentration of the drug was observed in trikatu pretreated group as compared to levofloxacin alone group. Significantly higher values were observed in distribution and elimination half- life in trikatu pretreated group as compared to levofloxacin alone group. Which is also supported by significantly decrease in total body clearance in trikatu pretreated against the levofloxacin alone group. Volume of distribution is also higher in trikatu pretreated group. Whereas significantly higher value of extrapolated zero time concentration during elimination phase, area under curve and area under first moment curve were observed in trikatu pretreated calves as compared to levofloxacin group.All calculated data for loading and maintenance doses for different therapeutic levels (0.2, 0.3 µg/ml) at different dosage intervals (8, 12 h) were significantly lower in trikatu pretreated against the levofloxacin alone administration in crossbred cow calves.

Key Words: Levofloxacin, Trikatu, Crossbred cow calves, Intravenous administration

INTRODUCTION

Trikatu is a herbal bioenhancer compound having Indian long pepper, black pepper and ginger. It provides a natural and safe support system for impaired gastric function associated with gaseous distension. Trikatu is a safe digestive, carminative, anti-flatulent and is effective in dyspepsia. It also improves gastric function.Pharmacokinetic interaction is result of alterations of drug absorption, distribution, metabolism and elimination in combination therapy. The present study is planned to determine effect of pretreated trikatu on the pharmacokinetics of levofloxacin following intravenous administration in crossbred cow calves.

MATERIALS AND METHODS

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

Experimental animals

The study was conducted on six healthy crossbred cow calves (C1, C2, C3, C4, C5 and C6) ranging 9-12 months of age and weighing between 70-90 kg. The animals were maintained at the Instructional Farm of the College under good management conditions with *ad libitum* availability of feed and water. Fifteen days before the start of experiment deworming carried out with broad spectrum anthelmintics. The experimental protocol was approved by IAEC.

Experimental design

Levofloxacin infusion (500 mg/100 mL; ZILEE®, Axa Parenterals Ltd. Roorkee, India and Trikatu (60 capsules, each 250 mg Himalaya health care Ltd., India) were procured from Khandewal Chemist, Mhow. study was conducted in a cross over design with an interval of fifteen days between successive administrations of the drug/s in two different phases i.e., in phase I, levofloxacin was administered @ 4 mg/kg b wt, iv. whereas during phase II levofloxacin was administered at the same dose after 7 days trikatu pretreatment @ 0.2 g/kg b wt, po.

In each phase, the blood samples (4-5 ml) from each cow calf were collected from lateral jugular vein in clean sterilized previously added anticoagulant (10 % EDTA solution) test tube at 0 minute (before drug administration) 0.042, 0.083, 0.167, 0.25, 0.333, 0.50, 0.75, 1, 2, 4, 6, 8, 10, 12, 16 and 24 h. Plasma was separated soon after collection by 10 min centrifugation at 5000 rpm and stored in deep freezer at -20°C.

Estimation of levofloxacin

The concentration of levofloxacin in plasma was determined by microbiological assay technique (Arret *et al*, 1971) using *E. coli* (ATCC 25922) as test organism. The plasma concentration-time profile of levofloxacin for each animal was used to determine the pharmacokinetics. The data of levofloxacin was subjected to two compartment open model (Gibaldi and Perrier, 1982).

RESULTS AND DISCUSSION

Concentrations of levofloxacin at all time intervals of post i.v. administrations of levofloxacin alone (4 mg/kg i.v.) and in trikatu pretreated group (0.2g/kg, orally for seven days) differed significantly (except 8 h). Higher concentrations were found in trikatu pretreated levofloxacin group in comparison with its alone administration in crossbred cow calves (Figure 1).

Table 1 reveals the comparison of kinetic parameters of levofloxacin without and with pretreated trikatu in crossbred cow calves.

The comparison of calculated dosage regimen of levofloxacin without and with pretreated trikatu group for different therapeutic level (C_P^0 min of 0.2, 0.3 μg/ml) and different dosage intervals (τ) of 8, 12 h have been shown in Table 2. All calculated data for loading (D*) and maintenance (D_0) doses for different therapeutic level at different dosage interval (τ) are significantly lower in trikatu pretreated group as compared to levofloxacin alone group.

In the present study, after single intravenous dose of levofloxacin in crossbred cow calves, mean peak plasma concentration of levofloxacin at 2.5 min was 17.02 ± 0.03 μg/ml and it was detected up to 24 h. Concentrations of levofloxacin in pretreated trikatu group at 2.5 min was 29.08 ± 0.131 μg/ml, which declined rapidly 14.92 ± 0.15 μg/ml (50%) at 10 min in crossbred cow calves are in accordance with the findings of Dumka (2007), who also reported 1 min value of 17.2 ± 0.36 ìg/ml rapidly declined to 6.39 ± 0.16 ìg/ml (64% reduction) at 10 min in combined administrations of levofloxacin with paracetamol in calves.

The higher concentration of levofloxacin in pretreated trikatu group indicates that pretreatment with trikatu may influence the plasma levels of levofloxacin all time intervals in crossbred cow calves and also influenced the metabolism of levofloxacin.

No kinetic study pertaining to influence of pretreatment with trikatu on pharmacokinetics of levofloxacin has been carried out in crossbred cow calves so far.

The peak plasma level of levofloxacin was attained in the present study was approximately 58 fold higher in pretreated trikatu levofloxacin group. The mean therapeutic concentration ($\geq$ 0.1 μg/ml) of levofloxacin was maintained from 2.5 min to 16 h in plasma of calves when it was given alone however, in pretreated trikatu group it was maintained for longer duration up to 24 h. In contrast to present findings (24 h), Dumka (2007) observed that therapeutic concentration of levofloxacin in plasma was maintained only up to 10 h after i.v. administration of the drug (5 mg/kg) with paracetamol in crossbred calves. Goudah and Abo el-sooud (2008) noted 19 fold higher than the MIC of levofloxacin and the drug was detected above the minimum plasma level up to 24 h after administration in crossbred cow calves. Dumka *et al.* (2008) stated that levofloxacin level above MIC_{90} ($\geq$ 0.1 μg/ml) in plasma which was detected up to 10 h on single intravenous administration of levofloxacin (4 mg/ kg) concurrently with meloxicam (0.5 mg / kg) in calves.

Plasma levofloxacin concentration *versus* time disposition curves after intravenous administration was best fitted to the two compartment open model reported in calves (Dumka and Srivastava, 2007). Most of the kinetic parameters of levofloxacin when given alone (4 mg/kg, i.v.) and with pretreated trikatu (0.2 g/kg, orally for seven days) differed significantly except mean residence time (Table 1). The above findings clearly indicate that pretreated trikatu influences kinetic processes of levofloxacin in crossbred cow calves.

In the present study, significantly lower value for extrapolated zero time concentration of levofloxacin during distribution phase and theoretical zero time concentration were observed in trikatu pretreated group as compared to levofloxacin alone group. In contrast, the mean value for theoretical zero time concentration during elimination phase was significantly lower when levofloxacin administered alone as compared to trikatu pretreated crossbred cow calves.

In trikatu pretreated calves the distribution rate constant (α) was significantly lower while distribution half life ($t_{1/2}$ α) was significantly higher (Table 1). The above facts of lower distribution rate constants (α) and higher distribution half life ($t_{1/2}$ α) denote that higher rate of distribution of the drug occurred when levofloxacin given alone and slower rate of distribution of drug in pretreated

Table 1:
Comparison of pharmacokinetic parameters of levofloxacin (4 mg/kg, i.v.) without and with pretreated trikatu (0.2 g/kg, orally for seven days) in crossbred cow calves.

Parameters (Unit)	Levofloxacin without pretreated trikatu	Levofloxacin with pretreated trikatu
A (μg/ml)	65.34±5.56	28.38±0.43 **
B (μg/ml)	0.848±0.008	1.07±0.032 **
Cp^0 (μg/ml)	66.19±5.56	28.44±1.112**
α (h^{-1})	13.77±0.386	4.569±0.03**
$t_{1/2\alpha}$ (h)	0.051±0.001	0.151±0.001**
β (h^{-1})	0.121±0.002	0.108±0.001**
$t_{1/2\beta}$ (h)	5.72±0.08	6.40±0.08**
AUC (μg/ml.h)	10.43±0.14	15.88±0.422**
AUMC (μg/ml.h^2)	58.21±1.77	96.00±5.011**
MRT (h)	5.56±0.158	6.00±0.161
K_{12} (h^{-1})	7.98±0.049	2.571±0.048**
K_{21} (h^{-1})	0.299±0.006	0.269±0.003*
K_{el} (h^{-1})	5.61±0.366	1.836±0.029 **
F_c	0.022±0.001	0.059±0.001**
TH"P	45.06±2.59	15.95±0.165**
Vd_C (L/kg)	0.06±0.005	0.14±0.001**
Vd_B (L/kg)	4.702±0.043	3.77±0.118**
Vd_{area} (L/kg)	3.17±0.051	2.33±0.038**
Vd_{ss} (L/kg)	2.14±0.062	1.518±0.007**
Cl_B (L/kg/h)	0.38±0.005	0.252±0.007**
AUC/MIC	104.33±1.406	158.80±4.22**

* = $p < 0.05$, ** = $p < 0.01$

Table 2:
Comparison of dosage regimens of levofloxacin without and with pretreated trikatu group for intravenous route in crossbred cow calves

C_P^∞ min (μg/ml)	τ (h)	Dose (mg/kg)	Levofloxacin without pretreated trikatu	Levofloxacin with pretreated trikatu
0.2	8	D*	1.67± 0.02	1.11± 0.03**
		D_0	1.04± 0.02	0.64± 0.02 *
	12	D*	2.72± 0.04	1.71± 0.05**
		D_0	2.08± 0.04	1.25± 0.05**
0.3	8	D*	2.51± 0.03	1.67± 0.04**
		D_0	1.56± 0.03	0.97± 0.03 **
	12	D*	4.08± 0.06	2.57± 0.08**
		D_0	3.13± 0.06	1.87± 0.07**

* = $p < 0.05$, ** = $p < 0.01$; D*= Priming or Loading dose; D_0 = Maintenance dose; τ =Dosage interval; C_P^∞ min=Minimum therapeutic concentration in plasma

trikatu crossbred cow calves.

The present findings are more or less in close agreement with the findings of Dumka (2007) who also reported a very low $t_{1/2}\,\alpha$ of 0.04 ± 0.01 h in levofloxacin with paracetamol and 0.06 h when drug given alone in buffalo calves.

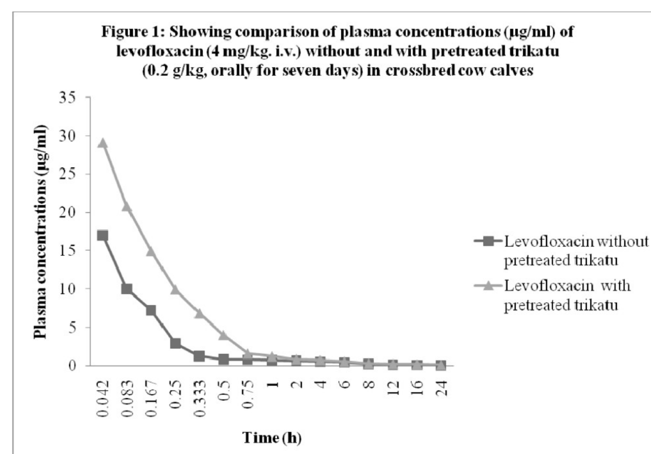
Significantly higher value (6.40 ± 0.08) was observed during elimination half life in trikatu pretreated group as compared to 5.72 ± 0.08 h in levofloxacin alone group , which is also supported by significantly decrease in total body clearance (Cl_B) as observed in trikatu pretreated group than levofloxacin alone group. It indicates that retention time of levofloxacin is more in trikatu pretreated group as compare to levofloxacin alone group. This is further supported by significantly lower value of elimination rate constant of drug from central compartment (Kel) for levofloxacin with pretreated trikatu groups as well as non-significant difference in MRT values among both the groups (Table 1).

In contrast to present findings, poor elimination half life of 2.58 ± 0.51 h after i.v. and 2.94 ± 0.78 h after i.m. administration of levofloxacin in stallions (Goudah *et al.*, 2008), 2.95 ± 0.27 h in crossbred cow calves (Goudah and Abo el-sooud, 2008). The difference in elimination half life value in the present study as compared to other species may be due to differences in biotransformation and excretory processes of different species.

The values of rate constant of drug transfer from central to peripheral compartment (K_{12}), peripheral to central (K_{21}) compartment and rate constant of drug elimination from central compartment (Kel) were significantly lower in trikatu pretreated calves as compare to levofloxacin alone group (Table 1). This denotes that rate of drug transfer from central to peripheral compartment and vice versa gets reduced when levofloxacin with and without pretreated trikatu was administered in crossbred cow calves.

The value of area under plasma concentration time curve (AUC) and area under first moment of plasma drug concentration time curve (AUMC) of levofloxacin were significantly higher in pretreated trikatu group as compared to levofloxacin alone intravenous administration in crossbred cow calves (Table 1). It reflects coverage of a vast body area by the drug when given in trikatu pretreated calves than levofloxacin alone group. Mean residence time (MRT) was differed non-significantly between both the groups (Table 1). A low value of AUC of 9.02 ± 0.34 μg/ml.h and MRT (2.58 ± 0.11 h) were recorded by Dumka *et al.* (2008) in crossbred calves after i.v. administration of levofloxacin with meloxicam. The MRT value of healthy crossbred cow calves (5.56 ± 0.158 h) was higher as compared to MRT of levofloxacin in crossbred cow calves (3.74 ± 1.21h (Goudah and Abo el-sooud, 2008) while lower as compared to MRT of 12.99 ± 2.12 h in cats (Albarellos *et al.,* 2005) after i.v. administration of levofloxacin which indicates that levofloxacin remains more time in body of cat than crossbred cow calves.

Present study revealed that in trikatu pretreated group, fraction of drug available for elimination from central compartment (Fc) was significantly higher while tissue to plasma concentration ratio (T ≈ P), was significantly lower as compared to levofloxacin alone administration (Table 1). All values of volume of distribution calculated by different methods (Vd_B, Vd_{area} and Vd_{SS}) were significantly lower in trikatu pretreated group than levofloxacin alone group except Vd_c which was significantly higher (Table 1). The findings indicate a relatively less wide distribution of



levofloxacin after i.v. administration in trikatu pretreated calves than levofloxacin alone. Indicating good penetration of levofloxacin into various body fluids and tissues was noted after intravenous administration in crossbred cow calves. The statement is further supported by higher tissue to plasma concentration ratio (45.06 ± 2.59) in levofloxacin alone and 15.95 ± 0.165 in trikatu pretreated calves as observed in the present study (Table 1).

The total body clearance (Cl_B) values of levofloxacin in present study was significantly lower in trikatu pretreated calves as compared to its alone administration in crossbred cow calves (Table 1). The higher total body clearance (Cl_B) values of levofloxacin in present study revealed that levofloxacin mainly excreted through glomerular filtration (GFR) and tubular secretion (Okazaki *et al.,* 1991).

The ultimate objective of the study of disposition kinetics is to determine an appropriate dose regimen of drugs. For any antimicrobial agent the dosage regimen is calculated to maintain the minimum therapeutic concentration (MIC) throughout the course of infections. An average plasma concentration of 0.032-0.5 µg/ml has been reported to be the minimum therapeutic concentration (MIC_{90}) of levofloxacin against most gram positive, gram negative and atypical bacteria (Chulavatnatol, *et al*., 1999). Keeping in view of synergistic effects of the immune system and other *in vivo* factors as well as to cover most of the susceptible organisms, in this discussion, the MIC_{90} of 0.1 µg/ml of levofloxacin has been taken into consideration. Levofloxacin possessed excellent antibacterial activity (MIC for 90% of tested strains i.e. MIC_{90} d” 0.5 µg/ml) against most common gram-negative aerobic pathogens, including *E. coli* reported that most of veterinary fluoroquinolones are active at MIC_{90} d” 0.17 µg/ml against sensitive strains isolated from field of veterinary importance. Thus, in the present study dosage regimen was derived at MIC of 0.1, 0.2, and 0.3 µg/ml for levofloxacin at dosage interval of 8, 12 and 16 h.

Significantly lower loading (D*s) and maintenance (D_0s) doses were observed for levofloxacin at all dosage intervals in levofloxacin with trikatu pretreated as compared to its alone intravenous administration in crossbred cow calves (Table 2) which suggested that levofloxacin dose has to be reduced for safe and effective combination with trikatu pretreatment for treating systemic microbial infections.

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EFFECT OF *EMBLICA OFFICINALIS* ON EGG CHOLESTEROL AND PRODUCTION PERFORMANCE TRAITS IN BIRDS

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ABSTRACT

The present study clearly indicated that dietary supplementation with *Emblica officinalis* at 1 and 2 percent levels in birds for forty two consecutive days caused a significant (P<0.05) reduction in egg cholesterol, triglycerides and total lipids. Production performance traits namely egg production, body weight and feed consumption in birds treated with *E. officinalis* (2 percent) showed a significant (P<0.05) and gradual increase in the above traits, however, *E. officinalis* (1 percent) did not show any significant change.

Keywords: *Emblica officinalis*, cholesterol, egg, laying hen

Chicken eggs are well established as an excellent source of all essential nutrients for persons of all ages. However, it is recommended that people should limit the consumption of eggs because of their high cholesterol (208 mg/egg) content (Kritchevsky and Kritchevsky, 2000). Therefore, it would be beneficial for health to provide a low-cholesterol egg and research efforts should be directed toward this goal. Several indigenous plants including *Emblica officinalis* (Amla and Amlaki) have been claimed to possess bypolipidemci and hypocholesteremic properties.

The study was conducted on a total of thirty six healthy Jabalpur colored birds of thirty two weeks old. Birds were procured from All India Coordinated Research Project on Poultry, Department of Poultry Science, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur. Birds were kept in individual cages under standard management conditions. All the experimental birds were kept under constant observations during the entire period (42 days) of study. Fruit of *Emblica officinalis* (Amla) was obtained from the Department of Aromatic and Medicinal Plants, Agriculture College, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur. Fruit pulp of *E. officinalis* were dried, crushed, powdered and used for supplementation of the diet in birds. Birds were divided into three groups each containing 12 birds. Group I was used as control while group II and III were supplemented with *Emblica officinalis* fruit pulp powder at the rate of 1% and 2%, respectively.

Eight eggs were collected from each group on day 0 (pre treatment), 14, 28 and 42 (post treatment) of the experiment. Lipid was extracted from the egg yolk and total lipid were estimated (Folch *et al.,*1957; Fringe *et al.*,1972). *In vitro* determination of cholesterol and triglycerides was done by using diagnostic reagent kits from Reckon Diagonostics P. Ltd., Baroda and Transasia Bio- medicals Ltd., Solan.

In unsupplemented control group of birds (Group I) total lipids, cholesterol and triglycerides in egg yolk showed no significant change throughout the experiment. However, group of birds supplemented with 1 percent *E. officinalis* fruit pulp (Group II) showed a significant (P<0.05) reduction in levels of egg yolk total lipids, cholesterol and triglycerides on day 42 of the experiment which was recorded to be 16.81, 19.35 and 18.15 percent, respectively, (Table 1).

In control group (Group I) body weight, feed consumption, egg production and egg weight did not show any significant change. However, in group III significant (P<0.05) increase in body weight was recorded on day 42 of the experiment as compared to day 0 (Table 1). Similarly, feed consumption and egg production were significantly (P<0.05) increased during period III (last two weeks) as compared to period I (first two weeks) in group III however, in group II body weight, feed consumption and egg production did not alter significantly (Table 1).

Various research workers have reported hypocholesteremic activity of *Emblica officinalis* in mammals such as rats and rabbits, however, scientific literature keeps paucity with regards to their hypocholesteremic effects in birds. *E. officinalis* used in the present study has also been suggested for hypolipidemic activity in rats. The study of Anila and Vijayalakshmi (2002) reported hypolipidemic effect of *E. officinalis* in serum and tissues of hyperlipidemic rats. The aforementioned reports of various coworkers are in close agreement to our findings.

Groups of birds supplemented with 2 percent *E. officinalis* showed significant increase in production performance traits namely; average feed intake, body weight and egg production during last two weeks of the experiment. The results of the present study are in agreement with the findings of Sapcota and Islam (2005), who reported improvement in body weight and feed consumption in broiler chicks when diet was supplemented

Table 1 :
Effect of dietary supplementation with *E. officinalis* on egg yolk total lipids (mg/g yolk), cholesterol (mg/g yolk), triglycerides (mg/g yolk), Feed consumption (g/day), Egg production (number of eggs/bird) and Body weight (g).

Group		I	II	III
Egg yolk total lipids (mg/g yolk)	**Day 0**	298.46	301.00^{a}	305.61^{a}
	Day 14	303.21	285.45^{b}	283.39^{b}
	Day 28	300.75	265.24^{c}	262.43^{c}
	Day 42	306.20	250.39^{d}	240.54^{d}
	SEM	4.37	3.54	4.30
CD at P<0.05	NS	10.28	12.47	
Egg yolk cholesterol (mg/g yolk)	**Day 0**	17.92	17.88^{a}	18.22^{a}
	Day 14	18.63	17.16^{ab}	16.88^{a}
	Day 28	18.92	15.83^{bc}	14.72^{b}
	Day 42	18.97	14.42^{c}	12.48^{c}
	SEM	0.53	0.56	0.52
CD at P<0.05	NS	1.61	1.52	
Egg yolk triglycerides (mg/g yolk)	**Day 0**	182.15	185.75^{a}	182.74^{a}
	Day 14	185.00	174.99^{a}	170.70^{b}
	Day 28	180.43	160.14^{b}	154.93^{c}
	Day 42	187.20	152.03^{b}	140.50^{d}
	SEM	4.73	4.02	4.09
CD at P<0.05	NS	11.67	11.86	
Feed consumption (g/day)	**Period I**	94.89	97.24	94.65^{b}
	Period II	92.74	98.50	95.96^{a}
	Period III	93.96	98.69	95.78^{a}
	SEM	0.90	0.53	0.35
CD at P<0.05	NS	NS	1.02	
Egg production (No. of eggs/bird)	**Period I**	3.58	3.42	3.67^{b}
	Period II	4.67	5.33	5.67^{a}
	Period III	4.08	5.33	5.25^{a}
	SEM	0.49	0.73	0.53
CD at P<0.05	NS	NS	1.53	
Body weight (g)	**Day 0**	1955.00	1916.67	1856.67^{b}
	Day 14	1984.17	1963.33	1861.67^{ab}
	Day 28	1988.33	1990.00	1908.33^{ab}
	Day 42	2001.67	2018.33	1973.33^{a}
	SEM	56.19	54.03	41.98
CD at P<0.05	NS	NS	119.73	

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

with 0.25 percent *E. officinalis* fruit powder. Wadhwa *et al.* (2007) also reported significant increase in body weight and feed consumption in birds supplemented with *E. officinalis* fruit powder in broilers at the level of 250, 500 and 750 g/ton of feed. The aforementioned researchers have clearly indicated that *E. officinalis* exert beneficial effect on production performance traits.

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Short Communication

EFFECT OF KETOPROFEN ON PHARMACOKINETICS OF LEVOFLOXACIN IN BUFFALO CALVES

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ABSTRACT

The present study was conducted to determine the effect of intramuscularly administered ketoprofen @ 3 mg/Kg on the pharmacokinetics of the intravenously administered levofloxacin in buffalo calves. Drug concentration in the plasma was determined by using high performance liquid chromatography. After i.v. administration of levofloxacin alone and in ketoprofen treated buffalo calves, there was no significant difference in pharmacokinetic parameters of levofloxacin. This indicated that the plasma concentration of the drug after iv administration in ketoprofen treated buffalo calves was not significantly higher during the phase of elimination as compared to the level in normal buffalo calves. The level of levofloxacin was maintained up to 0.090µg/ml till 10 hours. Thus, it is concluded from this study that drug is extensively distributed and is suitable for bacterial infections in buffalo calves.

Key Words : Levofloxacin, ketoprofen, buffalo calves, pharmacokinetics.

Levofloxacin is recently introduced broad spectrum third generation fluoroquinolone for veterinary use which shows favourable pharmacokinetic profile and excellent tissue penetration body tissues in man and animals (North *et al*.,1998). Antimicrobial agents and non steroidal anti-inflammatory are often used concomitantly to treat bacterial diseases. Ketoprofen is a non-steroidal anti-inflammatory, analgesic and antipyretic properties (Booth,2001).There are reports of interaction among NSAID and antimicrobials which is likely to affect pharmacokinetics of levofloxacin (Taniguchi *et al.,*1996). Therefore, the present study was undertaken to evaluate the effect of ketoprofen on the pharmacokinetic of levofloxacin in buffalo calves.

Six healthy buffalo calves of 1-1.5 years of age and with a body weight of 100-140 kilograms were maintained at instructional farm of the College of Veterinary Sc. & A.H, Mhow. Calves were subjected to clinical examination and deworming to exclude possibility of any disease. Animal were housed in stress free environment and given standard ration and water *ad libitum* and. Ketoprofen was procured from the local market and levofloxacin technical grade powder from Moxi Lab Pvt Ltd Gujrat. Levofloxacin was given alone @ 4mg/kg, i.v. in the jugular vein after the interval of 15 days of wash out period and along with ketoprofen @ 3mg/kg, intramuscularly. Blood samples were collected from the calves in clean sterilized anticoagulant mixed test tube with the help of intravenous catheter at 0 (before administration of drug),1, 2, 5, 10, 15, 30 and 45 minutes and at 1, 2, 4, 6, 8, 10, 12, 18, 24, 36 and 48 hours in both the groups. Soon after the collection of the blood, plasma was separated by centrifugation at 5000 rpm for 10 minutes at 10p c and stored at -40p c untill assayed using HPLC. The experimental protocol and work of present study was approved by the Institutional Animal Ethical Committee (IAEC).

Levofloxacin was assayed in plasma with HPLC (Ishiwata *et al*., 2007). Chromatogrphic separation was performed using reverse phase c_{18} column (Thermo, ODS; 250 x 4.6 mmID) at room temperature. The HPLC data integration was performed using software clarity (Version 2.4.0.190).The mobile phase consisted of a mixture of 1% triethylamine in water and acetonitrile (85:15v/v) adjusted to pH 3 with orthophosphoric acid. Mobile phase was filtered by 0.45µ size filter and degassed by ultrasonication. Standard curve was prepared by using the final dilution in mobile phase or plasma. Linearity was obtained from 0.01 to 50 µg/ml with mean correlation coefficient (r >0.999).The lower limit of the quantification of the drug with coefficient of variation of less than 8.36% was 0.01µg/ml. Quantification was done using standard curve. Precision and accuracy of the assay was assessed with the linearity for each standard concentration. The intraday assay precision and accuracy was estimated by analyzing six replicates at five different QC levels where as inter-day assay precision was estimated by analyzing five level QC samples on three different runs. The assay values on both occasion was seen with in the acceptable variable limits. The various pharmacokinetic parameters were calculated from plasma concentration of levofloxacin by software PK solution (version 2.0) which relies on the use of non compartmental model of analysis for the estimation of pharmacokinetic parameters. The pharmacokinetic parameters were calculated by student 't' test using SPSS software (version 12.0.1).

The plasma level of levofloxacin were plotted in semi logarithm scale. After intravenous administration of levofloxacin the mean peak plasma drug concentration of 13.48±0.23 μg/ml was observed at 0.0166h, which rapidly declined to 1.53±0.039 μg/ml at 1 hr. Plasma concentration of drug was detectable upto 10 h in ketoprofen treated buffalo calves. The peak plasma levels of drug did not differ significantly as compared to buffalo calves with levofloxacin alone at one minute. The high values of distribution rate constant and low values for elimination rate constant (β=0.36±0.008/h) seen after single i.v. dose of levofloxacin alone indicated rapid distribution in the body and slow elimination of the drug from the body. The distribution half life ($t_{1/2}\alpha$) in the buffalo calves with levofloxacin alone was 0.040±0.019/h which was same as reported by Dumka and Shrivastav (2007). The elimination half life of levofloxacin after i.v. administration was 1.92±0.04 hr which was less than the value as reported in cow calves by Dumka and Shrivastav (2007). The mean apparent volume of distribution (Vd_{area})and volume of distribution at steady state (Vd_{ss}) at the dose rate of 4mg/kg i.v. was 1.45±0.01 L/Kg and 1.30±0.01L/kg respectively. The present findings confirmed extensive penetration of levofloxacin into various body fluids and tissues due to high lipid solubility and low plasma protein binding. The MRT value after the i.v. dose in normal buffalo calves was 2.49±0.07 h very close to cow calves (Dumka and Shrivastav, 2007). Lower value of MRT in the levofloxacin treated buffalo calves showed that drug remained for shorter span of time in the body of buffalo calves due to faster elimination of the drug. All the pharmacokinetic parameters like AUC, Vd_{area}, elimination half life, distribution half life, apparent volume of distribution, total body clearance and MRT of levofloxacin did not differ significantly in levofloxacin alone and ketoprofen treated buffalo calves. Thus, it showed that ketoprofen, 3mg/kg, i.m., did not produce significant influence on the pharmacokinetics of levofloxacin, 3mg/kg, i.v, in buffalo calves.

Table-1:
Comparison of plasma concentration (μgm/ml) of levofloxacin, 4 mg/kg, i.v. alone and in ketoprofen (3mg/kg) treated buffalo calves.

Time after drug administration (hr)	Mean± S.E.Levofloxacin and ketoprofen	Mean±S.E. Levofloxacin alone
0.0166	13.87±0.14	13.48±0.23
0.0333	9.26±0.08	9.13±0.018
0.0833	6.07±0.016	6.02±0.019
0.1667	4.54±0.02	4.45±0.039
0.25	3.18±0.006	3.21±0.014
0.5	2.50±0.03	2.23±0.013
0.75	1.92±0.01	1.86±0.11
1	1.43± 0.01	1.53± 0.039
2	0.88±0.008	0.93±0.020
4	0.45±0.009	0.51±0.026
6	0.28±0.011	0.27±0.016
8	0.14±0.002	0.15±0.0012
10	0.080±0.002	0.090±0.09
12	ND	ND

*Significant at p<0.05 ** Highly significant at p<0.01

Table-2:
Comparison of pharmacokinetic parameters (μgm/ml) of levofloxacin, 4 mg/kg, i.v. alone and in ketoprofen (3mg/kg) treated buffalo calves.

Pharmac-okinetic Parameters	Units	Mean±S.E. Levofloxacin alone	Mean± S.E. Levofloxacin and ketoprofen)
Cp0	μg/ml	15.49±0.26	15.96±0.16
A	μg/ml	12.72±0.26	13.18±0.17
B	μg/ml	2.76±0.01	2.78±0.025
A	h^{-1}	16.41±0.31	15.28±0.59
B	h^{-1}	0.36±0.008	0.37±0.002
$t_{1/2}\alpha$	h	0.040±0.009	0.04±0.0003
$t_{1/2}\beta$	h	1.92±0.0147	1.85±0.017
AUC(0-”)	μg.h/ml	7.68± 0.0147	7.39± 0.03
AUMC	$μg.h^2/ml$	19.23±0.82	19.85±0.20
Vd(area)	L/Kg	1.45±0.01	1.45±0.011
Vdss	L/Kg	1.30±0.017	1.27±0.01
Vdc	L/Kg	0.26±0.004	0.25±0.002
Kel	h^{-1}	1.84±0.03	1.86±0.012
K12	h^{-1}	11.70±0.029	11.95±0.14
K21	h^{-1}	3.22±0.028	3.23±0.027
K12/K21	Ratio	3.58±0.09	3.53±0.064
Cl_B	L/h/kg	0.52±0.01	0.54±0.002
MRT	h	2.49±0.07	2.35±0.02

*Significant at p<0.05 ** Highly significant at p<0.01

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