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Short Communications

Review Article

HAEMATO-BIOCHEMICAL PROFILE AFTER ACUTE TOXICITY OF *TRIANTHEMA PORTULACASTRUM* LINN. IN WISTAR RATS

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ABSTRACT

In this study, acute repeated dose toxicity of hydroethanolic extract of *T. portulacastrum* (TPHE) was investigated in Wistar rats following oral administration at the doses of 400, 800 and 1600mg/kg for 14 consecutive days. The clinical observations, haematological and biochemical analysis revealed signs of toxicity in animals exposed to *Trianthema* extract at the dose 1600mg/kg. Mild non-significant alterations were seen at the dose of 800 mg/kg whereas no change was found in any of the haematological and biochemical parametes at the dose of 400 mg/kg body weight. It is concluded from this study that hydroethanolic extract of *T. portulacastrum* (TPHE) was toxic only at doses higher than 800 mg/kg in rats.

Key words: *Trianthema portulacastrum,* hydroethanolic extract, haematological, biochemical alterations, toxicity, Wistar rats.

INTRODUCTION

Trianthema portulacastrum Linn. (family: Aizoaceae) commonly known as Biskhapara or horse purselane is an exotic plant of Africa, Southeast Asia and tropical America. The plant grows in sunny desert areas as well as abundantly as a "weed" in high rainfall areas, especially in India and neighbouring countries. The plant is used as a valuable herb in the traditional and Ayurvedic medicine (Kirtikar and Basu, 2003).The herb is commonly used as vegeTable dishes in India and other South-East Asian countries during the rainy seasons. In Africa, especially Ghana and Tanzania, the young leaves of the herb are consumed as cooked vegeTables and in soups (Jansen, 2004). Recent study showed nutritional potential of this weed as it is a good source of proteins, fiber, riboflavin, potassium, sodium and iron (Khan *et al.,* 2013). The plant is traditionally used as analgesic, laxative and stomachic and also useful in the treatment of anemia, asthma, alcohol poisoning, bronchitis, corneal ulcers, heart diseases, edema, inflammation, liver ailments, migraine, piles and rheumatism (Kirtikar and Basu, 2003; Khare, 2006). Based on scientific investigation, various extracts of *T. portulacastrum* have been found to possess a variety of pharmacological actions, including analgesic, antipyretic, antioxidant, anti-inflammatory, hypolipidemic, hypoglycaemic, antibacterial, antifungal and anticancerous activities (Anreddy *et al.,* 2010; Shivhare *et al.,* 2012; Bishayee and Mandal, 2014).

Studies regarding toxicity of various extracts of this plant are very few and a much needed area of investigation. Keeping in view of the potential pharmacological activities as well as limited information about toxicity profile of this plant, the present study was conducted with the objective to assess the acute multiple dose toxicity of hydroethanolic extract of this plant following oral administration in Wistar rats.

MATERIALS AND METHODS

Experimental Animals

Healthy adult male Wistar rats (albino) of 2 to 3 months of age, weighing between 200 to 250 gm were procured from Laboratory Animal Resource Centre, IVRI, Izatnagar. The animals were kept in polypropylene cages in the experimental animal shed of the Department of Pharmacology and Toxicology, C.V.A.Sc., Pantnagar. Balanced ration and fresh drinking water were given *ad libitum* throughout the study period. Animals were acclimatized in the laboratory house for two weeks and kept in standard managemental conditions before start of the experiment. Animals were kept under constant observation during entire period of study. Experiments were carried out as per the guidelines of Institutional Animal Ethics Committee (IAEC), Pantnagar accredited by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi vide approval number IAEC/VPT/CVASc./189 dated 25.02.2015 (CPCSEA Reg. No. 330/GO/ERe/SL/01/CPCSEA 03.01.2001).

Collection and authentication of plant materials

The aerial parts of the plant *Trianthema portulacastrum* (Biskhapara) were collected before flowering stage from nearby fields of Pantnagar from January to April 2015. The plants were taxonomically identified and authenticated first by the Department of Biological Sciences, C.B.S.H., G.B.P.U.A.andT., Pantnagar vide No. CBSH/ Biol. Sci./ 4274 dated 11.06.2015 and finally by the Botanical Survey of India (BSI), Dehradun vide No. BSI/NRC Tech./ Herb (Ident.)/

2016-17/ 89 dated 11.05.2016. Voucher specimens of the plant has been deposited in herbarium of Botanical Survey of India, Northern Regional Centre, Dehradun (BSD) with Accession No. 116105.

Preparation of plant extract

The plant materials were cleaned, shade dried under fan for 2 - 3 days and further dried under hot circulating air in oven at 37° C for 3 – 4 days. Finally dried plant materials were ground to obtain fine powder in mechanical grinder and stored in air tight glass jars in dry place till further use.

From the powder of this plant, 50% hydroethanolic extract was prepared by cold maceration method described by Handa *et al.* (2008) Green (2004) with slight modifications. 400 grams of powder was allowed to soak in 4000 mL of 50% ethanol in stoppered container for 24 hrs with frequent agitation at room temperature. The mixture was filtered by double layered muslin cloth in Buchner funnel and then through Whatman filter paper no. 42. The residue was again macerated with fresh solvent for another 24 hours and likewise the process was repeated thrice. The final extracts were obtained by drying the filtrate in flat glass trays in fan incubator at a temperature of 40ºC till constant weight. The dried extracts were stored in air tight glass containers in deep freeze at – 20º C.

Experimental design

The toxicity of hydroethanolic extract of *T. portulacastrum* (TPHE) was studied at three dose levels, a lower dose of 400 mg/kg, a moderate dose of 800 mg/kg and a higher dose of 1600 mg/kg body weight administered orally once daily for 14 days. The animals were divided into four groups of 6 animals each and the extracts were fed orally to each animal for 14 days. The animals were observed throughout the study period for signs of toxicity. Haematological and biochemical estimations were done after 14th day.

Body weights and organ weights

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

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

Haematological parameters

The blood samples from individual rats were collected from heart at the time of sacrifice. About 2 ml of blood was collected into dry sterilized vials containing anticoagulant, sodium-EDTA. The haematological estimations, such as hemoglobin concentration (Hb), packed cell volume (PCV), total erythrocytes count (TEC), total leucocytes count (TLC) and differential leucocyte counts (DLC) were carried out.

Serum biochemical analysis

Serum was separated from the blood and stored at -20ºC for estimations of the activity of ALT, AST, LDH and creatinine kinase by using commercially available diagnostic kits (Span Diagnostics Ltd; India).

Statistical analysis

Results are expressed as Mean ± SEM with n equal to number of animals. Data were analyzed applying one way anova using the GraphPad Prism v5.01 software program (San Diego, CA, USA), and the differences were considered statistically significant at * $p<0.05$ or lower (Snedecor and Cochran, 1989).

RESULTS

Clinical signs and symptoms of toxicity were observed daily during the study period. *T. portulacastrum* hydroethanolic extract (TPHE) fed animals at higher dose (TPHE @ 1600 mg/kg) exhibited signs of lethargy with reduced feed and water intake. 2 out of 6 animals died on day 8. There was no mortality in the moderate dose group (TPHE @ 800 mg/kg). Animals in this group were exhibiting normal activity and behaviour with normal feed and water intake. Animals in the lower dose group (TPHE @ 400 mg/kg) were healthy, active and exhibited normal activities and behaviour during the study period.

Body weight of rats was recorded at weekly interval *i.e.* at day 0 (initial body weight), day 7 and 14 (Table 1). There was a significant gain ($p<0.05$) in the final body weight (day 14) of rats in the control as well as TPHE 400 group ($p<0.05$) compared to their initial body weight (day 0) indicating normal growth of rats and absence of any toxicity. However, in case of TPHE 800 group, there was non-significant increase in body weight compared to day 0 indicating retarded growth of rats. But, animals in group TPHE 1600 showed non-significant decrease in body weight indicating toxicity of hydroethanolic extracts of *T. portulacastrum* at this dose level.

Absolute organ weights and relative organ weights after 14 days of experiment are presented in Table 2. The absolute and relative organ weights of all the organs of TPHE 1600 treated animals were found to be significantly higher ($p<0.05$) compared to control group. However, relative organ weights of spleen and heart were significantly increased ($p<0.05$) in TPHE 800 treated animals. None of the animals in TPHE 400 group showed significant change in weight of any organ.

The effect on haematological parameters in rats following 14 days oral administration of hydroethanolic extracts of *T. portulacastrum* for toxicity study are presented in Table 3. Percent haemoglobin (% Hb) and total erythrocyte count (TEC) were found to decrease significantly ($p<0.05$) in TPHE1600 treated animals compared to control group animals. Packed cell volume

Table 1
Effect of hydroethanolic extracts of *Trianthema portulacastrum* (TPHE) on body weight (g) of Wistar rats following oral administration for 14 days

Groups		Day 0 (Initial body weight)	Day7(Mid body weight)	Day 14(Final body weight)
I.	Control	201.33 ± 3.31	210.83 ± 2.47	222.33 ± 2.51*
II.	TPHE 400	206.19 ± 4.93	215.73 ± 5.27	224.52 ± 4.21*
III.	TPHE 800	205.33 ± 3.50	206.50 ± 3.66	208.67 ± 3.13
IV.	TPHE 1600	203.33 ±4.77	199.67 ± 4.12	195.67 ± 4.20

*Denotes significant difference at p<0.05 compared to Day 0 within row (Mean ± SEM, n=6).

Table 2
Effect of hydroethanolic extracts of *Trianthema portulacastrum* (TPHE) on organ weights (g) of Wistar rats following oral administration for 14 days

Group	Liver	Kidney	Brain	Heart	Spleen
Control	11.36 ± 0.39	1.16 ± 0.05	1.16 ± 0.04	1.02 ± 0.05	0.65 ± 0.05
TPHE 400	11.42 ± 0.52	1.19 ± 0.07	1.17 ± 0.06	1.04 ± 0.06	0.68 ± 0.04
TPHE 800	12.11 ± 0.26	1.28 ± 0.05	1.19 ± 0.05	1.19 ± 0.04	0.84 ± 0.03
TPHE 1600	13.62 ± 0.50*	1.53 ± 0.06*	1.39 ± 0.07*	1.48 ± 0.07*	0.89 ± 0.06*

*Denotes significant difference at p<0.05 compared to control within column (Mean ± SEM, n = 6)

Table 3
Effect of hydroethanolic extracts of *Trianthema portulacastrum* (TPHE) on haematological profile in Wistar rats following oral administration for 14 days

Groups		Hb (g/dl)	PCV (%)	TEC (10^6/µl)	TLC (10^3/µl)	Lympho cyte (%)	Mono cyte (%)	neutro phil (%)	Eosino phil (%)	Baso phil (%)
I.	Control	12.85±0.45	40.34±1.80	7.41±0.22	10.12±0.47	72.36±1.88	3.07±0.13	22.50±0.89	2.13±0.06	0.16±0.02
II.	TPHE 400	12.82±0.42	39.98±1.77	7.34±0.35	10.34±0.34	70.36±2.66	2.99±0.11	23.56±1.26	2.16±0.14	0.13±0.01
III.	TPHE 800	11.73±0.41	39.16±1.43	6.93±0.12	10.72±0.27	68.17±2.23	2.89±0.10	26.13±0.97	2.25±0.08	0.11±0.01
IV.	TPHE 1600	10.78±0.41*	38.24±2.19	6.11±0.19*	10.65±0.46	61.50±2.91*	2.02±0.09*	34.17±1.38*	2.47±0.08*	0.13±0.01

*Denotes significant difference at p<0.05 compared to control within column (Mean ± SEM, n=6)

Table 4
Effect of hydroethanolic extracts of *Trianthema portulacastrum* (TPHE) on serum biochemical profile of Wistar rats following oral administration for 14 days

Group	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)	A:G ratio	AST (IU/L)	ALT (IU/L)	Creatinine (mg/dl)	LDH (U/L)
Control	7.25± 0.16	4.32± 0.15	2.93± 0.08	1.48± 0.07	68.76± 3.17	32.33± 1.73	0.68± 0.02	248.58 ± 8.99
TPHE 400	7.22± 0.34	4.29± 0.22	2.88± 0.20	1.46± 0.15	69.45± 2.98	33.23± 1.45	0.70± 0.04	251.03 ± 7.45
TPHE 800	6.82± 0.21	4.01± 0.19	2.81± 0.19	1.47± 0.14	74.33± 2.87	36.83± 1.25	0.75± 0.03	252.50 ± 8.67
TPHE 1600	6.10± 0.30*	3.60± 0.16*	2.50± 0.17	1.47± 0.10	85.67± 3.57*	41.33± 1.86*	0.95± 0.03*	268.67 ± 5.19

*Denotes significant difference at p<0.05 compared to control within column (Mean ± SEM, n=6)

(PCV) also decreased in the TPHE1600 group but the decrease was non-significant.

There was no change in total leucocyte count (TLC) in TPHE treated animals although differential leucocyte count changed significantly (p<0.05) in case of lymphocytes, monocytes, neutrophils and eosinophils. Neutrophils and eosinophils were found to increase in TPHE 1600 treated animals while lymphocytes and monocytes were decreased compared to control. There were no alterations in values of haematological parameters in TPHE 800 and TPHE 400 treated animals.

The effect on serum biochemical parameters following 14 days oral administration of hydroethanolic extracts of *T. portulacastrum* are presented in Table 4. The levels of total protein and albumin were found to be decreased significantly (p<0.05) while significant increase in serum level of AST, ALT, creatinine and LDH activities were observed in TPHE 1600 treated animals as compared to control. However, TPHE 800 and TPHE 400 treated animals showed no significant alterations in biochemical profile.

DISCUSSION

There are a number of research findings of the pharmacological activities of different extracts of *Trianthema portulacastrum* but reports of its safety as well

as toxicity profile in experimental animals are lacking and requires investigation. Before conducting pharmacological investigation, it is necessary to evaluate the drug on toxicological profile. With this objective, hydroethanolic extract of *T. portulacastrum* was administered orally to rats for 14 consecutive days and the observations for clinical, haematological as well as biochemical parameters were recorded.

Signs of toxicity were observed mainly in the group in which *T. portulacastrum* extract was fed orally at a dose of 1600 mg/kg (TPHE 1600). Mortality, reduction in body weight, alterations in organ weights followed by changes in haematological and biochemical parameters were observed in this group. The levels of total protein and albumin were found to be decreased significantly while significant increase in serum enzyme levels of ALT, AST and LDH as well as serum creatinine were observed in TPHE 1600 treated animals as compared to control.

These findings suggest that *T. portulacastrum* hydroethanolic extract at a higher dose of 1600 mg/kg body weight is toxic to animals whereas at the lower dose of 800 mg/kg showed no signs of toxicity although relative organ weights were slightly increased for heart and spleen, non-significant changes in haematological profile and biochemical profile. However, doses of 400 mg/kg body weight did not induce any of the changes and animals were completely healthy.

Very few studies have been conducted regarding evaluation of toxicity of these two plants in animals. Asif *et al.* (2013) studied acute toxicity of aqueous extract of *T. portulacastrum* and reported non-toxic action upto the dose of 3000 mg/kg orally. Apart from this, no toxicity data of this plant could be found in literatures. Hence, lack of toxicity studies of this plant in experimental animals justifies the significance of our present work.

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Research Article

ANTIDIABETIC EFFECT OF METHANOL EXTRACT OF *CASSIA AURICULATA* ON EXPERIMENTAL DIABETES IN WISTAR RATS

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ABSTRACT

The present study was carried out to investigate the effect of *Cassia auriculata* bark extract on alloxan induced diabetes in Wistar rats. Forty Wistar rats of either sex were divided into 4 groups with 10 rats in each group. In group B, C and D hyperglycaemia was induced by administering a single dose of alloxan monohydrate (120 mg/kg b wt,i.p.). Group A rats served as normal control. Alloxan treated rats of group B served as a diabetic control and given distilled water orally. Group C rats were given methanolic bark extract of *C. auriculata* @ 500 mg/kg b.wt. for 30 days. Group D rats were injected insulin @ 6 IU/kg b. wt. S/C for 30days. Group B rats showed significant reduction in body weight, AST, ALT, BUN, serum triglyceride, total cholesterol, serum creatinine and glucose values. The rats treated with *C. auriculata* methanolic extract and insulin showed significant improvement in body weight and serum levels of AST, ALT, BUN, triglyceride, total cholesterol,creatinine and blood glucose values. Thus, methanolic extract of *C. auriculata* bark showed significant hypoglycemic effect.

Key Words: Alloxan, *Cassia auriculata,* diabetes, insulin, Wistar rats

INTRODUCTION

Diabetes mellitus is a metabolic syndrome caused due to absolute or relative deficiency of insulin secretion, insulin action or both. Based on the World Health Organization (WHO) report, the number of diabetic patients is expected to increase from 171 million in year 2000 to 366 or more by the year 2030 (Wild *et al.,* 2004). Diabetes mellitus is also observed in animals. There is a role of genetic and environmental factors in feline and canine diabetes and type II diabetes is most common form of diabetes in cats. High carbohydrate diets increase blood glucose and insulin levels and may predispose cats to obesity and diabetes (Rand *et al.*, 2004). The risk of development of diabetes increases about 2 fold in obese cats (Henson and O' brien, 2006). Regardless of underlying etiology diabetic dogs and cats are hyperglycemic and glycosuric which leads to the classic clinical signs of polyuria, polydipsia, polyphagia and weight loss. Increased fat mobilization leads to hepatic lipidosis, hepatomegaly, hypercholesterolemia and increased catabolism (Rucinsky, 2010). The present conventional drugs are not only costly but also associated with lots of adverse effects. (Adeneye and Agbaje, 2008). Many herbal medicines have been recommended for the treatment of diabetes (Mukherjee *et al.,* 2006).

Cassia auriculata is a shrub that is used as 'Avarai Panchaga Choornam' (mixture of five parts of the shrub i.e. roots, leaves, flowers, bark and unripe fruits) which establishes good control on sugar levels (Brahmachari and Augusti, 1961). The present study was planned with the objective to prepare methanolic extract of *C. auriculata* bark and study its anti-diabetic activity on wistar rats.

MATERIALS AND METHODS

Preparation of extract

Cassia auriculata bark from *Cassia auriculata* plant were dried under shade and then pulverized into fine powder by using mixer grinder. Methanolic bark extract of *Cassia auriculata* was prepared using Soxhlet apparatus and extractability percentage was determined as per the method suggested by Rosenthaler (1930).

Chemicals

Alloxan monohydrate powder, manufactured by Avra Synthesis Pvt. Ltd. Hyderabad, was used to induce diabetes in Wistar rats. Insulin inj. I.P. 40 IU/ml, 10 ml vial purchased and given at dose 6 IU/kg b. wt. once a day in alloxan induced diabetic rats.

Experimental design

The apparently healthy total 40 Wistar rats of either sex with an average body weight of 200 - 250 gm were procured from National Institute of Bioscienes, Pune. Rats were divided into 4 groups consisting of 10 rats in each group. After overnight fasting, hyperglycaemia was induced by administering a single dose of alloxan monohydrate (120 mg/kg b wt i.p.), prepared in sterile saline to all groups (group B, group C and group D) except group A which served as normal control. During this period, the animals were given free access to water. After 5 days of alloxan administration, fasting blood glucose levels of rats were checked by glucosometer. The animals having blood glucose levels more than 250 mg/dl were separated and selected for studies in antidiabetic activity of *Cassia auriculata* extracts and standard drug insulin. Blood samples from all the animals were collected in anticoagulant free vial for biochemical estimations.

Group A rats served as a normal control and given only distilled water orally daily. Alloxan treated Group B rats served as a diabetic control and injected only distilled water daily. Group C rats were given methanolic bark extract of *Cassia auriculata* @ 500 mg/kg b. wt. orally as a curative regimen for the period of 30 days. Group D rats were injected with insulin @ 6 IU/kg b.wt. S/C for 30 days. After the administration of last dose, all the rats from entire groups were fasted for 24 hours and blood samples from all the animals in two aliquots were collected.

Phytochemical analysis

Phytochemical studies of methanolic bark extract of *Cassia auriculata* were carried out as per standard method (Prabhuji *et al.,* 2005). The methanolic bark extract of *Cassia auriculata* was subjected to series of phytochemical tests for the presence of alkaloids, glycosides, proteins, reducing sugar, tannins, resins, sterols, phenolic compounds, saponins, flavonoids and anthraquinones.

Clinical examination

All the rats were monitored thrice daily (morning, afternoon and evening) for clinical signs, abnormal behavior and ill health. Any rat showing abnormal clinical signs was kept under close observation. Body weight of all the animals were recorded initially on day 0 and then 14th day and 30th days post treatment (DPT).

Biochemical analysis

The blood samples were collected on zero and thirty day post treatment (DPT) in clean, dried, sterilized and anticoagulant free test tubes. The blood samples were processed for separation of serum. The serum was analyzed for blood urea nitrogen (BUN), serum creatinine, serum alanine transaminase (ALT), serum aspartate transaminase (AST) and triglycerides. The biochemical estimations were carried out by using serum auto analyzer as per the protocol mentioned in the literature of respective kit.

Statistical analysis

The data collected for various parameters were statistically analyzed by using WASP 2 statistical software Completely Randomized Design (C.R.D. test). All the values in the text were expressed as Mean ± SE as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The percent extractability was found to be 15.2 %. The color obtained of the extract was dark brown. Tween 20 was used in 0.125 per cent (w/v) as vehicle for feeding of bark extract of *Cassia auriculata L*. in treatment groups. Results of qualitative tests conducted for the presence of different phytochemicals of the methanolic extract of *Cassia auriculata* bark showed the presence of alkaloids

Table 1:

The effect of methanolic extract of *Cassia auriculata* bark on body weight (g) in different groups of rats at various periods.

Group	Treatments	Body weight (g) (Mean SE)		
		Day 0	Day 14	Day 30
A	Control	227.6±1.88	229.4^{a}±1.6	230^{a}±1.299
B	Alloxan Monohydrate @120 mg/kg b.wt.(i/p)	228.40±1.43	178.8^{c}±0.77	158.30^{d}±0.730
C	Alloxan Monohydrate @120 mg/kg b.wt.+ C. auriculata extract @ 500mg/kg b.wt.	230.7±1.318	191^{b}±0.889	200.30^{c}±1.758
D	Alloxan Monohydrate @120 mg/kg b.wt.+ Insulin 6 IU/kg b. wt.	230.20±1.698	191.8^{b}±0.474	219.70^{b}±0.804

Note: 1. Figures bearing different superscripts within same column differ significantly at 5% (P<0.05). 2. a, b, c and d are the superscripts within the same column.

Table 2:

The effect of methanolic extract of *Cassia auriculata* bark on various biochemical parameters in different groups of rats.

Parameters	A		B		C		D	
	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30	Day 0	Day 30
AST (U/L)	80±0.474	80.1^{a}±0.375	80±0.474	118.2^{d}±0.488	80.4±0.454	92^{c}±0.474	80.4±0.498	86.9^{b}±0.488
ALT (U/L)	28±0.474	28.1^{a}±0.291	27±0.557	72^{d}±0.474	28±0.474	37.6^{c}±0.612	29±0.474	29.7^{b}±0.339
BUN (mg/dl)	18.16±0.066	18.06^{a}±0.066	18.18±0.053	35^{c}±0.474	18.09±0.041	20^{b}±0.474	18.16±0.066	19^{bc}±0.474
Serum triglycerides(mg/dl)	43.38±0.042	43.44^{a}±\0.039	43.24±0.039	64.72^{d}±0.089	43.72±0.046	55.26^{c}±0.464	43.6±0.089	51.65^{b}±0.500
Total Cholesterol (mg/dl)	71.0±0.474	71.40^{a}±0.710	70±0.474	190.90^{d}±1.624	69.40±0.161	140.3^{c}±1.087	70.20±0.574	79.2^{b}±1.20
Creatinine (mg/dl)	0.441±0.017	0.496^{a}±0.004	0.546±0.014	1.6^{d}±0.035	0.488±0.023	0.926^{c}±0.011	0.486±0.001	0.598^{b}±0.020
Blood glucose (mg/dl)	86.40±2.070	87.60^{a}±0.266	87.60±0.339	324.2^{d}±1.124	89±0.761	180.6^{c}±0.877	88.60±0.339	106.4^{b}±2.733

Note: 1. Figures bearing different superscripts within same row (on day 30) differ significantly at 5% (P<0.05). 2. a, b, c and d are the superscripts within the same row.

by Dragendorff's reagent test and Mayer's test, glycosides by Benedict's test, saponins by Foam test and flavonoids by Ferric-chloride test.

It was observed that animals of control group A did not show any abnormal sign and symptoms throughout the experimental period. The rats of group B, C and D showed varying degrees of clinical signs including restlessness, depression, lowered head, dullness, weight loss and weakness during the initial period of study. At the end of study period, behavioral pattern was nearly normal in the rats of group C and group D, while the rats from group B showed sluggish behavior with lethargic activity, weakness, depression and weight loss.

The data on body weights of the rats in group A, B, C and D at 0, 14th and 30th day is given in Table 1. The antidiabetic activity of methanolic extract of *C. auriculata* bark was assessed in Group C. The result of present study indicated that body weight of rats administered with alloxan monohydrate alone @120 mg/kg body wt. (i/p) were significantly lowered on 14thday compared to zero day. The body weights of the rats from the Group A, B, C and D were 230±1.299, 158.30±0.730, 200.30±1.758 and 219.70±0.804 gms, respectively, on 30th day. The average live body weights of rats of Group B fed with alloxan monohydrate (150 mg/kg) were significantly (P<0.05) lower as compared to the rats of Group A fed on basal diet and the rats of Group C given hypoglycemic treatment of *C. auriculata* and insulin treated rats of Group D. Body weights increased after 14 days in rats of Group C and Group D as compared to rats Group B. Body weights of rats from Group C were non-significant from Group A (control) on day 30. The administration of methanolic extract of *C. auriculata bark* was helpful to restore the body weight of animals. Body weights of rats from Group C were non-significant from Group A (control) on 30 day post treatment. The administration of methanolic extract of *Cassia auriculata bark* was helpful to restore the body weight of animals.

As shown in Table 2, the values of AST, ALT, BUN, serum triglyceride, total cholesterol, creatinine and blood glucose were increased significantly (P<0.05) in alloxan induced diabetic rats (Group B) as compared to normal control (Group A) rats. While the rats (Group C) treated with methanolic bark extract of *Cassia auriculata* showed a significant decrease (P<0.05) in the values of AST as compared to diabetic control rats and were close to normal control rats and rats treated with insulin. Pari and Latha (2002) showed the anti-hyperglycemic and hypolipidemic activity of aqueous extract of *C. auriculata* flowers in experimental diabetes using the rat model.

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Research Article

IN VITRO EFFECT OF *PUNICA GRANATUM* AND *OCIMUM SANCTUM* ON EXTENDED SPECTRUM BETA LACTAMASE ENZYME OF *E.COLI*

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ABSTRACT

In the present investigation inhibitory potential and per cent inhibition of β-lactamase enzyme obtained from the extended spectrum beta lactamase producing *E.coli* in broilers was undertaken. The study included fruit peel juice of *Punica granatum* (pontgrante) and fresh leaves juice of *Ocimum sanctum* (tulsi) and one test drug tazobactum. In case of fruit peel juice of *Punica granatum* and fresh leaves juice of *Ocimum sanctum,* 11.8 ± 0.31(Mean±S.E.) with 13.4%, and 13.0± 0.45 (Mean±S.E.) with 10.2% of zone of inhibition and inhibition per cent,($p<0.05$) were observed. *Punica granatum and Ocimum sanctum in* combination gave 13.0± 0.37 to 11.0±0.21 of inhibition zone with per cent inhibition of 27 to 19%.

INTRODUCTION

Antimicrobial resistance, within a large range of infectious agents is a rising health risk of broad concern to countries and multiple sectors. Most resistant phenotypes present in animal populations are present in *Escherichia coli,* therefore commensal *Escherichia coli* can be used as indicators of the gram-negative species. extended spectrum beta-lactamases (ESBLs) have been defined as plasmid-encoded enzymes found in the Enterobacteriaceae, (European Food Safety Authority, 2011) frequently in *Escherichia coli* and *Klebsiella pneumoniae*, that confer resistance to a variety of beta-lactam antibiotics.Antimicrobial resistance problem has forced to switch over to the use of plant herbs for various infectious conditions. Pomegranate peels are characterized by substantial amounts of tannins, gallic acid ,ellagic acid and punicalagin tannins which have been reported to hold antimicrobial activity against intestinal flora, particularly enteric pathogens, such as *Escherichia coli*, *Salmonella spp*. *Shigella spp*. and *Vibrio cholerae* (Al-Zoreky, 2009). Looking into the sensitivity and severity of the problem the present study was undertaken.

MATERIALS AND METHODS

Sample collection

Initially 400 caecal samples of freshly slaughtered broilers were collected randomly from every corners of poultry sale outlets of Jabalpur. Each sample was screened for ESBL producing *E.coli* using standard methods (European Food Safety Authority, 2011). Out of 400 samples 135 samples were positive for ESBL *E. coli*. Out of these positive samples 6 samples in triplicates were used for the *in vitro* study of inhibitory potential and percent inhibition effect for *Punica granatum, Ocimum sanctum* and tazobactum as standard control.

Preparation of beta lactamase enzyme

Fresh overnight cultures of bacteria were inoculated into broth and grown for 2 h at 35°C in a rotary shaker. Inducer (penicillin G 400 µg/ml) was added, and incubation was continued for an additional 4 h. The cell pellets were collected by centrifugation, resuspended, and washed with potassium phosphate buffer (0.05M, pH 7.0) at 4°C. The bacteria were recentrifuged and subsequently resuspended in the same buffer that is 10-fold concentrated. The bacteria were disrupted by sonic treatment for 5 minutes in an ice bath. Cellular debris was removed by centrifugation at 40,000 rpm for 20 minutes at 4°C. The resulting supernatants containing beta lactamase enzyme were stored in portions at -20°C until required.

Preparation of juice of *P. granatum* and *Ocimum sanctum*

Fruit peel of *Punica granatum* was obtained from the market authenticated from the Department of Botany, RDVV, Jabalpur fruit peel juice was obtained by grinding the fruit peel and was filter sterilized by 0.2micron filter. Further fruit peel juice was freeze dried in lyophilizer and used in the concentration of 10mg/ml

Fresh leaves of *Ocimum sanctum* were procured from the Department of Medicinal and Aromatic plants, JNKVV and crushed to obtain fresh leaves juice and was filter sterilized by 0.2 micron filter. Further fresh leaves juice was freeze dried in lyophilizer and used in the concentration of 10mg/ml.

Preparation of starch iodine plate

Starch iodine plate was prepared with hot agar solution mixed with soluble starch and distilled water and poured out into the plate, later 0.5 ml of iodine solution (composed of iodine and potassium Iodide) was immediately added into the plate that immersed the hot agar solution(Zaichang *et al.*, 2009). The mixed solution

was stirred evenly and allowed to solidify at room temperature. Wells (diameter 5 mm) were punched in the previously prepared starch-iodine plates using a sterile stainless steel borer. One well was filled with 100 µl of penicillin G (50 mg/ml, the solvent was phosphate buffer (pH 7.0)) and 100 µl of phosphate buffer (pH 7.0) and was considered as negative control. Second Well was filled with 50 µl of crude β-lactamase solution and 50 µl of phosphate buffer (pH 7.0), and filled with 100 µl of penicillin G (50 mg /ml). After 10 min was taken as positive control. Third Well was filled with 50 µl of crude β-lactamase solution and 50 µl of tazobactum (standard β-lactamase inhibitor), filled with 100 µl of penicillin G (50mg/ml) 10 min later. Fourth well was filled with 50 µl of crude β-lactamase solution and 100 µl of fresh fruit peel juice of *Punica granatum* and fresh leaves juice of *Ocimum sanctum* @ 10 mg/ml respectively in separate wells and filled with 100 µl penicillin G (50 mg/ml) 10 min later.In one well combination of fruit peel juice of *Punica granatum* and fresh leaves juice of *Ocimum sanctum* was included for the study. The plates were incubated at 35 °C for 30 min. The diameter (mm) of the transparent zones included the diameter (5 mm) of well. The experiments were carried out for 6 samples three times each with fruit peel juice of *Punica granatum* and fresh leaves of *Ocimum sanctum.*

RESULTS AND DISCUSSION

Inhibitory potential of fruit peel juice of *Punica granatum* and fresh juice of *Ocimum sanctum* leaves were undertaken alone and in combination of tazobactum is given in the Tables1 to 3.

The antimicrobial potency of plants is believed to be due to tannins, saponins, phenolic compounds, essential oil, and flavonoids (Serrano *et al.,* 2009). An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. It is expected that plant extracts showing target sites, more active against drug-resistant microbial pathogens and can be used as an alternative to antibiotics. Isolates positive both phenotypically and genotypically were used in triplicates to study inhibitory potential and antibacterial potentiation of *Punica granatum and Ocimum sanctum* against extended spectrum beta lactamase enzyme in triplicates and per cent inhibition was obtained on the mean inhibitory potential by Iodometric method. The inhibitory potential of fresh leaf juice of *Ocimum sanctum* showed 11.8± 0.41 to 13 ± 0.45 (Mean ±S.E.) whereas per cent inhibition ranged from 9.1 per cent to 10.3 per cent. In case of fruit peel juice of *Punica granatum,* slightly higher values of inhibitory potential (10.5 ± 0.22 to 11.8 ± 0.31) and per cent inhibition (12.4per cent to 13.4per cent) were exhibited(Table1). In case of Tazobactum, 5.2±0.17 to 5.8±0.31 (Mean± S.E.) zone of inhibition was observed with the maximum per cent inhibition in the range of 72.3 per cent to 86.8 per cent (Table 3).

Similar results were also observed by Lena and Tripathi (2013) where cinnamon pomegranate clove oil exhibited stronger antibacterial activity against these ESBL isolates than Tulsi, Garlic or Neem oil. Mishra and Mishra (2010) also reported that level of antibacterial effect of Tulsi against *E.coli* was not so high as compared with the other herbs which simulate with the present investigation where effect of fresh leaves juice of *Ocimum sanctum* was fairly less than the *Punica granatum.*

The principle of the Iodometric method is based on the fact that penicillin G hydrolyzes to penicillinoic acid by β-lactamase and penicillinoic acid combines with iodine to form a colorless complex. When beta lactamase inhibitors like tazobactum were used, there was no transparent zone as these agents inhibit the activity of β-lactamase completely. On the other hand the various plant herbs showed small transparent zone around the well, partly inhibiting the beta lactamase activity. Size of zone of inhibition/ inhibitory potential is inversely related to the per cent inhibition ability of an herb. Lower the zone of inhibition higher is the per cent inhibition capacity. By measuring the diameter of transparent zone activity of the herbs to inhibit β-lactamase activity was easily determined and also the inhibiting strength of the particular herb was measured. The results of the present work correlates with study performed by Shilpa *et al.* (2014) where pomegranate alone exhibited maximum antibacterial effect with the MIC values of 0.097 against ESBL producing *E.coli*. Approximately 86 per cent to 100 per cent of *E. coli* were sensitive to pomegranate and clove oil which exhibited stronger antibacterial activity against these ESBL isolates than tulsi, garlic or neem oil. A fair amount of antibacterial potentiation was depicted by fruit peel juice of pomegranate due to the presence of substantial amounts of polyphenols such as ellagic tannins, ellagic acid, and gallic acid (Negi *et al.*, 2003).

Ahmad and Beg (2001) also reported that the phytochemical components found in alcoholic extract of pomegranate are alkaloid, flavonoid, glycoside, phenol, and tannin. Li *et al.* (2005) also reported that phenolic compounds in pomegranate juice are punicalagin isomers, ellagic acid derivatives, and anthocyanins.

Machado *et al.* (2002) reported the antibacterial activity of punicalagin against various bacterial pathogens. Besides having high antioxidant activity, pomegranate also has antibacterial activity and may be used as medicine for poultry; this also reduces the cost and the risk of antibiotic consumption. Furthermore, added value from the peels which is the by-product could provide health benefits to poultry and may be employed in food preservation and pharmaceutical purposes.Similarity in our study ,as pomegranate fruit peel juice showed higher antibacterial activity than *Ocimum sanctum* leaves (Hayrapetyan *et*

Table 1

Comparative inhibitory potential (Mean ± SE) and per cent inhibition of fresh juice of *Ocimum sanctum* leaves and fruit peel juice of *Punica granatum* by Iodometric method.

Sample	Zone of inhibition *Ocimum sanctum*	% Inhibition *Ocimum sanctum*	Zone of inhibition *Punica granatum*	% Inhibition *Punica granatum*
1	12.3 ± 0.42	10.2	10.8 ± 0.31	12.4
2	12.5 ± 0.43	9.2	11.0 ± 0.26	13.4
3	12.3 ± 0.33	10.2	10.5 ± 0.22	12.4
4	13.0 ± 0.45	10.2	10.8 ± 0.40	13.4
5	12.8 ± 0.41	9.1	11.8 ± 0.31	13.4
6	11.8 ± 0.41	10.3	11.3 ± 0.33	12.4

Paired t test ($p < 0.05$) significant difference was seen between the two.

Table 2

Inhibitory potential and per cent Inhibition of combination of fresh juice of *Ocimum sanctum* leaves and fruit peel juice of *Punica granatum* and tazobactum by Iodometric method.

(Mean ±S.E. (N=3)

Sample	Zone of inhibition	% Inhibition
1	12.3±0.21	24.3
2	13.0±0.37	27.0
3	11.7± 0.21	21.7
4	11.0± 0.63	19.0
5	12.5± 0.22	25.0
6	12.0± 0.26	23.0

Table 3

Inhibitory potential and per cent inhibition of tazobactum as standard control by Iodometric method. (Mean ±S.E. (N=3)

Sample	Zone of inhibition	% Inhibition
1	5.8±0.31	77.9
2	5.7±0.21	80.7
3	5.7±0.21	86.2
4	5.5± 0.22	83.4
5	5.2±0.17	72.3
6	5.5±0.22	77.9

al.,2012). *Punica granatum* and *Ocimum sanctum* in combination exhibited 12.3± 0.26 to 11.7±0.21 of inhibition zone with per cent inhibition of 21 to 27 per cent showing better activity than *Ocimum Sanctum* and *Punica Granatum* alone. Statistical analysis was also done using paired t test which also showed ($p<0.05$) significant difference between the inhibitory potential and per cent inhibition of the two herbs (Table 01).

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Research Article

ISOLATION, MORPHOLOGICAL IDENTIFICATION AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC BACTERIA ISOLATED FROM *ALOE BARBADENSIS* (ALOE VERA) LEAVES

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ABSTRACT

The present study was done to isolate endophytic bacteria from *Aloe barbadensis* (aloe vera) leaves, their identification and investigate their antibacterial activity against three gram positive bacteria, *Staphylococcus aureus*, *Streptococcus pyogens* and *Bacillus cereus* and gram negative bacteria *Escherichia coli*, *Salmonella* Typhimurium and *Klebsiella pneumoniae.* A total of 25 leaves samples were taken, they were sterilized with 0.1 per cent sodium hypochlorite, 0.01 per cent bavistin, 0.05 per cent and 70 per cent ethanol. Sterilized leaves of the plants were embedded in King's B petri plates for the isolation of endophytic bacteria. Maximum isolated sample on King's B media were irregular in shape, flat elevation, entire colony margin, smooth growth surface, opaque and green in colour, the microscopic examination revealed that isolated endophytic bacteria were gram positive and rod shaped. The antibacterial effect was studied by the disc diffusion method with known antibiotic ciprofloxacin (Ci) as standard. The antibacterial activity of endophytic bacteria isolated from *Aloe barbadensis* (aloe vera) showed good antibacterial activity against *Staphylococcus aureus* and *Escherichia coli.*

Key words: Endophytic bacteria, *Aloe barbadensis* (aloe vera), leaves, antibacterial activity, ciprofloxacin.

INTRODUCTION

An increase in the number of people in the world having health problems leading to various types of cancers, drug-resistant bacteria, parasitic protozoans and fungal infection is a cause for alarm. An intensive search for newer and more effective agents to deal with these disease problems is now underway and endophytes are a novel source of potentially useful medicinal compounds. Endophytes are microorganisms including bacteria that live in the intercellular spaces of plant without showing any disease symptoms to the host plant (Compant *et al.*, 2005). Many studies have emphasized endophytes from medicinal plants and their application in different areas (Garcia *et al.*, 2012). Recently many known as well as new endophytic bioactive metabolites, possessing a wide variety of biological activities as antibiotic, antiviral, anticancer, anti-inflammatory, antioxidant etc., have been identified (Strobel and Daisy, 2003).

The objective of the present study was to isolate endophytic bacteria from *Aloe barbadensis* (Aloe vera) leaves, their identification and investigate their antibacterial activity against three gram positive bacteria, *Staphylococcus aureus*, *Streptococcus pyogens* and *Bacillus cereus* and gram negative bacteria *Escherichia coli*, *Salmonella* Typhimurium and *Klebsiella pneumoniae.*

MATERIALS AND METHODS

Plant material

Fresh leaves of *Aloe barbadensis* (Aloe vera) was procured from the Department of Botany, J.N.K.V.V., Jabalpur. Mature healthy plant leaves were collected by selected medicinal plants viz *Aloe barbadensis* (Aloe vera). Samples were immediately brought to laboratory and were used within 24 hrs and finally processed for isolation of endophytic bacteria. Five samples were taken and each sample was divided into 5 sub samples and separated for further isolation of endophytic bacteria.

Sterilization of leaves

The sterlization of leaves and isolation of endophytic bacteria from the leaves was done according to Mahajan *et al.,* (2014), with some modifications. Each leaves was treated with double distilled water for 2-3 minutes. Leaves were then surface sterilized with 0.1 per cent sodium hypochlorite for 5 minutes. Again the leaves were treated with double distilled water for 2-3 minutes. Later, surface sterilization was done with 0.01 per cent bavistin. The leaves were kept in distilled water for 5 minutes. For further sterilization the leaves were exposed to 0.05 per cent streptomycin followed by treatment with double distilled water for 5 minutes. Then the leaves were exposed to 70 per cent ethanol and again were kept in double distilled water for 5 minutes and then air dried in laminar flow.

To confirm that the surface of leaves were effectively sterilized 1 ml of the sterile distilled water that was used in final rinse of surface sterilization procedures were planted onto nutrient agar media and incubated at 37^0C for 24 hrs. Bacterial growths were observed after 24

hrs. Also surface sterilized leaves were rolled on nutrient agar plates and incubated at 37^{0}C for 24 hrs and checked for possible microbial growth.

Preparation and sterilization of media

King's B (KB) media, mueller hinton media, blood agar media and BHI broth were prepared by adding agar into the distilled water. Hot plate was used for the proper mixing of media and autoclaved at 121°C for 15-20 minutes at 15 lbs.

Inoculation of leaves

The media was poured into different autoclaved petri plates and leaves of the plants were embedded in petri plates. These plates were then incubated at 37°C for 24 hrs. Characterization of the bacteria was done according to its morphology and by grams staining. After that a single colony was transferred into BHI broth and incubated at 37°C for 24 hrs.

Purification of endophytic bacteria

For purification of endophytic bacteria subculturing was mainly done by streaking a loop full of BHI broth on the fresh pre solidified blood agar plates and then incubated at 37°C for 24 hrs. After incubation the colonies were transferred into BHI broth and then incubated at 37°C for 24 hrs and purity was checked by grams stain and stored for further work.

Preparation of inoculums

The known culture of bacteria were procured from Hi-media and are enlisted in Table 1.The above prepared bacterial inoculums were evenly spread on sterile mueller hinton agar plates as described by Bauer *et al.* (1969) and antibacterial effect was studied by the disc diffusion method in these plates. The known antibiotic ciprofloxacin (Ci) was simultaneously used and placed as control for antibiotic sensitivity. The dried discs were immediately used and incubated at 37^{0}C for 24 hrs.

Preparation of antibacterial disc

For determination of antibacterial activity of endophytic bacteria, broths were centrifuged at 4°C at 12000rpm for 30 minutes. Supernatant of each of these broths were taken, sterile discs were soaked in these broths in a sterile test tubes for 24 hrs and dried in laminar flow. After drying the discs were used immediately for disc impregnation in the inoculated plates as described by Kirubaharan *et al.* (1999) with slight modifications. Ciprofloxacin dics were used as control drug to compare the effect of treatment during *in vitro* study.

Antibacterial test

The prepared bacterial inoculums were evenly spread on a sterile mueller hinton agar plate as per method described by Bauer *et al.* (1969). The known antibiotic ciprofloxacin (Ci) was simultaneously placed as a control for antibiotic sensitivity. The dried disc was incubated at 37°C for 24 hrs. Results were recorded as positive (growth) or negative (no growth) and zone of inhibition of growth exerted by these impregnated discs.

RESULTS

The present study was conducted with a view to isolate and characterizes endophytic bacterial diversity from *Aloe barbadensis* (Aloe vera). Twenty five bacterial isolates from *Aloe barbadensis* (Aloe vera) were obtained and identified morphologically. These endophytic bacteria were evaluated for *in vitro* antibacterial activity. The sterilized leaves of *Aloe barbadensis* (Aloe vera) were put in the King's B media and incubated at 37^0 C at 24 hrs. The morphological characterisation of endophytic bacterial isolates exhibited diverse colony shape, colour margin and texture including round to irregular colonies and white and yellow colonies with irregular and wavy margins. The endophytic bacterial isolates recovered from kings B media showed soft and mucoid colonies.

Preliminary characterisation of isolated endophytic bacteria

Growth characteristics of endophytic bacteria isolated from aloe vera leaves on kings B medium indicated that 80 per cent were irregular in shape while 20 per cent circular in shape, 84 per cent were flat elevation on petri plate while 16 per cent raised elevation, margin of the 80 per cent colonies were entire while 20 per cent undulated, the surface of the growth was smooth in 88 per cent while 12 per cent glistening, the growth was opaque in 100 per cent isolates and 92 per cent isolates were green in colour (Table 2).

Growth characteristics of endophytic bacteria isolated from aloe vera leaves on 5 per cent sheep blood agar medium presented that 80 per cent samples were irregular in shape while 20 per cent circular in shape, 84 per cent had convex elevation on petri plate while 16 per cent raised elevation, margin of the 84 per cent colonies were undulated while 16 per cent entire margin. The surface of the all isolates was smooth and glistening. All the isolates were non haemolytic and non chromogenic (Table 2).

Endophytic bacteria from aloe vera leaves grown in BHI broth showed the following characteristics. All isolates showed turbidity, 88 per cent isolates without flocculent growth, 100 per cent isolates with pellicle formation, in 84 per cent isolates ring formation was seen, only 16 per cent isolates showed sediment formation (Table 2).

On microscopic examination, endophytic bacteria isolated from aloe vera revealed that 88 per cent isolates were gram positive and 12 per cent were gram negative, 84 per cent endophytic bacteria were rod shaped while 16 per cent were cocci, Microscopic examination showed that more than one type of endophytic bacteria were present in 88 per cent of isolates (Table 2).

In *in-vitro* studies, the antibacterial activity of endophytic bacteria was evaluated against various gram positive and gram negative pathogenic bacteria namely *Bacillus cereus, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae and Salmonella* Typhimurium. Results were recorded for zone of inhibition around the disc. The inhibitory zone around the disc indicated absence of bacterial growth reported as sensitive and absence of zone reported as resistant. The *in vitro* antibacterial activities of the endophytic bacteria against different gram positive bacteria are shown in Table 06. The endophytic bacteria isolated from *Aloe barbadensis* (aloe vera) shown antibacterial activity as 84 per cent of isolates inhibited growth of *Staphylococcus aureus,* 16 per cent of isolates inhibited growth of *Streptococcus pyogenes* and 16 per cent isolates inhibited growth of *Bacillus cereus.* The *in vitro* antibacterial activities of endophytic bacteria against different gram negative bacteria have been shown in Table 07. The endophytic bacteria isolated from *Aloe barbadensis* (aloe vera) shown antibacterial activity as 84 per cent of isolates inhibited growth of *Escherichia coli,* 12 per cent of isolates inhibited growth of *Salmonella* Typhimurium and 8 per cent isolates inhibited growth of *Klebsiella pneumoniae.*

Concludingly, Out of 25 isolates from *Aloe barbadensis* (aloe vera) 21 isolates were effective against *Staphylococcus aureus*, 4 against *streotococcus pyogens*, 4 against *Bacillus cereus*, 21 against *Escherichia coli,* 3 against *Salmonella* Typhimurium, 2 against *Klebsiella pneumoniae* (Table 2).

DISCUSSION

Twenty five strains of endophytic bacteria were isolated from leaves of *Aloe barbadensis* (aloe vera). Endophytic bacteria are found in virtually every plant on earth (Ryan *et al.* 2008). Different plant parts such as root, stem and nodule (Hung and Annapurna 2004), leaves, stems and root (Sobral *et al.* 2005) can also be used for isolation of endophytic bacteria. Costa *et al.,* (2012) had isolated culturable endophytic bacteria from common bean (*Phaseolus vulgaris*) leaves.

The preliminary identification of the bacterial isolates was done based on various morphological features of isolated endophytic bacteria. The colony characteristics of endophytic bacteria isolated from aloe vera leaves were irregular in shape, flat elevation on petri plate, margin of the colonies were entire, smooth growth surface, opaque and green in colour. The microscopic examination of endophytic bacteria isolated from aloe vera showed that 88 per cent isolates shown gram positive reaction and 12 per cent gram negative, 84 per cent endophytic bacteria were rod shape while 16 per cent were cocci, Microscopic examination showed that more than one type of endophytic bacteria were present in 88 per cent of isolates.

The isolation of endophytic bacteria was in agreement with the findings of (Hung and Annapurna 2004), had found equal percentages of gram positive 49 per cent and gram negative 51 per cent bacteria. Sobral *et al.*, (2005) and Ebrahimia *et al.*, (2010) has also found equal percentage of gram positive and gram negative bacteria. However, Bahgat *et al.*, (2014) found the 90 per cent of gram positive bacteria.

As summarized in results antibacterial activity of endophytic bacteria was calculated by the presence of zone of inhibition produce by endophytic bacteria against pathogenic bacteria. All the isolates from endophytic bacteria were screened for the antibacterial activity against pathogenic bacteria *Bacillus cereus, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae and Salmonella* Typhimurium.

The overall *in vitro* antibacterial results shown that maximum sensitivity was observed against *Staphylococcus aureus* and *Escherichia coli.* Isolates from aloe vera did not showed good antibacterial activity against *Bacillus cereus, Streptococcus pyogenes*, *Salmonella* Typhimurium and *Klebsiella pneumoniae*.

Verma *et al.,* (2009) observed antibacterial activity of endophytic actinomycetes from *Azadirachta indica*

Table 1:
List of culture procured from Hi media

S.No.	BACTERIA	ATCC Catalogue No.
1.	*Escherichia coli*	25922
2.	*Klebsiella pneumonia*	700603
3.	*Salmonella* Typhimurium	13311
4.	*Bacillus cereus*	11778
5.	*Staphylococcus aureus*	6538
6.	*Streptococcus pyogens*	12386

Table 2:
All *in vitro* antibacterial activity of endophytic bacterial isolates

Sl. No.	Samples	Activity against
1	A1a, A1c, A1d, A1e, A2a, A2b, A2d, A2e, A3a, A3b, A3c, A3d, A3e, A4a, A4c, A4d, A 4e, A5a, A5b, A5c, A5d	*Staphylococcus aureus*
2	A1e, A2d, A4a, A4b	*Streotococcus pyogens*
3	A1d, A2e, A3e, A4d	*Bacillus cereus*
4	A1a, A1c, A1d, A1e, A2b, A2c, A2d, A2e, A3a, A3b, A3c, A3d, A3e, A4a, A4b, A4c, A4e, A5a, A5b, A5d, A5e	*Escherichia coli*
5	A1d, A3b, A5a	*Salmonella* typhimurium
6	A1b, A4b	*Klebsiella pneumonia*

against *Escherichia coli*. Ebrahimia *et al.*, (2010) observed antibacterial activity of endophytic bacteria isolated from leaves of *Hypericum scabrum* against *S. aureus.* Jalgaonwala *et al.,* (2010) observed antibacterial activity of endophytic bacteria isolated from roots of *Aloe vera* possess strong antibacterial activity against *S. typhi* in dual culture assay. Roy and Banerjee (2010) isolated endophytic bacteria from a medicinal plant *Vinca rosea*. One of the isolated endophytes produced potential antimicrobial activity against *Bacillus cereus, Klebsiella pneumoniae, Escherichiae coli*. Pal *et al.* (2012) reported the antimicrobial activity of the bacterial endophytes of *P. foetida* indicating the inhibitory effect of majority of the isolates against *Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumoniae*. The present study is also very near to all the above authors.Thus, endophytic bacteria were present in leaves of *Aloe barbadensis* (aloe vera), gram positive and rod shaped bacteria were present in leaves of *Aloe barbadensis* (aloe vera). Endophytic bacteria from aloe vera possess maximum antibacterial activity against *Staphylococcus aureus* and *Escherichia coli.*

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Research Article

CYPERMETHRIN INDUCED NEUROTOXICITY AND AMELIORATIVE EFFECT OF VITAMIN E IN ALBINO RATS

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ABSTRACT

Pyrethroid has been widely used globally accounting for over 30% of insecticide use. It persists in environment. The present study was designed to investigate the neurotoxic effects of cypermethrin on behavioural and postural pattern of rats. Ameliorative effect of vitamin E in these neurotoxic pathological alterations was also examined. The study was conducted on 30, two months old albino rats which were randomly assigned to five groups with six rats in each. Group I rats received standard diet and water *ad libitum* and acted as control. Group II received oral supplementation of cypermethrin in feed daily @ 75mg/Kg body weight (b wt), Group III were given oral supplementation of cypermethrin in feed daily @ 150 mg/kg BW, Group IV and V rats were supplemented with vitamin E @ 100 mg/kg b wt along with cypermethrin in the doses indicated in group II and III, respectively. The cypermethrin treated rats showed typical behavioral changes and reduced reflexes. Staggering gait and dizziness were also observed in cypermethrin treated rats in a dose dependent manner. Serious hemorrhages and congestion were the constant feature in both liver and brain. Microscopically, the liver revealed degenerative changes in parenchyma, hepatocytes and hepatic cords and dilatation of sinusoids. Besides hemorrahges and congestion, brain revealed mild deformed areas of neurons and varying degrees of degenerative changes.There was a significant ($P< 0.05$) reduction in blood, liver and brain acetylcholinesterase enzyme activity in cypermethrin 75 and 150 mg toxicity groups. Marked ameliorative effect of vitamin E was observed on acetylcholinesterase activity in cypermethrin plus vitamin E supplemented rats when compared with their respective toxicity groups. On the basis of these results, it was concluded that cypermethrin is not safe for animals in high doses.

Key words: Acetylcholine esterase, cypermethrin, histopathology, neurological, toxicity

INTRODUCTION

The undesirable effects of pesticide and insecticides have been recognized as a serious public health concern during the past few decades, Cypermethrin, a type II pyrethroid insecticide has occupied a prominent place in agriculture and domestic veterinary application accounting for over 30% of the insecticide use globally (Shukla *et al.*, 2002). It has high bio-efficiency, enhanced stability and lower toxicity (Leng *et al.,* 1996). It has also been shown that cypermethrin persists in air, on walls and furniture for months after use (Cox, 1996). A serious aspect of insecticide use is the contamination of food and water which, if consumed, are toxic to the humen being as well as domestic and wild animals (Flickinger *et al.,* 1991).

Limited studies have been made to investigate the neuro-toxic effects of cypermethrin on behavioural, nervous and enzymatic changes in rats. Environmental contamination due to extensively used pyrethroid is believed to cause increasing oxidative stress in mammals (Yousef *et al.,* 2006). The objective of the present study was, therefore, not only to evaluate the cypermethrin induced neurological changes and status of blood, liver and brain cholinesterase activity but also to examine the protective role of vitamin E in countering the effects of free radicals. Histopathological changes in important vital organs like liver and brain which are the main targets of cypermethrine have also been studied.

MATERIALS AND METHODS

Experimental animals and design

The study was conducted on 30, two months old albino rats weighing around 80 to 150 grams. They were maintained under good hygienic conditions and provided with standard feed and water *ad libitum*. After one week period of acclimatization in the laboratory conditions, the animals were randomly assigned to the following five groups with six rats in each group. Group I rats received standard diet and acted as control. Group II received oral supplementation of cypermethrin in feed daily @ 75mg/kg b wt, Group III, was given oral supplementation of cypermethrin in feed daily @ 150 mg/kg b wt, Group IV and V rats were supplemented with vitamin E @ 100 mg/kg b wt along with cypermethrin in the doses indicated in group II and III, respectively.

On 28th day of experiment, all the rats were decapitated and whole blood, liver and brain were collected quickly and placed in ice-cold saline. Pieces of the tissues from liver and brain were fixed in 10% formalin for histopathological studies. The micro sections were cut and stained through haematoxylin and eosin for microscopic examinations.

Cholinesterase activity was measured in blood, liver and brain tissues of the rats at the end of the experiment (Ellman *et al.*, 1961). The analysis was done, using CRD (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The cypermethrin treated rats were dull, depressed with sluggish movements and showed reduced feed intake. The behavioural effects were huddling, hyperactivity and hump formation. The central nervous system (CNS) stimulation and other symptoms were more visible in rats fed with 150 mg cypermethrin. Reflexes like touch, pedal, corneal and tail were found reduced to variable degrees. Muscular tremors, staggering gait and dizziness were also observed in cypermethrin treated rats in a dose dependent manner.

Variable behavioural and nervous signs have been observed by various investigators to cypermethrin toxicity in different species ranging from rats to buffalo calves. Manna *et al.* (2004) working in rats using alpha cypermethrin observed nervous and postural symptoms identical to the signs in the present investigation. In contrast to the changes in cypermethrin - treated rats, the degree of nervous, behavioural and postural disorders were of far lesser order in rats of group IV and V which were supplemented with Vitamin E along with cypermethrin.Vitamin E is well known for its antioxidant properties on biological membranes where it acts to prevent the peroxidation of lipid membranes (Jiang *et al.,* 2000). These results suggest the ameliorative effects of vitamin E in opposing the toxic effects of cypermethrin.

Serious hemorrhages and congestion was the constant feature in both the organs. Grossly, liver showed hepatomegaly with rounded borders. Microscopically, the liver revealed congestion (Fig. 1), degenerative changes in parenchyma, hepatocytes and hepatic cords and dilatation of sinusoids (Fig. 2). Besides hemorrahge and congestion, brain revealed mild deformed areas of neurons (Fig. 3) and varying degrees of degenerative changes (Fig. 4). These changes were more prominent in rats given cypermethrin at the dose of 150 mg/kg b wt. Similar histopathological picture has been reported by Luty *et al.* (2000) in wistar rats exposed to 250 mg/Kg alpha-cypermethrin. Mani *et al.* (2004) also reported identical changes in the liver of mice given daily oral administration of cyclohalothrin at a dose level of 200 mg/dl. Serious insecticide- induced pathological abnormalities of brain has also been reported by Latuszynska *et al.* (2001) in rats. These investigators reported increased density of cytoplasm in neurones by just dermal application of chlorpyrifos and cypermethrin.

As shown in Table 1, a clear dose dependent reduction in cholinesterase (ChE) activity in blood, brain and liver in both the groups of cypermethrin treated rats. The ameliorative effect of vitamin E was also evident in group IV and V rats as indicated by near normal level of ChE activity when compared with the values in rats of control group. The data is presented in Table 1.

Acetylcholine esterase is an important enzyme regulating optimum concentration of acetylecholine at the synapses. It acts as "on and off switch" in releasing this transmitter and control the normal function of nerves directly (Burgees *et al.*, 1999). It is concluded that cypermethrin

Table 1

Mean values of cholinesterase activity (µmol/min/gm) in blood, liver and brain in different group after 28 days in rats. (Mean ±SE)

Groups	Blood	Liver	Brain
Group I	1.44^{b}±0.09	1.55^{c}±0.20	1.54^{a}±0.07
Group II	0.60^{d}±0.13	1.55^{c}±0.00	1.14^{b}±0.04
Group III	0.54^{d}±0.09	1.99^{b}±0.09	0.57^{c}±0.05
Group IV	1.00^{c}±0.08	1.98^{b}±0.02	1.29^{b}±0.06
Group V	1.68^{a}±0.06	1.98^{b}±0.02	$1,26^{b}$±0.08

Mean values bearing different superscripts differ significantly (P≥0.05)

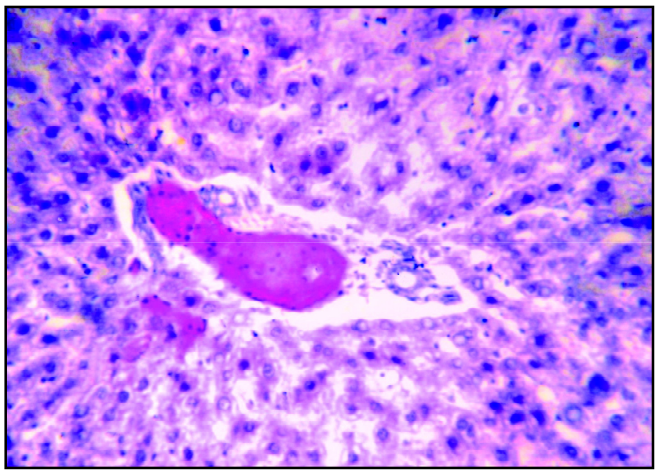

Fig. 1
Section of liver showing congestion and haemorrhage in parenchyma HandE(X400).

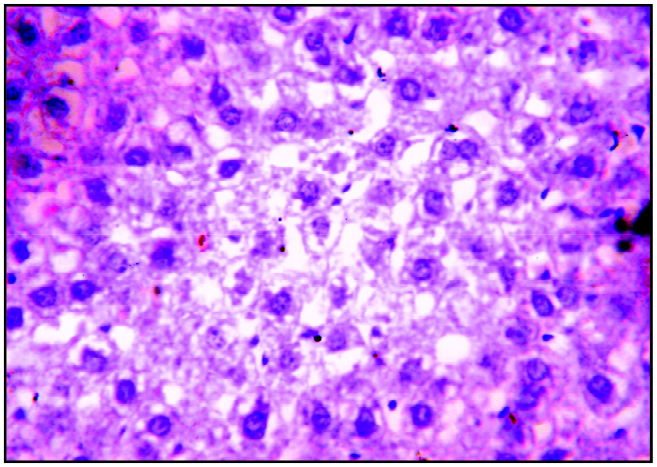

Fig. 2
Section of liver showing degenerative changes in the hepatocytes HandE (X400).

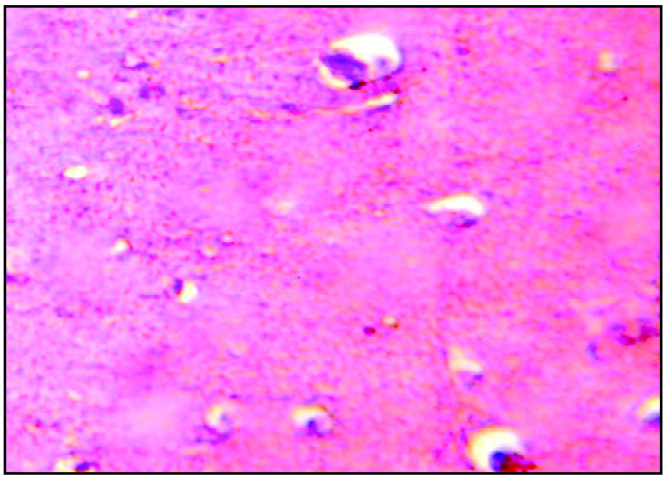

Fig. 3
Section of brain showing mild deformation areas due to shrinkage of neurons HandE (X400).

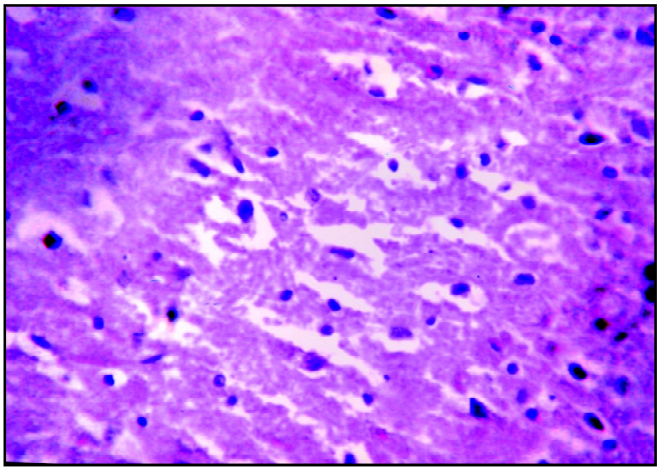

Fig. 4
Section of brain showing degenerative changes HandE (X200).

induced neurotoxicity and ameliorative by vit. E therapy in rats.

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Research Article

EFFECT OF LEAD TOXICITY ON HAEMATO-BIOCHEMICAL PROFILE AND AMELIORATIVE EFFICACY OF COW URINE DISTILLATE IN BROILERS

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ABSTRACT

The study examined the protective effect of cow urine distillate (CUD) on lead acetate induced toxicity in broiler chicken. One week old, healthy white Leghorn chicks were divided into four groups. Group I served as control, whereas other groups were assigned following treatments. Group II birds were given CUD @ 1 ml per bird per day , Group III birds, lead acetate @ 500 ppm mixed with feed and group IV birds, lead acetate plus CUD. Feeding of lead acetate resulted in significant decrease in the level of haemoglobin, packed cell volume, total erythrocyte count but significant increase in total leukocyte count and heterophils in group III birds. Serum levels of most enzymes were significantly elevated along with an increase in serum glucose, blood urea nitrogen and creatinine. On the contrary, there was a significant decrease in total serum protein and albumin levels. Cow urine distillate exerted benefecial effects on haematological and biochemical parameters as their values stayed near normal level in group IV birds.

Key words: Biochemical, cow urine distillate, haematology, lead acetate toxicity

INTRODUCTION

Lead is a highly toxic metal which commonly affects avian species by its ingestion through lead-based products, paints and sprays. It affects haemopoietic, nervous, gastrointestinal and urinary systems (Baykov *et al.,* 2005). Cow (*Bos Indicus)* urine has been elaborately explained in Ayurveda and described in Sushruta Samhita, Ashtanga Sangraha and other Ayurvedic literature to possess many medicinal properties and is used in curing number of diseases (Jain, 2006). During the past few years, claims have been made about theraputic efficacy of cow urine for the treatment of cancer and environmental pollutants. Therefore, the present study was designed to explore the beneficial effects of cow urine distillate against lead toxicity in chickens.

MATERIALS AND METHODS

Experimental animals and design of experiments

Twenty four, healthy, commercial broiler chicks were procured and reared in battery brooders under standard hygienic and management conditions. All the birds were fed standard feed *ad libitum* and given water during entire period of experiment which lasted for 45 days. After an initial acclimatisation period of one week, the birds were divided into 4 groups comprising six birds in each. Group I birds received only standard feed and water and served as control. Group II birds were given cow urine distillate @ 1 ml/ bird/day orally. Group III birds received lead acetate @ 500 ppm mixed with feed. Group IV birds were given lead acetate @ 500 ppm mixed with feed plus cow urine distillate @ 1 ml per bird /day by oral gavage. Birds were closely observed twice daily for development of clinical signs.

Study parameters

At the end of the experiment, the blood was collected from wing vein of birds into sterile vials containing sodium citrate (1:9 v/w) as anticoagulant before exsanguinations. For estimation of biochemical parameters, separate blood samples were collected without anticoagulant and serum was separated as per routine and established procedure (Calnek *et al.,* 1997). The following haematological parameters were examined.

Haemoglobin (Hb) estimation and packed cell volume (PCV) were done using Sahli's haemoglobinometer and capillary haematocrit method respectively as per the method given by Feldman *et al.* (2002). Total erythrocyte (TEC) and leukocyte counts (TLC) were done using standard Neubauer haemocytometer. Differential leukocyte count was done by standard blood smear method using Giemsa stain.

Serum enzymes viz alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN), creatinine, blood glucose, total serum protein, serum albumin and globulin were analyzed by semi automated serum analyzer. The amount of globulin was calculated by subtracting albumin concentration from the total protein level in serum. Albumin globulin ratio was calculated by dividing serum albumin level with globulin concentration.

The data was subjected to statistical analysis applying ANOVA as per the standard method of **c**ompletely randomized design (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

The results of the present investigation clearly show that lead acetate has toxic effects on body systems of

the birds which was evident due to significant alterations in haematological and biochemical parameters. Results have revealed that cow urine distillate has beneficial effects in restoring these parameters to the near normal values.

The changes in haematological and biochemical parameters observed are presented in Table 1 and 2 respectively. Lead acetate feeding (group III) caused a significant decrease in Hb, PCV and TEC resulting into an anaemic condition. On the contrary, it caused a significant increase in TLC and number of heterophils. Another important observation was that lead acetate caused lymphopaenia without any significant effect on eosinophils and basophils. Several other investigators have also reported toxic effects of lead on erythropoiesis, depressant action on bone marrow and inhibition of heme synthesis (Gurer and Ercal, 2000). It was interesting to note that significant fall in the level of Hb, PCV and TEC, and rise in TLC found in lead acetate group were not observed in the birds which were supplemented with cow urine distillate along with lead acetate feeding (group IV). This clearly indicated that supplementation of CUD had deterrent effect on lead acetate toxicity. The significant increase in TLC with heterophillia observed in present investigation in lead acetate fed group could be due to depressed haemopoiesis as a result of bone marrow suppression. These parameters were found significantly decreased in the birds of group IV who were supplemented with CUD. The findings of increase in lymphocytes in CUD treated group are in conformity with Kumar *et al.* (2004).

Feeding of lead acetate caused a significant increase in the level of all the three enzymes, glucose, BUN and creatinine activity, whereas, the concentration of serum proteins and albumin were significantly decreased. The increase in ALP might be due to damage to the liver, kidney and bones resulting into liberation of alkaline phosphatase (Kaplan and Reghetti, 1970). Significant decrease in the activity of ALT, AST ALP, and glucose level stayed near normal in group IV birds which were supplemented with CUD along with lead acetate feeding. Similar result of beneficial effect of CUD in the activity of ALT and AST was observed by Gururaja *et al.* (2009) in a study on toxic effects of carbon tetra chloride in rats. Significant increase in serum glucose level in lead acetate fed group of birds has been attributed to increased

Table 1:
Values (Mean±SE) of haematological parameters in lead intoxicated groups following treatment with urine distillate in broilers.

Observations	Control (Group I)	CUD Control (Group II)	Lead acetate (Group III)	Lead acetate + CUD (Group IV)
Hb conc(g/dl)	10.05^{b}±0.12	11.20^{a}±0.11	7.80^{c}±0.14	9.65^{c}±0.07
PCV (%)	31.17^{a}±0.51	33.00^{a}±1.12	23.53^{d}±0.91	28.72^{b}±0.79
TEC(10^{6}/µl)	2.96^{b}±0.08	3.39^{a}±0.08	1.81^{d}±0.03	2.71^{c}±0.02
MCH (pg)	35.56^{b}±1.13	33.08^{c}±0.54	43.07^{a}±1.31	35.58^{b}±0.22
MCV (fl)	105.67^{b}±4.22	97.67^{c}±4.43	129.49^{a}±3.16	89.11^{c}±1.58
MCHC (gm/dl)	33.75±0.89	34.13±1.21	33.29±1.65	33.73±0.99
TLC(Thousand/ µl)	27.03^{c}±0.16	27.63^{b}±0.17	29.06^{a}±0.34	28.69^{a}±0.21
Heterophils (%)	28.83^{c}±0.60	28.16^{c}±0.30	35.33^{a}±0.66	30.50^{b}±0.42
Lymphocytes (%)	64.00^{a}±1.00	65.16^{a}±0.70	53.00^{b}±1.03	62.00^{a}±0.63
Monocytes (%)	4.50^{c}±0.22	4.33^{c}±0.21	6.83^{a}±0.40	4.83^{b}±0.30
Eosinophils (%)	2.33±0.21	2.00±0.25	3.00±0.25	2.33±0.33
Basophils (%)	0.33±0.21	0.16±0.16	0.83±0.30	0.33±0.21

Values bearing different superscripts in the rows indicate significant differences (P< 0.05).

Table 2:
Values (Mean±SE) of biochemical parameters in lead intoxicated groups following treatment with urine distillate in broilers.

Observations	Control (Group I)	CUD Control (Group II)	Lead acetate (Group III)	Lead acetate+CUD (Group IV)
AST (U/L)	184.83^{c}±4.11	172.50^{c}±6.40	338.66^{a}±44.75	216.16^{b}±7.32
ALT (U/L)	5.00^{c}±0.36	4.66^{c}±0.33	8.66^{a}±0.33	5.83^{b}±0.30
ALP (U/L)	475.66^{c}±21.35	466.83^{c}±7.98	572.00^{a}±5.79	491.66^{b}±5.86
Total Serum Protein (g/dl)	3.33^{a}±0.21	3.50^{a}±0.22	2.33^{b}±0.21	3.00^{a}±0.25
Serum albumin (g/dl)	1.72^{a}±0.03	1.72^{a}±0.33	1.21^{c}±0.02	1.54^{b}±0.02
Serum globulin (g/dl)	1.61^{a}±0.01	1.65^{b}±0.03	1.12^{d}±0.03	1.46^{c}±0.02
Glucose (mg/dl)	251.00^{c}±3.78	242.66^{c}±6.15	357.00^{a}±3.45	265.33^{b}±6.04
BUN (mg/dl)	2.09^{c}±0.04	1.74^{d}±0.10	3.22^{a}±0.06	2.30^{b}±0.03
Creatinine (mg/dl)	0.56^{b}±0.05	0.39^{c}±0.03	0.88^{a}±0.06	0.61^{b}±0.02

Values bearing different superscripts in the rows indicate significant differences (P<0.05).

serection of glucocorticoids and decreased peripheral utilisation of glucose (Khan *et al.*, 1993). Lead acetate intoxication caused a significant decrease in serum protein and albumin level (group III). This could be attributed to increased catabolism of protein due to binding of lead to albumin causing excretion of protein through urine. Such a possibility and significant decrease in serum protein due to lead nitrate toxicity has also been noted by Sujatha *et al.* (2011). In the present study, blood urea nitrogen and creatinine levels were found significantly increased in the birds fed with lead acetate as compared to the control group (I). Similar results were also obtained in the studies of Sujatha *et al. (*2011). The increase in BUN and creatinine level induced by lead acetate was not observed in the chickens supplemented with CUD (group IV). In this group, the values of these parameters were significantly lower than group III which suggests the protective effect of cow urine distillate to overcome the lead acetate toxicity. However, further studies are required to unravel the modus operandi of action of cow urine.

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Research Article

PROTECTIVE NATURE OF HEMIN ON OXIDATIVE STRESS IN EXCISION WOUND MODEL OF STREPTOZOTOCIN-INDUCED CHRONIC DIABETIC RATS

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ABSTRACT

The present study was aimed to evaluate the antioxidative property of hemin in chronic diabetic wound. The two groups were formed containing five diabetic rats each. In both the groups 400 mm^2 excision wound were created 8 weeks after induction of diabetes with single intraperitoneal injection of streptozotocin (60 mg/kg/BW). Group I served as diabetic control and ointment base was applied topically to the wound twice daily (vehicle control). In group II rats, hemin ointment was applied topically to the excision wounds twice daily for 19 days. The granulation tissue was collected on day 19 post-wounding for estimation of oxidative-stress related parameters. Application of hemin ointment caused significant reduction in MDA and superoxide levels, as compared to diabetic control rats. A significant increase in level of reduced glutathione (GSH) with significant increase in the activity of catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were observed in hemin-treated rats, as compared to diabetic control rats. The data unveil the benefits of hemin in chronic diabetic wound and is suggestive of its protective mechanisms which include the suppression of oxidative mediators and upregulation of antioxidant status.

Key words: Diabetes, excision wound, hemin, oxidative stress

INTRODUCTION

Impaired wound healing is a complication of diabetes and a serious problem in clinical practice as they do not follow orderly and overlapping phases of healing process like inflammation, proliferation, maturation and reepithelialization; rather it becomes chronic due to persistent inflammation and increased oxidative stress play a crucial role in the pathogenesis of various diseas like diabetes, cancer, inflammatory disorders, etc. (Halliwell, 2012). Hence, the oxidants have to be detoxified in order to prevent damage to host cells. However, in diabetic wounds the detoxification process is hindered due to persistent and uncontrolled production of ROS and reactive nitrogen species (RNS) during the inflammatory phase (Schonfelder *et al.,* 2005).

Hemin (ferriprotoporphyrin IX chloride), an inducer of heme oxygenase-1 (HO-1); is a crucial component of the cellular stress response. HO cleaves the α-methene bridge of heme moiety to produce equimolar quantities of carbon monoxide (CO) and biliverdin. which elicit a cytoprotective, immunomodulator, anti-inflammatory, antioxidative and proangiogenic response (Ram *et al.*, 2014). Several studies have shown the protective effect of hemin in oxidative stress-induced damages (Ndisang *et al.,* 2010; Worou *et al.,* 2011). However, there is no report available regarding use of hemin in chronic model of diabetes. Therefore, the present study was undertaken in order to evaluate the role of topical hemin treatment on oxidative stress in chronic phase diabetic rats.

MATERIALS AND METHODS

Induction of diabetes

Ten adult male Wistar rats (180 to 220 gm) were used in the study which were divided into two groups. Group I (control) consisted of five and group II (hemin treated) consisted of five rats each. Before induction of diabetes, rats were kept for a week for acclimatization with free access to water and balanced ration and 12:12 h light and dark period . Rats were starved for overnight and their fasting blood glucose level was determined. Streptozotocin (STZ) solution was administered @ 60 mg/kg body weight. intraperitoneally to induce diabetes. After 72 hours of administration of streptozotocin, rats were again monitored for blood glucose and the rats showing more than 300 mg/dl blood glucose were selected for further study. The diabetic rats were kept under observation for eight weeks with intermittent glucose level determination before creation of wound the rats showing blood glucose >300 mg/dL are selected for wound creation.

Creation of wound in rats

The animals were anesthetized by intraperitoneal injection of pentobarbitone sodium (@ 50 mg/kg). Approximately 2 x 2 cm^2 (400 mm^2) open excision-type wound was created on the back (dorsal thoracic region) of the rats to the depth of loose subcutaneous tissue. After recovery from anesthesia animals were housed individually in properly disinfected cages.

Grouping of rats and application of ointment

Diabetic rats were divided into two groups-

1. Group I (control) was consisted of 5 diabetic rats. In this group wounds were treated with ointment base (hard paraffin, soft paraffin and lanolin).
2. Group II (hemin) was also consisted of 5 diabetic rats. In this group wounds were treated with hemin ointment (0.5% hemin in ointment base).

Collection of Tissue

The healing granulation tissue was collected immediately after euthanizing the rats with over dose of diethyl ether on day 19 post-wounding and preserved at -80°C.

Antioxidant parameters

Antioxidant status in granulation tissue of both the groups on day 19 post-wounding was assessed by lipid peroxidation (malondialdehyde /MDA) (Shafiq-ur-Rehaman ,1984), superoxide anion generation (Wang *et al.*, 1998), reduced glutathione (GSH) (Sedlak and Lindsay,1968), glutathione peroxidase (GPx) (Rotruck and coworkers ,1973), super oxide dismutase (SOD) (Madesh and Balasubramanian, 1998) and catalase (CAT) (Aebi,1984).

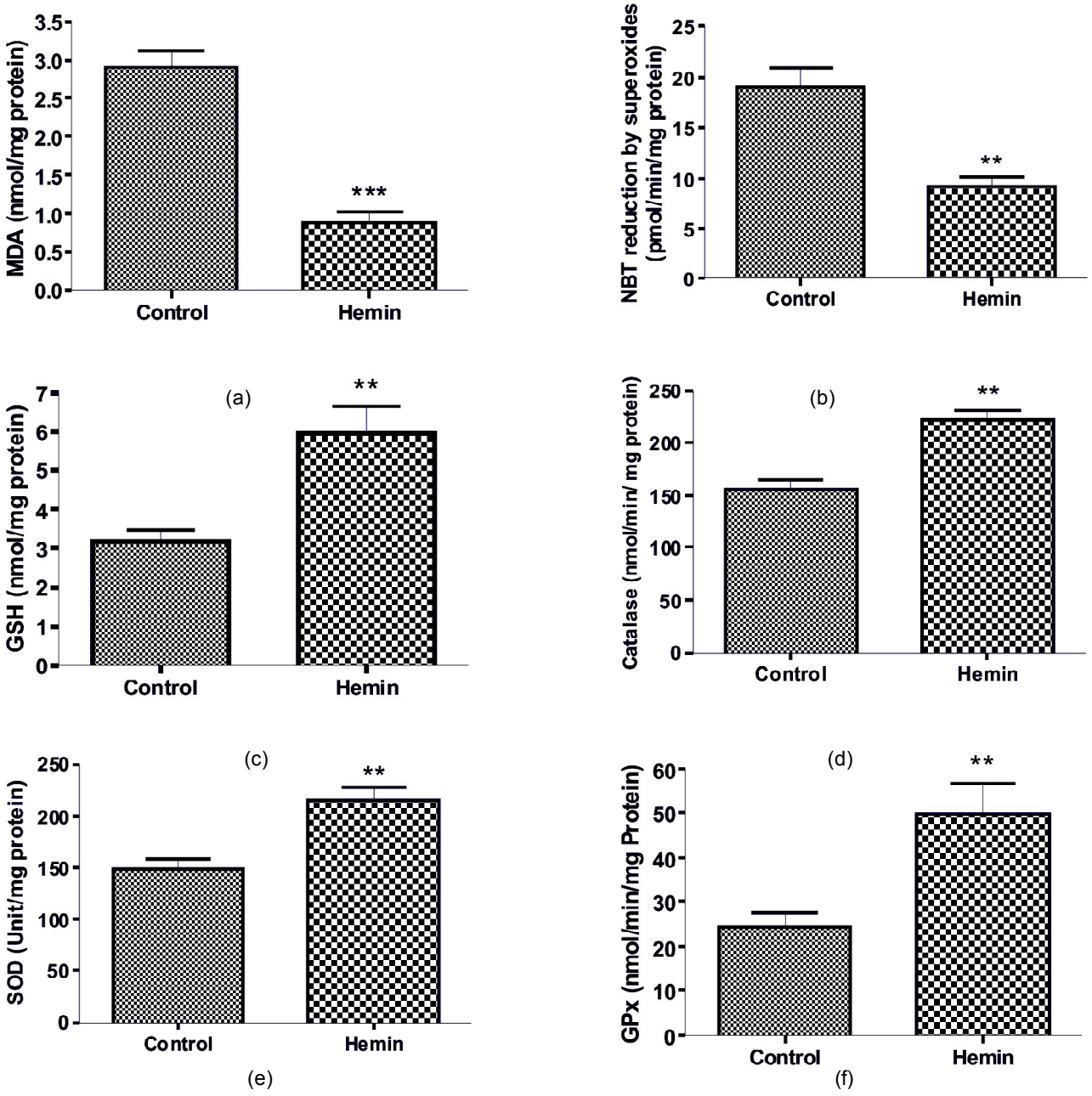

Fig. 1 (a-f):
Changes in activity of MDA (a), superoxides (b), GSH (c), catalase (d), SOD (e), and GPx (f) in healing tissue of different groups on day 19 post-wounding. Data are expressed as Mean ± SE; n=5; ** P<0.01 and *** P<0.001 vs. control.

Statistical analysis

Results are expressed as mean ± S.E ($P<0.05$). of five animals in each group. The statistical significance between the treated and control values was analyzed by applying unpaired student 't' test using GraphPad Prism v4.03 software program (San Diego, CA, USA)

RESULTS AND DISUSSION

Hemin treatment significantly ($p<0.01$) decreased the MDA (Fig. 1a) and superoxide production (Fig. 1b) in wound, as compared to diabetic control. Malondialdehyde (MDA) production is an index of lipid peroxidation (Kakkar *et al.,* 1998). The tissue lipid peroxidation in diabetic rats was increased, which might be due to an increase in the level of blood glucose (Manna *et al.,* 2010). Lipid peroxidation mediated tissue damage has been detected during the progress of diabetes mellitus; this is one of the specific features of chronic diabetes. The lipid radical and peroxide are risky to the body cells and allied with tissue damage. In the present study, it was observed that the hemin treatment significantly decreased the level of MDA in granulation tissue, as compared to control diabetic rats which is in agreement with previous reports (Taye and Ibrahim, 2013).

Glutathione protects cellular system against toxic effects of lipid peroxidants. The level of reduced glutathione was decreased in diabetes (Baynes and Thorpe, 1999). Reduced glutathione (GSH) is a major non protein thiol antioxidant compound present in living organisms, which serves a significant role in antioxidant defense mechanism (Venukumar and Latha 2002). In this study, there was significant increase in the level of GSH in hemin-treated group compared to control (Fig. 1c). Hemin compensated deficits in reduced glutathione, and catalase and superoxide dismutase activities, and suppressed lipid peroxidation in tissue xenobitics-induced oxidative stress in rats (Fouad *et al.*, 2009).

Topical application of hemin for 19 days on excision wound caused significant ($p<0.01$) increase in catalase and SOD activity (Fig. 1d and Fig. 1e) in granulation tissue, as compared to control diabetic rats. The superoxide dismutase (SOD) and catalase (CAT) are two key scavenging enzymes that eliminate the toxic free radicals induced by streptozotocin. SOD is considered to be an inducible key enzyme and its activity depends on $O_2^>$ concentration in the biological system (Heck *et al.*, 1992). SOD catalyzes the dismutation of $O_2^>$ into oxygen and H_2O_2, thus, decreases ROS generation and oxidative stress (Ponrasu *et al.* 2013). Catalase is a haeme containing enzyme, protects the tissues from highly reactive hydroxyl radical through catalyzing the reduction of hydrogen peroxide. The decrease of SOD and CAT activities might result from the inactivation by glycation of the enzyme. The most effective defense mechanism against diseases is the removal of superoxide and hydroxyl radicals. Ndisang *et al.* (2010) have shown the ameliorative effects of hemin by increasing catalase, SOD and total antioxidant capacity in diabetic rats which is in agreement with the present study.

The activities of GPx antioxidant enzyme were increased significantly ($p<0.01$) in granulation tissue by the topical application of hemin in STZ-induced diabetic rats. In diabetes, the activities of catalase and GPx are significantly decreased by superoxide radical and by glycation reactions. The glutathione peroxidase along with glutathione catalyzed the reduction of hydrogen peroxide into nontoxic metabolites. During diabetes, there is a decrease in the concentration of GSH that reduced the activities of GPx (Sayed *et al.*, 2012). Reduced activities of enzymatic antioxidants have been observed during diabetes, and this may result in a number of deleterious effects due to the accumulation of free radicals (Omotayo *et al.*, 2010).

In this study, decreased level of oxidants and increase level/activity of antioxidants in hemin-treated group might be due to induction of HO-1 enzyme. HO-1 induction has been recognized as a sensitive consequence of oxidative stress, and overexpression of HO-1 protects against oxidative damage in several cell types (Benjamin and McMillan, 1998) by producing bilirubin and ferritin from heme metabolism (Foresti *et al.,* 2013) which was also observed in our study.

In conclusion, the results of the present study suggests that hemin protects the granulation tissue from the oxidative stress by marked upregulation of the antioxidant profile of the diabetic wound and thus promote might healing.

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Research Article

AMELIORATING EFFECT OF *ZINGIBER OFFICINALE* LINN RHIZOME ON DMBA INDUCED MAMMARY GLAND TUMOUR IN RATS

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ABSTRACT

The present study was undertaken to substantiate the anti-cancer activity of ethanol extract of *Zingiber officinale* against 7,12-dimethlybenz[a]anthracene (DMBA) mammary tumour (MT) model in Wistar rats. 20 female rats (170-200g) were divided into 4 groups of 5 animals, healthy control, mammary tumour (MT) control, Z. officinale treated and cisplatin treated, were induced for tumour using DMBA (60 mg/kg b.wt.). *Zingiber officinale* (400mg/kg, p.o.) and cisplatin (8mg/kg , i.p.) were administered for 14 days after development of mammary tumor. *Z. offcinale* and cisplatin showed a steady increase in body weight (g), significant reduction in tumour volume (mm) and tumour weight (g) following treatment. The hematological profile (hemoglobin, RBC, WBC and platelets) were found to be reverted towards normal. *Z. officinale* and cisplatin elevated the level of antioxidant enzymes, SOD and GSH and reduced the level of elevated MDA significantly ($p < 0.001$). The level of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-2) decreased and anti-inflammatory (IL-10) cytokines in serum and tissue homogenates increased significantly ($p < 0.001$). Histopathological study revealed abundant mitotic figures in MT control group. Cisplatin treated group was almost free from neoplastic changes but *Z. officinale* treated rats showed less numbers of mitotic figures. The study evidently confirmed the anticancer activity of ethanolic extract of *Z. officinale* in mammary tumor in rats, presumably due to its antioxidant, anti inflammatory property and anti mutagenic properties.

Key Words: Cytokines; DMBA; Histopathology; Mammary Tumour; *Zingiber officinale*.

INTRODUCTION

Chemotherapy is the most effective method in cancer treatment in which drugs like cisplatin, carboplatin, cyclophosphamide, doxorubicin, melphalan, mitomycin-C, and gemcitabine are used. However, therapeutic efficacy of most of them is limited due to the development of various side effects in the host and/or the acquired drug resistance by the cancer cells. Thus to overcome these limitations traditional approach to treatment can prove beneficial and even cost effective. Ginger is one of the plants used by local people as a preventive to cancer but limited number of studies have been conducted on traditional use of ginger for the treatment of cancer. Along with the Khasi and Jaintia people of Meghalaya traditionally used to chew tobacco along with ginger found in their states as a preventive to oral ulcers. But this fact lacked scientific validation. Hence, in order to validate the claim, we initiated an *in vivo* study against breast cancer in Wistar rats.

Ginger (*Zingiber officinale* Rosc., rhizome), is one of the most widely used species of India. Amongst the 400 compounds, the anti-cancer properties of ginger are attributed to the presence of certain pungent vallinoids, viz. [6]-gingerol and [6]-paradol, [6]- shogaols, zingerone etc. [6]-Shogaol, was reported to be an active constituent of ginger, inhibited breast cancer cell invasion (Ling et al., 2010). Studies suggest that ginger and its active constituents suppress the growth and induce apoptosis of variety of cancer types including skin, ovarian, colon, breast, cervical, oral, renal, prostate, gastric, pancreatic, liver, and brain cancer (Srinivasan, 2014). With this back ground, *Z. officinale* was collected from Meghalaya for studying its anti-cancer property in mammary tumour in rats and substantiate the traditional claim.

MATERIALS AND METHODS

Plant, drugs and chemicals

Ginger was collected from a village of West Jaintia Hills District, Meghalaya. The plants were authenticated by taxonomist of NEIST, Jorhat, Assam and a voucher specimen was deposited in Herbarium of Botanical Survey of India, Meghalaya (Specimen No: AAU/CVSC/PHT/14-15/14).

Cisplatin and DMBA were purchased from Sigma (St. Louis, MO, USA). IL-β, IL-6, IL-2, IL-10 and TNF-α ELISA kits were purchased from Ray Biotech, Inc. U.S.A. Cisplatin solution was prepared in normal saline (0.9% NaCl). All the solutions were prepared freshly before the experiment.

Preparation of extract and phytochemical analysis

The shade dried coarsely powdered (250 g) *Z. officinale* was extracted by using ethanol (70%) as solvent in a rotary evaporator (Rotavapor R-210, Buchi). Finally, the liquid was transferred to a lyophilizer (Heto Power Dry LL3000, Thermo Electron Corporation). It was then stored at -20 ^{o}C till further use. The yield obtained was 6.51% (w/w). The extract was subjected to qualitative phytochemical

Table 1:
Effect of ethanol extract of *Z. officinale* and on body weight (g), tumour volume (mm) and tumour weight (g) in DMBA

Parameters	Healthy Control			MT control			*Z. officinale* (400 mg/kg)			Cisplatin (8mg/kg)		
	0 day	7th day	14th day	0 day	7th day	14th day	0 day	7th day	14th day	0 day	7th day	14th day
Body weight (g)	183.32 ±2.8^{a}	183.42 ±2.3^{a}	183.8 ±2.7^{a}	180.82 ±0.0^{b}	178.07 ±1.2^{b}	172.77 ±14^{b}	171.12 ±0.09^{c}	172.42 ±0.22^{cd}	179.02 ±0.58^{c}	171.22 ±0.26^{ec}	174.92 ±0.78^{e}	179.95 ±0.24^{ec}
Tumour volume (mm)	-	-	-	30.51 ±1.77^{a}	34.97 ±1.93^{a}	38.04 ±3.42^{a}	38.28 ± 3.02^{b}	34.25 ± 3.56^{a}	26.71 ± 2.31^{b}	38.35 ±1.92^{b}	30.02 ±1.28^{b}	22.33 ±1.82^{c}
Tumour weight (g)	-	-	-		0.801 ± 0.02^{a}			0.636 ± 0.040^{b}			0.389 ± 0.051^{d}	

Values were represented as mean ± SD (n = 4). Treatment means of different groups bearing common superscript did not differ significantly ($P < 0.05$).

screening for the presence of various active phytoconstituents (Harborne, 1991).

Experimental animals

Adult female Wistar rats of age 6-8 weeks weighing between (150-160g) were kept under the experimental housing (22 ± 2°C, 60 ± 5 % relative humidity, and 12:12 hrs light–dark cycle). and normal feeding conditions for 7 days prior to the test, fed with normal pellet feed, water in *ad libitum*. Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Science, Khanapara, Guwahati, Assam (No.770/ac/CPCSEA/FVSc/AAU/IAEC/13-14/217, Dated10.2.2014) approved the conducting of the study.

Tumour induction

7, 12-dimethyl benz [a] anthracene (DMBA in olive oil) at a dose rate of 60 mg/kg body weight, orally, a dose sufficient to cause 100% tumour incidence in the control group over the course of the study.

Experimental design

After an initial pilot study for selection of dose and duration, a total of 20 adult female Wistar rats were used for the study taking into consideration of mortality during study period. The rats were divided into four groups: Group I, healthy control where female rats received a single dose of olive oil (1 ml) , Group II, mammary tumour (MT) control female rats with DMBA (60 mg/kg) treatment only, Group III, MT induced female rats treated with ethanolic extract of *Z. officinale* (EEZO) @ 400mg/kg, for 14 days through oral gavages; Group IV, Standard group treated with cisplatin (8 mg/kg) , i.p. for 14 days. After 24 hours of administration of the last dose blood samples were collected (Serological study) and the animals were sacrificed humanely for collection of mammary tumour.

Body weight

The body weights of all the animals were recorded. The tumour mass was measured in all dimension (horizontally and vertically). Volume of tumour (V) was calculated by the formula determined by Carlsson: $V= (ab^2)/2$, where 'a'and 'b' is the longest and shortest diameters of the tumour, respectively (Carlsson et al., 1983).

Hematological and bichemical parameters

Estimation of hematological parameters like Hemoglobin (Hb), Red blood cell (RBC), White blood cell (WBC) and platelets count were done using Hematology Analyzer at Diagnostic Laboratory, Department of Animal Husbandry & Veterinary, Meghalaya, Shillong.

Antioxidant parameters

For the antioxidant assays, the mammary tumour from each animal was taken and homogenized in 0.1M Phosphate buffer, (pH 7.4) and centrifuged at 8000-10000 rpm at 4ºC for 15 min. (Cooling microfuge-Remi-CM-12). The supernatant was used for estimation of protein (Lowry et al., 1951), superoxide dismutase (SOD) (Marklund and Marklund, 1974), reduced glutathione (GSH) and lipid peroxidation (LPO) (Ohkawa et al., 1979).

Cytokine Estimation

The blood samples were kept for 1h at 4°C. Whole blood samples were centrifuged at 15000 rpm for 10min at 4°C. Serum and plasma were carefully removed and stored at -20°C until assayed. Serum TNF-α, IL-1β, IL-6, IL-2, IL-10 levels were assayed by an enzyme-linked immunosorbent assay (ELISA) kits (Ray Biotech., USA) following the instruction manual.

The mammary gland tumours were fixed in 10% buffered formalin solution, (Lee and Luna, 1968).

Acute toxicity study

The acute toxicity study of *Z. officinale* was evaluated in rats using a fixed dose procedure (OECD Guidelines No. 420, pages 1/14-14/14(2001). The test substance at a single dose of 2000 mg/kg body weight was administered to five animals orally by gavage. The animals were observed for toxic symptoms continuously for the first 4 hr after dosing (24h for 13 days).

Statistical analysis

Statistical analysis was carried out by using one-way ANOVA followed by Dunnet's and Tukey test with GraphPad Prism 5.0 (San Diego, CA, USA) and the values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Phytochemical screening of ethanol extract of *Z. officinale* showed presence of alkaloid, glycosides saponins, tannin and terpenoids by standard methods.

A significant decrease ($P < 0.001$) in body weight

was observed in mammary tumour (MT) control group when compared with healthy control group. However, rats treated with EEZO (400 mg/kg) and Cisplatin (8 mg/kg) treated group showed significant ($P < 0.001$) gain in body weight. (Table 1). Gradual increase in tumour volume of MT control group was evident when compared to the control. Significant reduction in tumour volume was observed in EEZO (400mg/kg) treated group and cisplatin (8mg/kg) treated group on 0, 7th ($P < 0.05$) and 14th day ($P < 0.001$) respectively when compared with the MT control group (Table 1). Likewise, the tumour weight showed significant reduction ($P < 0.001$) in rats treated with EEZO (400 mg/kg) and cisplatin treated (8 mg/kg) when compared with the MT control group (0.801 ± 0.02 g) (Fig. 1).

Decrease in RBC, haemoglobin (13.14 ± 0.09^{b} g/dl) and increase in platelets, WBC counts were observed in the mammary tumour (MT) control group when compared to the healthy control group. In EEZO (400 mg/kg) and Cisplatin (8 mg/kg) treated groups, the values were significantly increased Hb, RBC, WBC and platelets, respectively (Table 2).

GSH and SOD levels decreased ($P < 0.001$) and a significant ($P < 0.001$) increase in MDA level was observed in MT control on comparison to healthy control group. While GSH and SOD levels were elevated significantly ($P < 0.001$) and MDA level decreased significantly ($P < 0.001$) in *Z. officinale* (400mg/kg) and Cisplatin (8mg/kg) treated groups, respectively as compared with MT control group. (Table 2).

Cytokine levels in serum and tissue homogenates

The levels of both pro-inflmmatory and anti-inflammatory cytokines decreased significantly ($P < 0.001$) in EEZO (400mg/kg) in serum and tissue respectively . Cisplatin (8mg/kg) treated group also showed significant decrease in cytokine levels when compared with MT control group (Table 3).

Healthy control group displayed normal histology (Fig. 2a). In the mammary tumour control group, the cells lining showed hyperchromatic epithelium. Proliferating epithelial cells partially filled the lumen of the ducts, with abundant mitotic figures. The tumour cells had hyperchromatic nuclei (Fig. 2b). In *Z. officinale* treated rats, most of the ductules showed empty lumen with a few showing presence of desquamated lining epithelial cells with hyperchromatic nuclei. The oedematous structures of the alveoli were less in number. Mitotic figures were less in number (Fig. 2c). In Cisplatin treated group, most of the alveoli were devoid of any cellular exudates, they were lined by only one or two epithelial cells having hyperchromatic nuclei. The intraductular space showed presence of collagen and the tissue bearing the ductules areas was almost free of neoplastic changes (Fig. 2d).

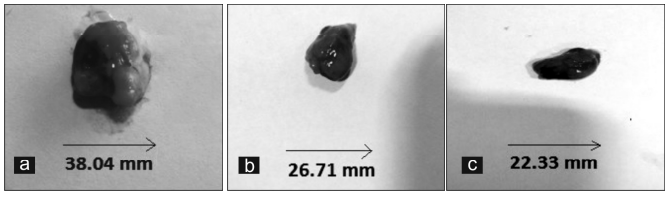

Fig 1.
Tumour volume measured after sacrifice of animals a) Mammary tumour control, b) *Zingiber officinale* treated, c) Cisplatin treated group. (Image not upto scale)

Oral administration of *Z. officinale* upto 2000mg/kg did not produce any toxic effect in the normal behaivor of the rats. No mortality was observed and the extract was found to be safe even at the given dose.

DISCUSSION

The aim of this study was to evaluate the anti-tumor property of a native species of ginger, collected from Meghalaya based on its ethnomedicinal use by the local tribes.

The phenolic compound is considered to be one of the most active constituent of *Z. officinale* and is responsible for all its anti-tumour activity (Srinivasan, 2014). Loss of body weight is one of prominent symptom of cancer, as shown by MT control group rats and in *Z. officinale* (400 mg/kg) and cisplatin (8 mg/kg) treated groups showed a steady weight gain when compared to control group (Habib *et al*., 2008). There was reduction in tumour volume in *Z. officinale* (400mg/kg) treated animals when compared to MT control group, possibly due to the positive effect of the extract on mammary tumour and is in close accordance with results obtained by other researcher (Samy et al., 2006). Tumour weight was also found to reduce in *Z. officinale* (400mg/kg) and Cisplatin (8mg/kg) treated groups. The above findings was also in support with Roomi *et al.* (2005). Hemolysis and other myelopathic condition lead to loss of RBC count and increased WBC count in MT control group, but following treatment with EEZO or cisplatin, they were found to be increased (RBC) and decreased (WBC) due to haemopoetic activity of the compound. Most of the plants exert their activity due to anti-oxidant property. We have estimated various antioxidant enzyme levels during the period of study. Levels of SOD and GSH were increased while the levels of MDA were declined following treatment with EEZO (400mg/kg) and Cisplatin (8mg/kg) as compared with MT control group. The above findings were in close concordance with other studies where they claimed that the phenolic substances of ginger played an important role in reduction of MDA and elevation of SOD due to its strong anti-inflammatory and antioxidant properties (Sharma *et al.,* 2011).

As reported by Sato *et al.* (2011), IL-10 is an immunomodulatory cytokine that is frequently upregulated in various types of cancers which justified our finding. Fang

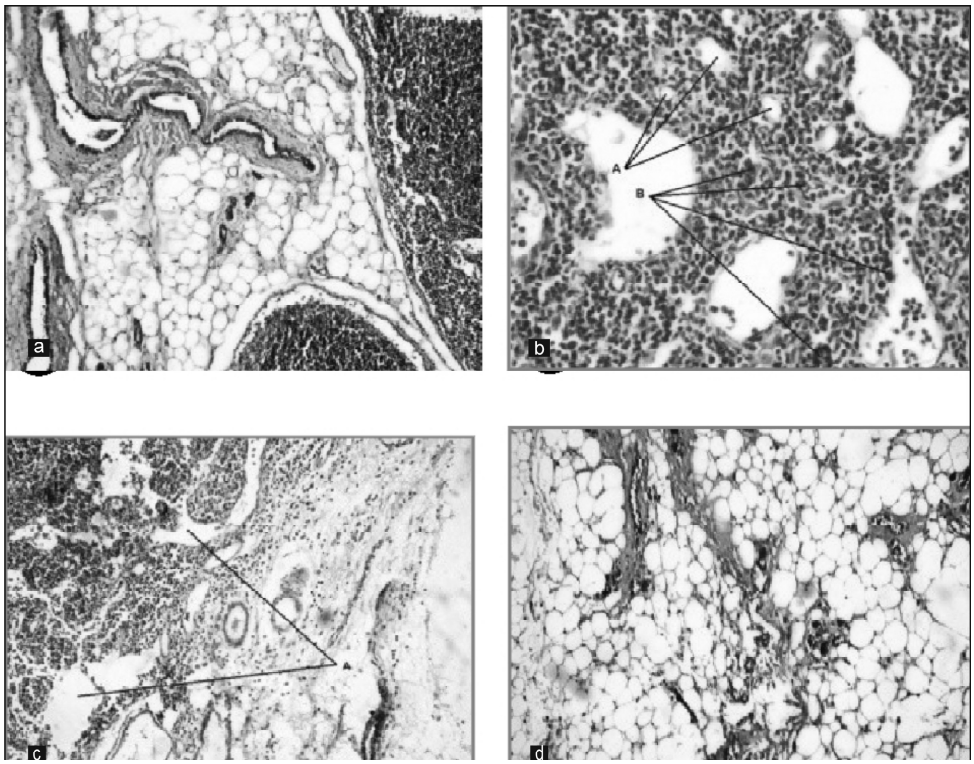

Fig. 2 (a-d):
Histological structure of mammary gland tumour in H & E (100x) of (a) Terminal ducts by loose connective tissue healthy control (b) proliferation of epithelial cells, formation of microglandular space (A) and mitotic figures (B) in MT control (c) ductules with empty lumen (A) and less number of tumour cell population in *Z. officinale* group and (d) less number of mitotic figures and proliferating cells in DMBA induced mammary tumour in Cisplatin group.

Table 2:
Effect of ethanol extract of *Z. officinale* on hematological and antioxidant parameters in DMBA induced mammary tumour.

Haematological parameters	Healthy control	MT control	*Z. officinale*(400 mg/kg)	Cisplatin(8mg/kg)
Haemoglobin (g/dl)	16.45 ± 0.44^{a}	13.14 ± 0.09^{b}	14.46 ± 0.29^{ac}	15.95 ± 0.58^{ac}
RBC (10^3/mm)	6.44 ± 0.16^{a}	4.90 ± 0.14^{b}	5.54 ± 0.16^{c}	5.97 ± 0.02^{e}
WBC (10^3/mm)	6.42 ± 0.41^{a}	8.12 ± 0.09^{b}	7.11 ± 0..03^{c}	6.59 ± 0.28^{a}
Platelets (%)	0.25 ± 0.006^{a}	0.29 ± 0.018^{b}	0.26 ± 0.012^{a}	0.25 ± 0.001^{a}
SOD (U/mg protein)	4.51 ± 0.34^{a}	1.58 ± 0.12^{b}	2.34 ± 0.10^{c}	3.76 ± 0.19^{e}
GSH (mg/g of protein)	5.61 ± 0.38^{a}	1.49 ± 0.14^{b}	2.47 ± 0.15^{c}	4.11 ± 0.12^{d}
MDA(nmol /g of protein)	0.72 ± 0.06^{a}	1.55 ± 0.16^{b}	1.13 ± 0.10^{c}	0.94 ± 0.03^{c}

Values were represented as mean ± SD (n = 4). Treatment means of different groups bearing common superscript did not differ significantly (P <0.05).

Table 3:
Effect of ethanol extract of *Z. officinale* on cytokines expression (serum and tissue) in DMBA induced mammary tumour.

Cytokines level (Serum)	Healthy control	MT control	*Z. officinale*(400 mg/kg)	Cisplatin(8mg/kg)
TNF-α (pg/ml)	16.50 ± 0.1.91^{a}	121.00 ± 1.82^{b}	117.75 ± 0.96^{c}	112.25 ± 1.26^{d}
IL-1 β (pg/ml)	49.00 ± 2.45^{a}	322.25 ± 10.63^{b}	216.25 ± 5.06^{c}	205.00 ± 3.56^{ce}
IL-6 (pg/ml)	9.25 ± 2.10^{a}	59.50 ± 1.58^{b}	56.25 ± 0.29^{c}	53.87 ± 0.75^{cd}
IL-2 (ng/ml)	0.79 ± 0.016^{a}	0.98 ± 0.030^{b}	0.86 ± 0.015^{c}	0.82 ± 0.010^{e}
IL-10 (pg/ml)	9.00 ± 1.63^{a}	24.00 ± 2.16^{b}	17.75 ± 0.58^{c}	14.25 ± 1.5^{d}
Cytokines Level (Tissue)				
TNF-α (pg/ml)	22.25 ± 0.96^{a}	138.5 ± 2.08^{b}	122.00 ±1.63^{c}	112.75 ± 0.96^{e}
IL-1 β (pg/ml)	52.75 ± 3.60^{a}	140.25 ± 8.14^{b}	109.75 ± 4.35^{c}	104.50 ± 0.58^{c}
IL-6 (pg/ml)	8.12 ± 2.29^{a}	57.87 ± 0.48^{b}	54.25 ± 0.29^{c}	49.87 ± 0.85^{d}
IL-2 (ng/ml)	0.13 ± 0.03^{a}	0.36 ± 0.03^{b}	0.31 ± 0.01^{c}	0.30 ± 0.014^{c}
IL-10 (pg/ml)	8.25 ± 1.50^{a}	24.25 ± 1.71^{b}	20.00 ± 0.82^{cd}	12.75 ± 0.96^{e}

Values were represented as mean ± SD (n = 4). Treatment means of different groups bearing common superscript did not differ significantly (p <0.05).

et al. (2007) recorded that the serum concentration of IL-2 in patients with painful bone metastases increased, whereas serum TNF-α concentrations decreased, which is in partial agreement to our finding. Reports demonstrated that serum cytokine IL-1 β levels were elevated in untreated breast cancer patients and significantly reduced after tamoxifen therapy for more than one year (Premkumar *et al.,* 2007). In the present study, both the levels of pro-inflammatory and anti-inflammatory cytokines were elevated. Treatment group for *Z. officinale* (400 mg/kg b.wt.) showed less proliferating cells and mitotic figures and cisplatin (8mg/kg) showed alveoli devoid of any cellular exudates which was prominent in MT control group. One or two epithelial cells having hyperchromatic nuclei and ductules areas were almost free of neoplastic changes in treated group. Its phytoconstituents such as vallinoids, viz. [6]-gingerol, [6]-paradol (Rahman *et al.*, 2011) and [6] shogaol might be responsible for its anti mutagenic activity.

Hence, from the above results it can be concluded that ethanol extract of *Z. officinale* showed anti-cancer activity against mammary tumor in rats. The in vivo antioxidant and anti inflammatory activity of the extract might contribute to its anti- neoplastic activity among many factors.

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Research Article

AMELIORATIVE EFFECTS OF *GYMNEMA SYLVESTRE* IN DIABETIC DOGS

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ABSTRACT

Gymnema sylvestre (GS) is an indigenous herb, used as anti diabetic drug in herbal medicine. GS is one of the most efficient antioxidants and hypoglycemic agent. So, present investigation was conducted to evaluate the efficacy of leaf powder of GS in diabetic dogs. Twelve diabetic dogs were randomly assigned to two treatment groups consisting six each. Group I (control) dogs were treated with Insulin @ 1.0 I.U./kg b.w. and second group was supplemented with leaf powder of Gurmar (*Gymnema sylvestre*) @ 1.0 gm as total dose along with Insulin @ 1.0 I.U./kg b.w. Blood samples were collected on day 0, 15, 30 and 45 and assessed for biochemical and oxidative parameters. The control group showed a linear increase in blood glucose level, serum cholesterol, serum triglycerides, biomarkers of oxidative stress (Malondialdehyde, MDA) and reduction in reduced glutathione, catalase and SOD which were significantly reversed by GS supplementation. It is concluded from the study that the leaf powder of *Gymnema sylvestre* can be used as adjunct therapy along with insulin in canine diabetes.

Key words: *Gymnema sylvestre*, diabetes, dogs hypoglycaemia, oxidative stress

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease with high levels of glucose in blood and changes in carbohydrate, lipid and protein metabolism that has a significant impact on health, quality of life, and life expectancy. Diabetes in animals shares many similarities to diabetes in man and dog. Oxidative stress has been suggested as a potential patho-physiological mechanism underlying diabetic complications (Fields, 1998).

Gymnema Sylvestre is an indigenous herb, belongs to Kingdom Plantae with Division Angiospermae and Class Dicotyledoneae. It is also known as 'gurmar' or 'sugar destroyer' is used in traditional herbal medicine (Thakur *et al.* 2012). Its hypoglycaemic effects are mainly due to improving secretion of insulin by regeneration of islet cells (Daisy *et al.*, 2009). Its antiantherosclerotic potential were almost similar to that of a standard lipid lowering agent clifibrate (Bishayee and Chatterjee, 1994). Therefore, this study was conducted to evaluate the effect of GS leaf powder in diabetic dogs.

MATERIALS AND METHODS

Twelve clinically presented diabetic dogs were randomly assigned to two treatment groups consisting of six replicates at teaching veterinary clinical complex (TVCC), O.U.A.T Bhubaneswar. All the diabetic dogs were treated with insulin (as therapeutic control) @ 1.0 I.U./kg b.w. The second group was supplemented leaf powder of Gurmar (*Gymnema sylvestre*) @ 1.0 gm as total dose along with insulin @ 1.0 I.U./kg b.w. The blood samples about 2-3 ml were collected from diabetic dogs on 0 day, 15th, 30th and 45th day and analyzed for biochemical and oxidative stress biomarkers by autoanalyser (Turbochem 100) using commercial reagent kits as a regular monitoring of diabetes.

Assessment of oxidative stress in diabetic in dogs was done by assaying of erythrocyte oxidant – antioxidant status. The lipid peroxides level in the RBC hemolysate was determined by the method of Placer (1967). Reduced glutathione was estimated by DTNB method of Prins and Loos (1969). Catalase activity in hemolysate was estimated by using H_2O_2 as a substrate as per the method of Bergmayer (1983). Superoxide dismutase was estimated as per the method described by Madesh and Balasubramanian (1998). The data were analyzed by two way analysis of variance (ANOVA) followed by Bonferroni's post test using the Graph Pad Prism v4.03 software program (San Diego, CA, USA) and the differences between the experimental and control groups were considered statistically significant at $p<0.05$ or lower.

RESULTS AND DISCUSSION

Blood glucose level was significantly lower in GS treated dogs as compared to control on day 30 and 45 (Table 1). Gymnemic acids from GS exert its hypoglycaemic effects by increasing utilization of glucose by enzymatic (phosphorylase) stimulation of glucose utilization by insulin-dependent pathways, suppressing gluconeogenic enzymes like sorbitol dehydrogenase. It causes inhibition of glucose absorption from intestine due to effect of Gurmarin and Gymnemic acid which are responsible for the antihyperglycemic effect (Nakamura *et al.*, 1999; Kang *et al.,* 2012).

There was significant reduction serum triglyceride on day 30 and 45 and cholesterol level on day 45 in GS treated dogs compared to control (Table 1). Lipid lowering effect of GS may be due to dose-dependent increase in faecal cholesterol and cholic acid-derived bile acid

excretion has been demonstrated in diabetic rats. GS (gymnemic acid) decreased the apparent fat digestibility and promoted the excretion of neutral sterol and acidic steroids to faeces (Nakamura, *et al.,* 1999). Mall *et al.* (2009) and Kang *et al.* (2012) reported that the feeding of GS leaf extract resulted in significant decrease in total cholesterol and serum triglycerides and significantly increased HDL could be beneficial in preventing atherosclerotic conditions, thereby reducing the possibility of coronary heart disease in general.

BUN, creatinine, AST, ALT and ALP levels were decreased non-significantly in GS group compared to control dogs (Table 1). The hydro-alcoholic extract of GS showed a significant restoration of the altered liver profile parameters in hepatic cell culture model (Srividya *et al.*, 2010).

Lipid peroxidation was significantly less in GS treated dogs in comparison to control group on day 45 (Table 2). Babujanarthanam *et al.* (2011) reported that antioxidants inhibited lipid peroxidation in STZ-induced diabetic rats. There was significant increase in GSH level in GS group, as compared to control on day 45 (Table 2). Meister *et al.* (1988) reported that Glutathione is also known to be the first line of defence against in vivo oxidation. Anderson *et al.* (1987) also reported that GS significantly increased the glutathione levels. Catalase activity was significantly increased in combination group, as compared to control on 45th day (Table 2). Catalase is a major antioxidant defense enzyme that detoxifies reactive oxygen radicals by catalyzing the decomposition of H_2O_2 to H_2O with GSH (Cheng *et al.,* 1981). The present results indicate that GS leaf powder increases catalase activity and consequently increases the antioxidant activity of RBC. GS treated dogs showed significant increase in SOD activity as compared to control group (Table 2). SOD present in the body is known to inhibit the reaction of

Table 1:
Effects of leaf powder of *Gymnema sylvestre* on biochemical parameters in insulin-treated diabetic dogs.

Bioiochemicalparameters	Group	Days			
		0	15	30	45
Blood glucose	Insulin	353.00±19.72	309.50±19.08	256.83±13.34	205.83±13.28
	Insulin+GS	344.83±18.28	287.67±18.41	200.00±8.64*	147.33±5.91*
Triglyceride (mg/dl)	Insulin	364.50±13.25	310.17±11.49	293.67±9.85	266.17±8.57
	Insulin+GS	380.17±10.82	274.67±8.21	239.33±17.28 **	199.67±9.35***
Cholesterol (mg/dl)	Insulin	506.17±27.28	465.16±43.75	389.67±32.69	306.67±26.60[a]
	Insulin+GS	526.00±49.66	424.17±29.33	284.67±18.56	211.83±11.82*
ALP (IU/L)	Insulin	506.17±27.28	465.16±43.75	389.67±32.69	306.67±26.60
	Insulin+GS	526.00±49.66	424.17±29.33	284.67±18.56	211.83±11.82
ALT (IU/L)	Insulin	174.33±9.30	159.83±9.69	145.50±8.12	127.33±8.12
	Insulin+GS	164.17±8.43	149.83±7.32	140.00±7.00	122.88±5.32
AST (IU/L)	Insulin	193.17±4.41	172.50±5.36	155.17±7.18	131.83±5.00
	Insulin+GS	200.17±7.06	177.83±4.23	155.33±3.53	133.17±4.58
Creatinine (mg/dl)	Insulin	1.88±0.05	1.60±0.06	1.41±0.03	1.23±0.15
	Insulin+GS	1.78±0.05	1.43±0.04	1.22±0.03	1.03±0.11
BUN (mg/dl)	Insulin	56.00±3.67	45.33±4.14	39.67±2.62	37.67±4.30
	Insulin+GS	60.50±2.26	48.83±2.76	38.50±1.43	35.33±1.82

Data are expressed as mean ± SE; n=6; data bearing superscript *,**, ***differ significantly on the same day at P<0.05,0.01 and 0.001, respectively.

Table 2
Effects of leaf powder of *Gymnema Sylvestre* on oxidative stress biomarkers in insulin-treated diabetic dogs.

Oxidative stress biomarkers	Group	Days			
		0 day	15 day	30 day	45 day
LPO (nmolMDA/mg Hb)	Insulin	8.17±0.33	7.92±0.26	7.52±0.26	6.80±0.26
	Insulin+GS	8.15±0.24	7.57±0.34	6.75±0.13	5.80±0.19*
GSH(µmol/mL of packed RBC)	Insulin	0.30±0.03	0.32±0.02	0.35±0.02	0.38±0.02
	Insulin+GS	0.31±0.03	0.36±0.02	0.43±0.03	0.50±0.02*
Catalase (µmol H_2O_2 decomposed/min/mg Hb)	Insulin	3.43±0.013	3.98±0.14	4.58±0.18	5.50±0.12
	Insulin+GS	3.50±0.24	4.13±0.20	5.38±0.20*	6.13±0.20**
SOD (µmolMTT formed/mg Hb)	Insulin	0.31±0.02	0.35±0.04	0.40±0.02	0.41±0.01
	Insulin+GS	0.32±0.04	0.42±0.02	0.48±0.01*	0.55±0.02**

Data are expressed as mean ± SE; n=6; data bearing superscript *, ** differ significantly on the same day at P<0.05 and P<0.01 respectively.

converting active oxygen into hydrogen peroxide in cells (Halliwell *et al.,*1992). Kang *et al.* (2012) reported that the extract of GS leaf exhibited strong antioxidant activity in the assays, including TBA (56%), SOD-like (92%), and ABTS (54%). Lipid peroxidation levels were decreased by 31.7% in serum, 9.9% in liver, and 9.1% in kidney in the diabetic rats fed the extract. Ethanolic leaf extract of *GS* effectively alleviates the deleterious effects produced by high fat diet in diabetic rats (Rathore *et al.*, 2015). GS leaf extract effectively reduces oxidative stress in myocardial cells diabetic rats (Rathore *et al.*, 2015). Antioxidant (SOD, catalase) enzymes levels were significantly increased by water soluble fraction of GS extract treated group as compared to the high fat diet fed diabetic rats (Ye *et al.,* 2000). Thus, powder of *G. sylvestr*e was found useful as adjunct to antidiabetic therapy.

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Research Article

DETERMINATION OF CHLORTETRACYCLINE RESIDUES IN CHICKEN MEAT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In the present study, the residual concentration of chlortetracycline was determined in chicken meat using ultra high performance liquid chromatography (UHPLC). In all 180 chicken meat samples including muscle, liver and kidney were analyzed. Out of 180 samples, 48 samples were found positive for residual concentration of chlortetracycline in chicken meat. Positive meat samples of chlortetracycline were further analyzed for quantitative estimation. Eight samples (16.66 per cent) were found positive for chlortetracycline residue at violative levels while 40 samples (83.33 per cent) were found below maximum residue limits recommended by Commission Regulation of European Union for chlortetracycline in chicken muscle, liver and kidney samples. Positive meat samples were further subjected for boiling and results indicates a significant reduction in residual concentration of chlortetracycline.

Key words: UHPLC, chlortetracycline, antibiotic, chicken, residue analysis

INTRODUCTION

Antibiotics are used in the poultry to enhance growth and feed efficiency and reduce the incidence of disease. A survey (Resurreccion and Galvez, 1999) revealed that 77% of consumers responding considered animal drug residues in meats to be an extreme health concern. Antibiotics are used as growth promoters at concentration lower than therapeutic concentration for a longer period of time. This is potentially dangerous practice since it provide strong selective pressures for emergence of antibiotic resistant strain of bacteria (Simonsen *et al.*, 1998), induction of allergic reactions in human and technological problem of fermented meat products (Pavlov *et al.*, 2005).

Chlortetracycline (CTC), a member of tetracycline family, is a broad-spectrum antibiotic which is commonly used in poultry. Chlortetracycline can be used against *Escherichia coli*, *Salmonella* and *Staphylococcus* spp. Chlortetracycline is given orally in drinking water and by intramuscular injection.

The present study was aimed to detect the residual concentration of chlortetracycline in chicken meat in and around Jabalpur district of Madhya Pradesh.

MATERIALS AND METHODS

Sample collection

A total of 180 broiler meat samples were collected from 10 target area in and around Jabalpur district of Madhya Pradesh from each target area 18 samples were collected, 6 each of muscle, liver and kidney. Approximately 5 g of muscle, liver and kidney sample, each of the same bird were aseptically collected for detection of antibiotic residues.

Chemicals and standard

The chemicals and standards used for extraction, detection and quantification of residual concentration of chlortetracycline, were acetonitrile (Sigma – Aldrich), methanol (Sigma – Aldrich), 0.01M oxalic acid (Hi-media), water (Sigma Aldrich) and standard chlortetracycline (Sigma – Aldrich). All the chemicals and standard were of HPLC grade.

Quantification of Positive Samples

Chromatography conditions

The (UHPLC) unit of Liquid Chromatography Mass Spectrometer (LCMS- 8030, Shimadzu, Japan), consisted of Mobile phase reservoir, degasser, HPLC pump, sample injector, guard column, main column, detector, data collection unit, waste or fraction collector with NEXERA software.

The HPLC system was equipped with photodiode array UV- Vis detector. Chromatography conditions were maintained as described by Adewuyni *et al.* (2011) with slight modification. Particle separation was done using with C18 column (Supelco, USA, column dimensions: 150× 2.1 mm, particle size: 1.9 µm) and the temperature of column was set at 30°C. The mobile phase comprised of 0.01M oxalic acid, acetonitrile and methanol respectively in 70:20:10 ratio (HPLC grade) with flow rate adjusted at 1ml/min. Peak separation was accomplished after 3.10 minutes and 20 µl treated samples were required for injection.

Stock standard solution of chlortetracycline was prepared by dissolving 10 mg of compound in 10 ml of methanol (1 mg/ml) stored at -20^0 C which was stable for at least 4 weeks. These solutions were diluted to give a series of working standard solutions (Cinquina *et al.*, 2003).

The extraction of chlortetracycline was done (Cinquina *et al.*, 2003) with slight modification. Briefly, five gram of muscle/liver/kidney of chicken meat sample were homogenised, placed in a glass centrifuge tube and 2 ml of 20 per cent trichloroacetic acid (TCA) added. Sample

was extracted with 20 ml McIlvaine buffer and centrifuged at 4000 rpm for 20 min. The supernatant was then applied to a SPE cartridge, previously activated with 3 ml of methanol and 2 ml of water. After sample loading, the cartridge was washed with 2 ml of methanol 5 per cent in water. Finally chlortetracycline was eluted with 3 ml of methanol. The solvent was removed under a nitrogen stream and the residue was dissolved in 1 ml of methanol and ûltered with a 0.2 mm ûlter. An aliquot (20 ml) was injected into the UHPLC system.

RESULTS AND DISCUSSION

Mean residue concentration of chlortetracycline in chicken meat samples (muscle, liver and kidney) from different target areas of Jabalpur district was analyzed using UHPLC. Total 180 chicken meat samples were analyzed including 18 samples each from Jabalpur-1 to Jabalpur-10 target areas. Among the 180 samples, 48 samples (26.66 per cent) were detected positive for chlortetracycline residues. Out of these 48 positive samples, 8 samples (16.66 per cent) were quantified with chlortetracycline residue at violative levels while 40 samples (83.88 per cent) were detected with residue below the recommended MRLs. Chlortetracycline residue concentration was detected in meat samples (muscle, liver and kidney) of target area 1, 3, 7, 8 and 10 (Table 1 and Fig. 1a, 1b, 1c).

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

Target area	Mean residual concentration(µg/kg) ± S.E.		
	Muscle	Liver	Kidney
TA-1	77±30.51	191±80.81	335.75±183.85
TA-2	0.00	0.00	0.00
TA-3	65.42±18.82	140±48.41	278.28±121.04
TA-4	0.00	0.00	0.00
TA-5	0.00	0.00	0.00
TA-6	0.00	0.00	0.00
TA-7	51.4±17.26	119.6±54.35	206±113.39
TA-8	47.62±15.67	148.87±38.34	231.37±89.32
TA-9	0.00	0.00	0.00
TA-10	83.33±38.36	237.66±90.43	458.66±201.32

Table 2:
Effect of boiling on mean residual concentration of chlortetracycline in muscle, liver and kidney samples of broiler chicken

Experimental group	Muscle (µg/kg)	Liver (µg/kg)	Kidney (µg/kg)
Raw samples	65.91[a]± 13.87	151.16[a]± 36.01	280.91[a]± 85.48
Boiled samples	45.94[b]± 9.74	102.23[b]± 24.36	183.49[b]± 55.85

(a, b) Values in the column with different superscript differ significantly ($p<0.01$)

Effects of boiling on chlortetracycline residue

Out of 48 positive samples, Mean residue concentration of chlortetracycline was 65.91±13.87 µg/kg in muscle, 151.16±36.01 µg/kg in liver and 280.91±85.48 µg/kg in kidney samples. After boiling the same samples, the mean residual concentration significantly reduced to 45.94±9.74 µg/kg in muscle, 102.23±24.36 µg/kg in liver and 183.49±55.85 µg/kg in kidney samples (Table 2).

Mean residue concentration of chlortetracycline

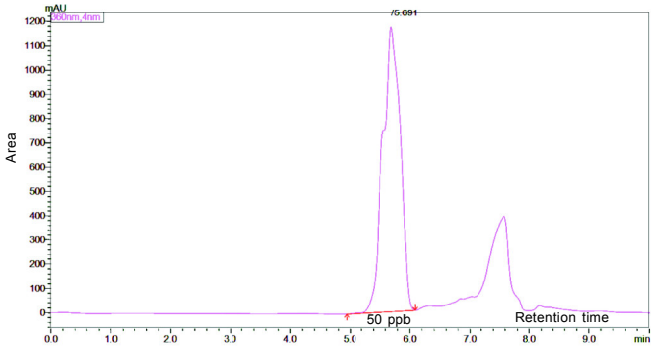

Fig 1 (a)
Chromatogram of chlortetracycline in liver by using High Performance Liquid Chromatography

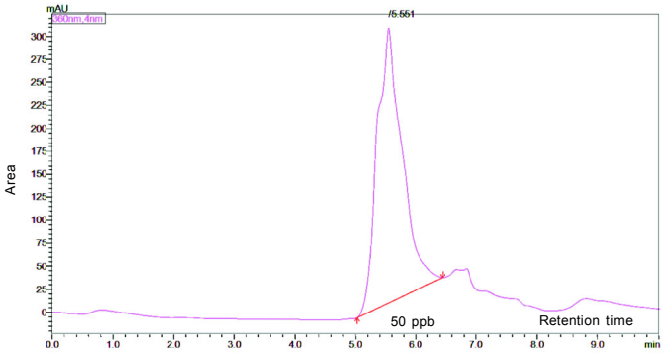

Fig 1(b)
Chromatogram of chlortetracycline in kidney by using High Performance Liquid Chromatography

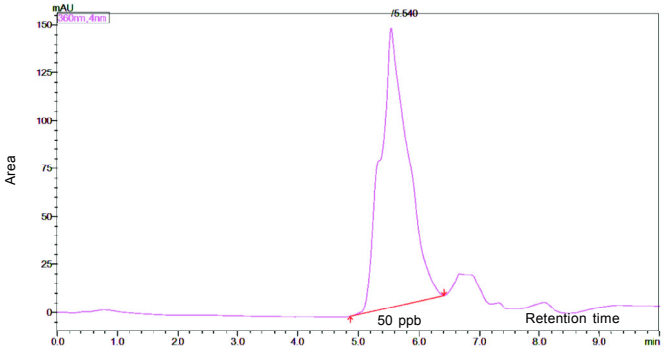

Fig 1(c)
Chromatogram of chlortetracycline in muscle by using High Performance Liquid Chromatography

significantly reduced to 30.12 per cent, 32.37 per cent and 34.67 per cent in muscle, liver and kidney samples respectively.

Mean residue concentration of chlortetracycline in muscle, liver and kidney samples were analyzed by HPLC in different target areas of Jabalpur district. Total 180 chicken meat samples were analyzed including 18 samples each from 10 target areas. As revealed under the study, 48 samples (26.66 per cent) showed detectable levels of chlortetracycline residues. The mean residue concentration of chlortetracycline in muscles, liver and kidney samples are given in Table 1. Liver and kidney samples showed more positive results with higher residual concentration as compared to the muscle samples. Out of 180 samples, 8 samples (4.44 per cent) were detected with residue level above maximum residue limit. The findings are in accordance with Cinquina *et al.*, 2003 who also quantified the residual concentration of chlortetracycline in bovine meat.

In the present study mean residual concentration of chlortetracycline was reduced significantly after boiling to the extent of 30.12 per cent, 32.37 per cent and 34.67 per cent in muscle, liver and kidney samples respectively.

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Research Article

PHARMACOKINETICS OF FLUCLOXACILLIN IN CISPLATIN INDUCED CHRONIC RENAL FAILURE GOATS

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ABSTRACT

Pharmacokinetics of flucloxacillin (10 mg kg^{-1}, i.v.) was studied in both healthy and chronic renal failure (CRF) female black Bengal goats. Cisplatin (30 mg m^{-2}) was administered intravenously, once daily consecutives for seven days to induce chronic renal failure (CRF) grade I. The pharmacokinetic parameters were calculated in both cases. CRF grade I caused significant changes in the determined variables of flucloxacillin. The C_{max} and C_{min} of FLX observed at 0.08 and 12 h in healthy goats were 77.76 ± 7.50 and 0.34 ± 0.11 μgml^{-1} respectively, while the same in CRF grade I goats at 0.08 and 24 h were 115.51 ± 4.12 and 0.21 ± 0.05 μgml^{-1} respectively. CRF grade I condition caused significantly increased $t_{1/2}\beta$ (3.61 ± 0.030 h), $t_{1/2}\alpha$ (0.06 ± 0.01), AUC (166.14 ± 5.18 μg ml^{-1} h) and significantly decreased V_{darea} (0.31 ± 0.03 L kg^{-1}), V_{dss} (6.45 ± 1.11 L kg^{-1}) and Cl_B (90.36 ± 5.30 L kg^{-1} h^{-1}) values of flucloxacillin (FLX) compared to healthy goats.

Keywords: chronic renal failure, cisplatin, flucloxacillin, goats, intravenous, pharmacokinetic

INTRODUCTION

Flucloxacillin (FLX) is a narrow spectrum beta-lactam antibiotics. It is a semi-synthetic penicillnase resistant penicillin. It is commonly used in human for treating most streptococcal and staphylococcal diseases (Hardman *et al.,* 2007, Rang and Dale, 2007) Like other β-lactam antibiotics, flucloxacillin acts by inhibiting the synthesis of bacterial cell walls. It inhibits cross-linkage between the linear peptidoglycan polymer chains that make up a major component of the cell wall of Gram-positive bacteria.In most Western countries with a relatively low incidence of MRSA, flucloxacillin (or cloxacillin, methicillin and nafcillin) will be the drug of choice, because of their good in-vitro activity, low toxicity, good clinical efficacy and relatively low cost. The pharmacokinetics of antimicrobial drugs in various types of organ function impairment are reviewed. The influence of renal function impairment on antimicrobial agents is well-known, but kinetics of flucloxacillin are scarcely available in animal renal damage in goats particular. Therefore, the present study investigates the alteration of disposition kinetics of FLX, if any, in healthy and chronic renal failure (CRF) goats following single intravenous administration.

MATERIALS AND METHODS

Chemicals

FLX Sodium was used as the test drug. FLX Sodium sterile analytical grade was obtained as gift from M/s Astral pharmaceutical industries Ltd, Vadodara, Gujarat, India. All the chemicals used for the experiments were purchased from E.Merck (India) and Sigma Chemicals Co., (USA), Rankem (India) and SRL Ltd., (India). The purity of the compound was 98.7 percentage. Cisplatin (Kemoplast®) used for induction of chronic renal failure (Mishra *et al.,* 2013).

Experimental animals and design

Six clinically healthy adult Black Bengal female goats weighing between 10-12 kg were utilized in this experiment. The animals were kept in individual custom made stainless steel cage (48''×4''×36'') in a temperature (25±2^{0}C) controlled animal room having provision of artificial light. They were acclimatized with the laboratory condition for 7 days. They were stalled-fed and water was supplied *ad libitum*. The goat feed was supplied by EPIC, Kalyani, West Bengal. Before starting the experiment, the animals were dewormed once with a Fluzan™ (mixture of levamisol hydrochloride I.P. 1.5% w/v and oxyclozanide I.P. vet 3.0% w/v) at the total dose rate of 1 ml/2kg body weight. The lower part of the neck of each animal was shaved and the jugular vein was exposed. The animals were kept overnight fasting prior to the start of experiment. All procedure involved in the study was approved by the Institutional Animal Ethics Committee (IAEC) of West Bengal University of Animal and Fishery Sciences.

FLX sodium dissolved in 2ml of pyrogen free sterile water was administered intravenously into right jugular vein @10 mg kg^{-1}. The blood samples (2 ml each) were collected from the left jugular vein in heparinized test tubes at 0 (pre-drug) and 0.08, 0.16, 0.25, 0.33, 0.5, 1, 2, 4, 6, 8, 12, 18, 24 and 48 h of post drug administration.

Plasma was separated by centrifugation at 3000 rpm for 20 minutes. 1ml of plasma was utilized for estimation of FLX concentration. After a period of rest for one month, cisplatin was administered at 30 mg m^{-2} once daily for consecutive 7 days by jugular veinpuncture to produce kidney damage. Confirmation of CRF was done

by monitoring blood urea nitrogen (BUN), creatinine (CRT), urine gamma glutamyl transaminase (GGT), glomerular filtration rate (GFR) and ultrasound trucut biopsy of kidney (Mishra *et al.,* 2013) After confirmation and persistence of CRF for 90 days, FLX Sodium at 10 mg kg^{-1} was administered intravenously to each goat and blood samples (2 ml each) were collected from left jugular vein in heparinized test tubes at 0 (pre-drug), 0.08, 0.16, 0.25, 0.33, 0.5, 1, 2, 4, 6, 8, 12, 18, 24 and 48h of post drug administration.

The urine samples of individual goats were collected using folly's catheter. The catheter was fixed inside the bladder and allowed to drain its urine content at the time of sampling. The sampling times for collection of urine were 0 (control), 0-12, 12-24, 24-48 and 48-72 h post-drug administration. The urine excretion were measured and cleaned-up thrice by separatory funnel with ethyl acetate and stored at -20 ^{0}C prior to extraction.

FLX was estimated by the method described in British Pharmacopoeia (2008). Plasma samples were subjected to liquid-phase extraction (Wilson and Walker, 2006). To one ml plasma, 3 ml of acetonitrile was added and mixed by vortexing for 20 second and they placed on ice for 15 minutes to enhance protein precipitation. It was centrifuged at 3000 rpm for 15 minutes by cooling centrifuge (R24C). The supernatant was transferred to another centrifuge tube. Dichloromethane (6 ml) was added to the supernatant and the contents were mixed by vortexing for 20 seconds followed by centrifugation at 2000 rpm for 15 minutes. Take the aqueous phase and filtered through 0.2 micron membrane filter and 20 micro liters was injected into the HPLC column.

Recovery

The recovery of plasma FLX was carried out *in vitro* to ascertain the reliability of the method after fortifying with analytical grade FLX at 0.01, 0.05, 0.5, 1.0, 5.0, 10.0, 25.0 and 50.0 µg ml^{-1} and after necessary work up. A 20 µl of sample was injected to the High Performance Liquid Chromatography (HPLC) and the area of peaks against several concentrations of FLX was plotted on graph paper and linearity was found to be maintained. One chromatogram of FLX standard has been presented in Fig.1. The linearity of the calibration curve was checked. The limit of detection (LOD) of FLX was 0.05 µg ml^{-1} and the recovery percentage was 84.75 to 89.50%.

Calculation

A stock solution of 10 µg ml^{-1} of analytical grade of FLX was prepared on sterile water as standard. The FLX concentration in plasma and urine was calculated using the following equation:

$$\text{Concentration of FLX in plasma/urine (µg ml}^{-1}) = \frac{a_2 \times v_2 \times C}{a_1 \times v_1}$$

Where a_1, is the area of standard chromatogram; a_2 the area of sample chromatogram; v_1 the initial volume of sample before processing (ml); v_2 the final volume of sample after processing (ml) and C, is the standard concentration of FLX (10 µg ml^{-1})

Apparatus

The HPLC system includes a Shimadzu LC-20AT solvent system, a SPD-M10AVP photo diode array detector and a 7725i; rheodyne injector with 20µ loop volume. The class *VP*6.01 data system software was utilized for integration. Separation was achieved using a phenomenex C18 column (250mm×4.6mm, 5µ ID, USA). The solvent system consisted of 20 mM potassium dihydrogen orthophosphate (pH adjusted to 4.5 using acetic acid) : acetonitrile (70:30 v/v) was pumped isocratically at wavelength 230nm and flow rate of 1 ml/minute. Pharmacokinetic parameters of FLX were determined from computerized curve fitting programme "PHARMKIT" supplied by the Department of Pharmacology, JIPMER, Pondicherry, India and also from standard formula. Pharmacokinetic parameters were determined for each animal individually and the mean and standard error (SE) were calculated.

Statistical analysis of data

Statistical analysis of data were carried out using standard methods (Snedecor and Cochran, 1968).

RESULTS

Mean plasma concentration of FLX at different time interval after single dose intravenous administration at 10 mg kg^{-1} in healthy goats has been shown in Fig. 2. The peak plasma concentration of FLX was at 0.08 h (77.76±7.50 µg ml^{-1}) which was followed by decline in concentration of drug. The minimum concentration was recorded (0.21±0.05 µg ml^{-1}) at 12 h post dosing (pd). The disposition kinetic parameters of FLX in goats after i.v. administration have been present in Table 1. The mean value of zero time plasma concentration (C_p^o) was found to be 105.97±4.53 µg ml^{-1}. The K_{12}, K_{21} and K_{el} values were 2.75±0.76 h^{-1}, 1.03±0.20 h^{-1} and 1.55±0.30 h^{-1}, respectively. On other hand, $t_{1/2}\alpha$ and $t_{1/2}\beta$ values were 0.13±0.01 h and 2.15±0.05 h (Table 1). The Vd_{area} and Vdc values were 0.5±0.08 and 0.30±0.02 L kg^{-1} while Cl_B values were 266.54±8.67 L $kg^{-1}h^{-1}$. The f_c and T~P values were 0.20±0.03 and 3.874±0.23.

Mean plasma concentration of FLX at different time intervals after single dose i.v. administration at 10 mg kg^{-1} in induced chronic renal failure (CRF) goats have been presented in Fig.3. Maximum mean plasma concentration of FLX was attained at 0.08 h which was found to be 115.51±4.12 µgml^{-1}followed by a decline with a minimum plasma concentration of 0.21±0.05 µg ml^{-1} at 24 h post dosing and could not be detected thereafter. FLX persisted in blood of induced CRF goats for longer period with higher concentration compared to healthy goats.

The kinetic parameters of FLX in induced CRF grade

l goats after i.v. administration are shown in Table 1. Table 1 reveals that mean value of plasma concentration at zero time (C_p^o) was 243.76±5.40 µgml^{-1}. More than two fold increase of plasma concentration at zero time was observed in induced CRF grade I goats compared to healthy goats. The $t_{1/2}\beta$ and Cl_B values were 3.61±0.03 h and 90.36±5.30 L kg^{-1}h^{-1} respectively (Table 1). The higher $t_{1/2}\beta$ along with lower Cl_B values indicated that FLX persisted in the body for long time with lesser excretion in induced

Table 1:
Kinetic parameters of flucloxacillin in healthy and induced chronic renal failure goats after single dose i.v. administration at 10 mg/kg body weight(Mean of 6 replicates with SE).

Kinetic parameters	Flucloxacillin	
	Healthy	Chronic renal failure Grade I
	Group I	Group II
C_p^0 (µg ml^{-1})	105.97 ± 4.53	243.76** ± 5.40
α (hr^{-1})	5.01 ± 0.64	11.43** ± 0.28
β (hr^{-1})	0.32 ± 0.05	0.19* ± 0.02
$t_{1/2}\alpha$ (hr)	0.13 ± 0.01	0.06* ± 0.01
$t_{1/2}\beta$ (hr)	2.15 ± 0.05	3.61* ± 0.03
AUC (µg×hr×ml^{-1})	61.43 ± 3.52	166.14** ± 5.18
Vdc (L kg-1)	0.3 ± 0.02	0.90^m ± 0.05
Vd_{area} (L kg^{-1})	0.5 ± 0.08	0.31* ± 0.03
Cl_B (ml kg^{-1} hr^{-1})	266.54 ± 8.67	90.36** ± 5.30
K_{el} (hr^{-1})	1.55 ± 0.30	1.36* ± 0.10
K_{21} (hr^{-1})	1.03 ± 0.20	1.59 ± 0.08
K_{12} (hr^{-1})	2.75 ± 0.76	8.67** ± 1.29
T~P	3.87± 0.23	6.19* ± 1.58
f_c	0.20 ± 0.03	0.13 ± 0.01

*Values differ significantly at P<0.05; **Values differ significantly at P<0.005

Abbreviation: C_p^0, zero-time blood drug concentration; α and β, distribution and elimination rate constant; $t_{1/2}\alpha$ and $t_{1/2}\beta$, distribution and elimination half lives; *AUC*, total area under the blood drug concentration versus time curve; Vd_c, apparent volume of distribution in central compartment; Vd_{area}, apparent volume of drug distribution based on area method; Cl_B, total body clearance; K_{el}, First order elimination rate constant for disappearance of drug from the central compartment; K_{21}, rate constant for drug transfer from tissue to central compartment; K_{12}, rate constant for drug transfer from central to tissue compartment; *T~P*, tissue/plasma ratio; F_c, fraction of drug in the central compartment.

Table 2:
Mean urine concentration of FLX (µg ml^{-1}) in healthy and induced chronic renal failure grade I goats after mono dose intravenous administration at 10 mg kg^{-1} body weight. (Mean of 6 replicates with SE)

Time (h)	Urine concentration of FLX (µg ml^{-1}) in goats	
	Healthy (group I)	CRF (group II)
0-12	193.51±28.00	301.90**±21.26
12-24	9.69±1.10	80.12**±2.41
24-48	BDL	13.30±0.86
48-72	BDL	1.05±0.27
72-96	BDL	BDL

**P≤0.005; BDL= Below detection limit

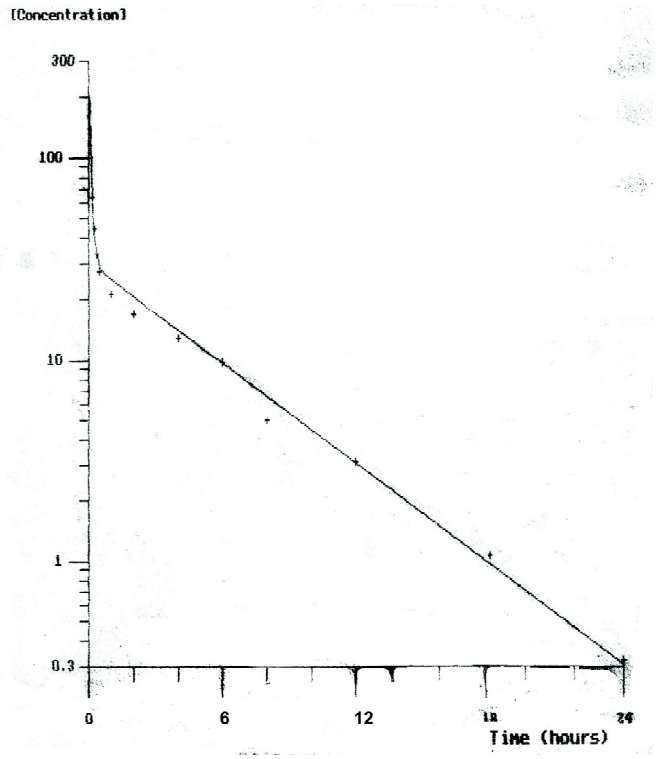

Fig 1:
Semilogarithmic plot of mean concentration of FLX against time with computerized best-fit line after administration of single intravenous dose @10 mg/kg in healthy goats.

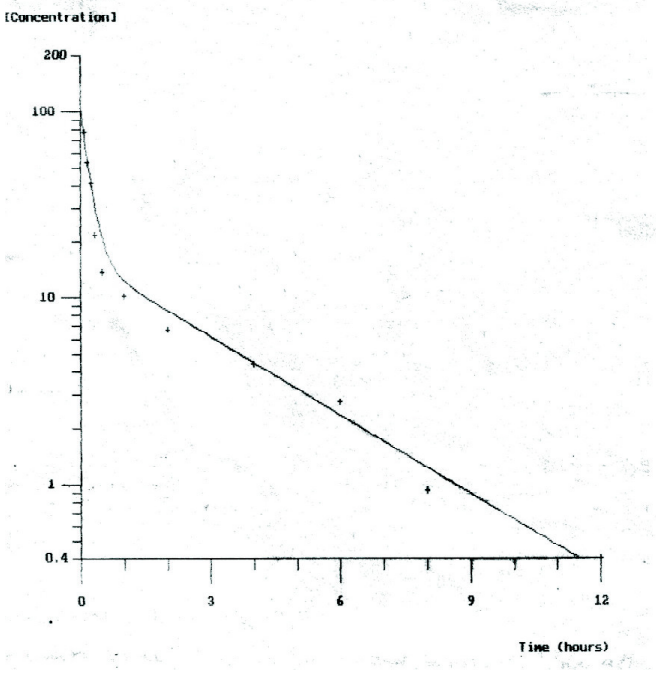

Fig 2:
Semilogarithmic plot of mean concentration of FLX against time with computerized best-fit line after administration of single intravenous dose @10 mg/kg in chronic renal failure (CRF) goats.

CRF goats compared to healthy goats. The K_{el} value was 1.36±0.10 h^{-1} while Vd_{area} and Vdc 0.31±0.0.03 and 0.90±0.05 L kg^{-1} respectively. Cl_B and K_{el} values were significantly lower that of the values of healthy goats.

Mean urine concentration of FLX in healthy and induced CRF grade I goats after mono dose intravenous administration at 10 mg kg^{-1} have been presented in Table II. The maximum urine concentration in urine in group I (healthy) and group II (CRF grade I) observed 193.51±28.00 and 301.90±21.26 µg ml^{-1}, respectively, at 0-12 h, which declined to 9.69±1.10 and 80.12±2.41 µg ml^{-1} respectively in both groups at 12-24 h but the concentrations at different time intervals were altered significantly between healthy (group I) and CRF goats (group II). FLX could not be detected in urine 24 h onward in group I and 48 h in group II.

Computerized semilogarithmic plot of plasma concentration of FLX against time showed "Two Compartment open model" for both healthy and induced CRF goats following intravenous administration (Fig. 1 and Fig. 2).

DISCUSSION

The value of α (distribution rate constant) was higher in CRF goat compared to healthy goat suggesting rapid distribution in tissues which is substantiated with significantly higher K_{12} value in CRF goats. The significantly higher AUC and $t_{1/2}\beta$ values along with significantly lower Cl_B value of FLX indicates longer persistence in CRF goats. Though Vdarea value decreased significantly but the drug had a tendency to accumulate in tissues at higher concentration which is reflected from significantly higher T~P ratio in CRF goats. Cisplatin damaged kidney structure such as Bowman's capsule, proximal convoluted and distal convoluted tubules and in compliance FLX excreted slowly from the body. Kinetic behavior of beta-lactam antibiotic like cephalosporin derivatives were altered significantly in acute renal goats as their major excretory pathway were directed through kidney particularly by glomerular filtration and active tubular secretion (Dutta *et al.*, 2003; Shakthidevan *et al.*, 2005;). Experimentally produced uremia coupled with elevation of creatinine levels induces progressive metabolic alkalosis and slow metabolism of drug (Redenberg, 1971; Radostitis *et al.*, 1994). Therefore, the low body clearance and prolonged blood disposition might be the sequel of slow excretion of FLX through kidney tubules and diminished metabolism of drug in kidney damage goats. Besides, it is expected that acidic drug FLX (pH 5-7) will remain in ionized form that can not pass the biological membranes. Thus, it can be concluded from the above findings that the kinetic behavior of FLX was altered between healthy and grade I CRF goats and the dosage schedule can be used in field of FLX may be reduced in nephropathic goats.

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Research Article

DETERMINATION OF MAXIMUM TOLERATED DOSE OF THIAMETHOXAM AND SUBACUTE TOXIC EFFECT OF THIAMETHOXAM ON FEMALE WISTAR RATS AND AMELIORATION WITH QUERCETIN

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ABSTRACT

The maximum tolerated dose (MTD) of thiamehoxam (TMX) determined through oral route in adult female Wistar rat was 3700 mg /kg body weight. The sub acute toxic effect of thiamethoxam (Actara®) on body weight gain and relative organ weight was determined at two dose levels i.e. 7.5% of MTD (277.5 mg/kg b wt) and 15% of MTD (555 mg/kg b wt). The ameliorative potential of quercetin (Qu 100 mg/kg, orally) was studied against toxic effects produced at both dose levels of TMX. In this study, 144 adult female Wistar rats were used divided in 6 groups, each group comprising of 24 rats. 12 rats from each group were killed on 15th and 29th days of experiment. In 14 days schedule, relative weight gain was significantly ($p<0.05$) lower in animals treated with TMX at 277 mg/kg b wt and 555 mg/kg b wt level as compared to control and quercetin treated groups. Increase in body weight gain was significantly ($p<0.05$) lower in animals treated with lower dose of TMX+Qu and animals treated with higher dose of TMX+Qu as compared to quercetin treated groups. Quercetin treated group showed significant increase in weight gain than control animals. In 28 days schedule, relative weight gain was significantly ($p<0.05$) lower in TMX-treated groups at both doses as compared to control and quercetin treated groups. Significant reduction in weight gain was also observed in lower dose of TMX+Qu and higher dose of TMX+Qu treated groups in comparison to control and quercetin-treated groups. There was significant increase in body weight gain in animals treated with lower dose of TMX+Qu and animals treated with higher dose of TMX+Qu as compared to animals treated with lower dose and higher dose of TMX alone, respectively. In 14 days treatments schedule, significant ($p<0.05$) increase in relative liver weight was observed at both doses in TMX-treated groups and TMX+Qu treated groups as compared to control and quercetin-treated groups. The relative weight of various organs, viz. uterus, ovary, adrenal gland, heart, and spleen was not changed among various treatment groups of both the treatment schedules.

Key words: Thiamethoxam, MTD, body weight, organ weight.

INTRODUCTION

Pesticides are poisonous chemicals that can injure or kill non-target plants and animals or humans and are widely used in agricultural ecosystems (Ecobichon, 2001). Thiamethoxam (Actara® 25WG) has been used for the protection of crops and vegeTables against bed bugs, the Colorado potato beetle, trips, aphides, flea beetles, whiteflies etc. (Kulkarni and Patil, 2012; Alina *et al.*, 2010).

Quercetin, a flavonoid found in fruits and vegeTables, has diverse pharmacological actions viz.anti-inflammatory, antiallergic, antitumour and antioxidant properties. Biological properties quercetin improve mental/ physical performance and reduce infection risk (Tutelian and Lashneva, 2013).The monitoring of body weight provides information on general health level of animals which can also be an important interpretation for reproductive effects. Body weight and relative organ weights are important criteria for evaluation of organ toxicity (Crissman *et al.*, 2004 and Heikal *et al.*, 2011).Present study was aimed to determine the maximum tolerated dose (MTD) of thiamethoxam in female rats and to study the sub acute toxic effect of TMX and TMX along with quercetin on body weight and organ weight.

MATERIALS AND METHODS

184 female Wistar rat weighing 120-140 g were procured from Disease Free Small Animal House, LUVAS, Hisar. Prior approval of Institutional Animal Ethical Committee was obtained for the use of animals in this study. These were housed in the Departmental Animal House in hygienic polyacrylic cages. The animals were provided with feed and water *ad libitum* and were maintained at 22 to 27°C with a natural lightdark cycle. The animals were acclimatized to laboratory conditions for 2-3 days before the experiment. Thiamethoxam (Actara® 25WG), purchased from local market, was used in the present study. Aqueous suspension of thiamethoxam in 2%gum acacia was prepared and administered orally at 7.5% of MTD (277.5 mg/kg b.wt.) and 15% of MTD (555 mg/kg b.wt.) in female rats. Quercetin at a dose rate of 100mg/ kg body weight was given orally as a co-treatment with both doses of TMX in female Wistar rats.

Determination of MTD

MTD was determined in female Wistar rats following oral route for 14 and 28 days. For this, the pilot dose range finding study were conducted in small groups of rats (n=3) using several doses including few lethal doses. Each group of rats was administered single oral dose. Out of these doses, a maximum dose was selected which could produce clear sign of toxicity but not result in lethality i.e. MTD.Subacute toxicity of thiamethoxam and ameliorative effect of quercetin was studied in 144 adult

female rats weighing between 120-140 g. Rats were divided in 6 groups, each comprising of 24 rats.12 rats from each group were killed on 15th and 29th days of treatment to collect the blood and different vital organs for further toxicological studies.

Relative body weight gain

Body weight of female rats was recorded on day 0 and at an interval of two days till the completion of experiment and relative weight gain (gram/100g b wt) and was calculated as:

$$\frac{\text{Final body weight (g) - Initial body weight (g)}}{\text{Initial body weight (g)}} \times 100$$

Determination of relative organ weight

After killing of animals under ether anaesthesia, vital organs viz. liver, heart, kidney, spleen, ovary, uterus, and adrenal gland were excised free from surrounding tissues and were weighed individually.Later on relative organ weights (per 100 g body weight) were calculated.

$$\text{Relative organ weight (g/100g)} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100$$

Statistical analysis

The results were expressed as mean ± standard error of means (S.E.M.) followed by one way ANOVA along with Bonferroni multiple comparison tests using Graph Pad PrismVersion-4.0 software and Microsoft Excel.P<0.05 was the critical criterion for the statistically significant differences between the data.

RESULTS AND DISCUSSION

The dose range values and corresponding mortality data in determining the Maximum Tolerated Dose (MTD) of thiamethoxam in female rats by conducting pilot dose range finding study are as presented in Table 1. The MTD of thiamethoxam (Actara®25WG) in female Wistar rats was determined to be 3700 mg/kg by oral route.Dyspnoea and prostration was observed just before death. Sole (2008) reported that maximum tolerated dose (MTD) of thiamethoxam formulation (Actara®) through oral route was 1600 and 4200 mg/kg in male mice and male rats, respectively. He reported that thiamethoxam produced ataxia followed by straub tail, tremors, convulsions on touch and sound, increased respiratory rate, and change in body posture with tilted head and decreased grip strength and spontaneous motor activity in mice.

Effect of sub acute oral exposure of thiamethoxam, quercetin and their combination on relative body weight gain (g/100g b.wt.) of female rats in different treatment groups of both schedules are presented in Table 2.

The body weight gain serves as an index of growth rate (Palani *et al.,* 1999). In 14 days schedule, relative weight gain was significantly (p<0.05) lower in animals treated with TMX at 277 mg/kg b.wt. and 555 mg/kg b.wt. level as compared to control and quercetin treated groups. Increase in body weight gain was significantly lower in animals treated with lower dose of TMX+Qu and animals treated with higher dose of TMX+Qu as compared to quercetin treated groups. Quercetin treated group showed significant increase in weight gain than control animals.

In 28 days schedule, relative weight gain was significantly (p<0.05) lower in TMX-treated groups at both doses as compared to control and quercetin treated groups. Significant reduction in weight gain was also observed in lower dose of TMX+Qu and higher dose of TMX+Qu treated groups in comparison to control and quercetin-treated groups. There was significant increase in body weight gain in animals treated with lower dose of TMX+Qu and animals treated with higher dose of TMX+Qu as compared to animals treated with lower dose and higher dose of TMX alone, respectively. Significant dose dependent decreasing trend in body weight gain was observed in thiamethoxam treated rats in sub acute toxicity study at both the dose levels. This may be due to decreased feed intake as result of toxic effect of thiamethoxam on motor co-ordination due to which access of animals to the feed must have abandoned or it may have affected feeding centre in brain which probably reduced appetite of the animal.

Effect of sub acute oral exposure of thiamethoxam, quercetin and their combination on relative weight of liver, heart, spleen, uterus, and kidney of animals of different treatment groups of both treatment schedules are expressed in organ wt. in g/100g b. wt., as presented in Table 3 and relative weight of ovary, and adrenal gland are presented in Table 4.

Liver is a target organ and primary site of detoxification and is generally the major site of intense metabolism and is therefore prone to various disorders as a consequence of exposure to the toxins of extrinsic as well as intrinsic forms. Liver plays important role in metabolism to maintain energy level and structural stability of body (Guyton and Hall, 2002).

Table 1:
Maximum tolerated dose (MTD) of thiamethoxam administered orally in female wistar rats.

Dose (mg/kg)	Number of rats died/ number of rats administered	Percent mortality
5500	3/3	100
5300	3/3	100
4700	2/3	66.6
4500	2/3	66.6
4200	2/3	66.6
3800	1/3	33.3
3700	0/3	0
3600	0/3	0
3500	0/3	0

Table 2:
Effect of sub acute oral exposure of thiamethoxam, quercetin and their combination on relative body weight gain (g/100g b.wt.) of female rats

	Days	Control	TMX (277.5 mg/kg)	TMX (555 mg/kg)	Quercetin (100mg/kg)	TMX (277.5 mg/kg) +Qu(100mg/kg)	TMX(555 mg/kg) +Qu(100mg/kg)
Relative weight gain(g/100g b. wt.)	14	20.07±0.70	17.18^{a}±0.42	15.42^{a}±0.71	26.76^{abc}±2.36	19.12^{cd}±1.64	17.68^{d}±1.68
	28	38.10±1.28	25.82^{a}±0.74	20.58^{ab}±0.91	43.29^{abc}±2.42	28.65^{abcd}±3.80	24.57^{acde}±3.38

Values are expressed as mean ± SE of 12 animals in each group.
Supercripts a, b, c, d, e (p <0.05) vs Control, TMX (277.5 mg/kg), TMX(555 mg/kg), Qu, TMX (277.5 mg/kg) +Qu, represent signficant difference (p<0.05).

Table 3:
Effect of sub acute oral exposure of thiamethoxam, quercetin and their combination on relative organ weight (g/100g b. wt.) of female rats.

Relative organ weight	Days	Control	TMX (277.5 mg/kg)	TMX (555 mg/kg)	Quercetin (100mg/kg)	TMX (277.5 mg/kg) +Qu(100mg/kg)	TMX(555 mg/kg) +Qu (100mg/kg)
Liver(g/100g b. wt.)	14	3.39±0.073	4.08^{a}±0.083	4.96^{ab}±0.081	3.41^{bc}±0.064	3.88^{acd}±0.086	4.87^{abde}±0.071
	28	3.43±0.054	4.92^{a}±0.022	5.86^{ab}±0.052	3.40^{bc}±0.083	3.56^{bc}±0.073	4.97^{acde}±0.075
Heart(g/100g b. wt.)	14	0.33±0.006	0.34±0.006	0.35±0.009	0.34±0.006	0.33±0.005	0.34±0.006
	28	0.34±0.007	0.35±0.007	0.36±0.005	0.34±0.001	0.34±0.001	0.35±0.004
Spleen(g/100g b.wt.)	14	0.23±0.004	0.24±0.004	0.25±0.010	0.24±0.005	0.24±0.007	0.24±0.009
	28	0.25±0.008	0.26±0.011	0.27±0.008	0.25±0.006	0.26±0.012	0.27±0.006
Uterus(g/100g b.wt.)	14	0.19±0.021	0.20±0.009	0.21±0.015	0.18±0.007	0.19±0.014	0.20±0.009
	28	0.20±0.017	0.21±0.014	0.22±0.010	0.19±0.009	0.20±0.009	0.20±0.007
Right kidney (g/100g b. wt.)	14	0.29±0.006	0.32^{a}±0.014	0.33^{a}±0.009	0.29^{bc}±0.007	0.30^{c}±0.010	0.31±0.007
	28	0.30±0.015	0.33^{a}±0.004	0.34^{a}±0.009	0.29^{bc}±0.002	0.32±0.010	0.33^{ad}±0.010
Left kidney (g/100g b. wt.)	14	0.31±0.006	0.33±0.008	0.34^{a}±0.006	0.29^{bc}±0.011	0.31±0.012	0.32±0.010
	28	0.31±0.007	0.32±0.007	0.34^{a}±0.009	0.29^{bc}±0.007	0.30±0.008	0.33^{cd}±0.007

Values are expressed as mean ± SE of 12 animals in each group.
Supercripts a, b, c, d, e (p <0.05) vs Control, TMX (277.5 mg/kg), TMX(555 mg/kg), Qu, TMX (277.5 mg/kg) +Qu, represent signficant difference (p<0.05).

Table 4:
Effect of sub acute oral exposure of thiamethoxam, quercetin and their combination on relative organ weight (g/100g b. wt.) of female rats.

Relative organ weight	Days	Control	TMX (277.5 mg/kg)	TMX (555 mg/kg)	Quercetin (100mg/kg)	TMX (277.5 mg/kg) +Qu(100mg/kg)	TMX(555 mg/kg) +Qu(100mg/kg)
Left ovary(g/100 g b.wt.)	14	0.035±0.0020	0.033±0.0017	0.031±0.0018	0.036±0.0021	0.034±0.0011	0.032±0.0021
	28	0.036±0.0006	0.034±0.0015	0.033±0.0017	0.036±0.0016	0.035±0.0018	0.035±0.0012
Right ovary(g/100g b. wt.)	14	0.034±0.0034	0.032±0.0028	0.031±0.0036	0.034±0.0015	0.033±0.0019	0.032±0.0015
	28	0.036±0.0161	0.034±0.0014	0.032±0.0020	0.035±0.0014	0.035±0.0017	0.035±0.0026
Left adrenal gland(g/100gb.wt)	14	0.015±0.0008	0.014±0.0006	0.013±0.0008	0.015±0.0005	0.015±0.0012	0.014±0.0017
	28	0.016±0.0016	0.015±0.0007	0.014±0.0009	0.016±0.0005	0.015±0.0007	0.015±0.0006
Right adrenal gland(g/100gb.wt))	14	0.015±0.0004	0.014±0.0003	0.014±0.0005	0.016±0.0016	0.015±0.0010	0.015±0.0009
	28	0.017±0.0005	0.015±0.0006	0.014±0.0005	0.017±0.0013	0.015±0.0031	0.016±0.0010

Values are expressed as mean ± SE of 12 animals in each group.

In 14 days treatments schedule, significant (p<0.05) increase in relative liver weight was observed at both doses in TMX-treated groups and TMX+Qu treated groups as compared to control and quercetin-treated groups, In 28 days schedule, relative liver weight was increased significantly (p<0.05) in TMX-treated groups at both doses, as compared to control and quercetin group. Relative liver weight was significantly decreased in animals treated with lower dose of TMX+Qu and in animals treated with higher dose of TMX+Qu in comparison to animals treated with lower dose and higher dose of TMX but the values were significantly higher in higher dose of TMX+Qu-treated group than the control and quercetin- treated groups

Kidney is the main organ of TMX excretion. Increased relative weight of both kidneys in TMX (277.5 and 555mg/kg)-treated rats during exposure days indicated the renal toxicity, which might be due to high concentration of this pesticide during excretion in this organ. These findings are in agreement with earlier reports of other pesticides like imidacloprid which significantly increased the relative weight of kidney at 38 mg/kg/day after 20 and 30 days exposure (Lohiya, 2013). Quercetin could not reverse increase in relative weight of kidney due to thiamethoxam treatment.

The relative wt. of various organs viz. uterus, ovary, adrenal gland, heart, and spleen was not changed among various treatment groups of both the treatment schedules.

Thiamethoxam produced toxic effect on body weight gain at both doses in 14 days and 28 days treatment schedules. Quercetin co-treatment resulted in significant

increase in body weight gain as compared to respective TMX treated groups in 28 days treatment schedule. Thiamethoxam produced significant toxic effects in organ of metabolism (liver) and (kidney) and quercetin could not protect these organ from toxic effects of thiamethoxam.

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Research Article

BIOCHANIN A IMPROVES LIPID PROFILE AND OXIDATIVE STRESS IN ISOPRENALINE-INDUCED MYOCARDIAL INJURY IN RATS

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ABSTRACT

The present study was undertaken to evaluate the effect of biochanin-A (BCA) in isoprenaline (ISP)-induced myocardial injury in rats. Evaluation of three doses (3, 10 and 30 mg/kg body weight) of BCA was done in ISP-treated rats. ISP was administered @ 85 mg/kg body weight subcutaneously for two subsequent days to induce myocardial injury in rats. Assessment of myocardial injury was done by estimation of different cardiac injury markers like serum LDH and CK-MB. Serum cholesterol, LDL, triglyceride and oxidative stress were also assessed. ISP administration significantly induced the myocardial injury in rats. Rats were pretreated with BCA showed significant (p<0.05-0.001) improvement in serum LDH, CK-MB, cholesterol, LDL, and triglyceride levels in comparison to isoprenaline-alone group. Pre-treatment with BCA showed significant reduction in MDA level in comparison to myocardial injured rats. Further, antioxidant enzymes level was also improved in terms of improved activity of superoxide dismutase and glutathione reductase with BCA pre-treatment. The study suggests that BCA pretreatment showed beneficial effect in ISP-induced myocardial injury in rats.

Keywords: Myocardial injury, isoprenaline, biochanin A, antioxidant, lipid.

INTRODUCTION

Cardiovascular diseases are the most important cause of death throughout the world including developed as well as in developing countries (Reeve *et al.* 2005). World Health Organization (WHO) reported that 17.5 million people died from CVDs in 2012 which was 31% of all global deaths. Biochanin A (BCA) is a flavonoid found in many edible plants such as soy, alfalfa sprouts, peanuts, red clover, broccoli, cabbage. Various epidemiological studies suggest that ingestion of foods containing flavonoids has a positive relation in reduction of cardiovascular diseases (Calderón-Montaño *et al.* 2011). BCA exhibits various pharmacological activities such as antihyperglycemic (Azizi *et al.* 2014), anti-inflammatory and antioxidant (Breikaa *et al.* 2013; Wang *et al.* 2015). One more *in vitro* study showed that BCA has free radical scavenging capacity in ischemia reperfusion injury (Bhandary *et al.* 2012). Recent study showed the therapeutic efficacy of biochanin A against arsenic-induced renal and cardiac damage in rats (Jalaludeen *et al.* 2015). However, there is no report in respect of preventive cardioprotective effect of biochanin A. Therefore, the present study was designed with the objective to evaluate the preventive effects of biochanin A against isoprenaline-induced myocardial injury in rats.

MATERIALS AND METHODS

Experimental animals and design

Adult male Wistar rats were obtained from Laboratory Animal Section, Indian Veterinary Research Institute, Izatnagar and kept in Divisional animal shed for acclimatization for a week in proper managemental condition and provided with *ad libitum* feed and water. The rats were divided into nine groups of six animals each. Isoprenaline (ISP) was administered subcutaneously to induce myocardial injury at a dose rate of 85 mg/kg body weight (bw) twice at an interval of 24 hrs in group-II animals. Biochanin A was administered to the rats of group-IV, V and VI for a period of 7 days at different doses 3, 10 and 30 mg/kg body weight, respectively, before administering isoprenaline on 6th and 7th day. Group-I animals served as control. Group III was administered BCA alone (30 mg/kg). On 8th day of the experiment, rats from different groups were killed under suiTable anaesthesia. Heart and blood were collected for the estimation of different parameters. All the experimental protocols were done under the guidance laid down by the Institutional Animal Ethics Committee (IAEC).

Biochemical parameters

Rats were sacrificed on 8th day under urethane (1.2 g/kg BW i.p) anesthesia and blood sample was collected. Serum was separated and preserved at -20°C for estimation of different biochemical parameters. The activity of serum lactate dehydrogenase (LDH), creatine kinase-myocardial band (CK-MB), total cholesterol, low density lipoprotein (LDL) and triglycerides were measured by using commercial kits from Span cogent and Coral Diagnostics, India.

Oxidative stress assessment

Oxidative stress parameters were done in the heart tissue and heart tissues were homogenized in ice cold PBS (pH 7.4) and centrifuged at 10,000 rpm for 15

min. Tissues were snap frozen in liquid nitrogen before making the homogenate for further investigation. In the homogenatesof lipid peroxidation was estimated superoxide dismutase (Madesh and Balasubramanium, 1998) and glutathione reductase activity was determined by assay kit.

Isoprenaline was purchased from Sigma Aldrich, USA. All other chemicals were of analytical grade and were purchased from SRL, India.

Statistical analysis

The mean values (± SEM) from different groups were analyzed by one way ANOVA followed by Newman-Keuls Post-hoc Test using Graph Pad Prism Version 4.0. software.

RESULTS

Figure 1 illustrates that there was a significant (p<0.001) increase (1.94 fold) in serum LDH activity in ISP-administered rats in comparison with control rats. However, there was a significant (p<0.001) improvement in serum LDH activity in biochanin-A pretreated (3, 10 and 30 mg/kg) rats in comparison with ISP-alone administered rats. However, BCA (30 mg/kg) did not show any effect on normal rats. There was significant (p<0.01) increase (1.47 fold) in serum CK-MB activity in ISP-administered animals in comparison with control rats. No significant improvement was observed at 3 and 10 mg/kg doses of BCA. However, a significant (p<0.01) decrease in serum CK-MB activity at 30 mg/kg of biochanin-A pretreated rats was observed in comparison with ISP-administered rats without pretreatment (Figure 2). Figure 3 depicts total cholesterol level was significantly (p<0.001) increased (74.71±3.39 mg/dl) in ISP-treated animals in comparison with normal rats (45.12±3.35 mg/dl). BCA (3, 10 and 30 mg/kg) pretreatment for seven days showed significant (p<0.05-0.001) improvement in total cholesterol level in comparison with ISP alone group without pretreatment. However, BCA alone did not show any effect on normal control rats.

Figure 4 depicts that there was a significant (p<0.001) increase (43.19±2.2 mg/dl) in serum LDL level in ISP-administered rats in comparison to normal rats (27.75±2.3 mg/dl). A significant (p<0.05-0.001) decrease in serum LDL level was observed at 10 and 30 mg/kg doses of biochanin-A treated animals in comparison with ISP-alone administered rats. BCA (3 mg/kg) had no effect on myocardial injured rats. Figure 5 depicts that there was a significant (p<0.001) increase in serum triglyceride level (112.7±5.1 mg/dl) in ISP-treated animals in comparison with control rats (57.34±4.2 mg/dl). BCA (10 and 30 mg/kg) pretreatment for seven days showed significant (p<0.01) improvement in serum triglyceride level in comparison with ISP alone-administered rats. However, BCA (3 mg/kg) did not show any significant improvement in myocardial injured rats. Figure 6 depicts that there was significant (p<0.05) increase in MDA level (25.32±2.0 nmol/g tissue) in ISP-treated animals in comparison with normal rats (14.02±1.7 nmol/g tissue). There was significant (p<0.05) decrease in MDA level at 30 mg/kg dose of biochanin-A treated rats in comparison with ISP-administered group without pretreatment. However, BCA (3 and 10 mg/kg) did not show any improvement in lipid peroxidation in myocardial injured rats.

A significant (p<0.05) decrease in SOD activity (3.10±0.34 Units) was observed in ISP-administered rats in comparison with control rats (6.33±0.79 Units). Pretreatment with BCA for seven days at 30 mg/kg followed by ISP administration showed significant (p<0.05) improvement in SOD activity in comparison ISP alone-treated rats. However, BCA (3 and 10 mg/kg) did not show any improvement in myocardial injured rats (Figure 7). Figure 8 depicts that there was significant (p<0.001) decrease in GR activity in ISP-administered rats in comparison with control rats. However, there was a significant (p<0.001) increase in GR activity at 10 and 30 mg/kg doses of biochanin A-treated rats in comparison with ISP-alone administered rats. No effect was observed at BCA (3 mg/kg) pretreated rats in comparison with cardiac injured group.

DISCUSSION

Isoprenaline induces cell damage of myocardium and releases LDH and CK-MB from the myocardial cells to the blood which leads to increase in their levels in the serum. Reduction in serum LDH and CK-MB levels with biochanin A (BCA) pre-treatment may be due decrease in myocardial cell injury and reduction in the leakage of the cardiac marker enzymes (Singh *et al.*, 2014).

Present study suggests that BCA pretreatment for seven days in rats leads to reduction in serum cholesterol, low density lipoprotein (LDL) and triglyceride levels. Present findings are in agreement with earlier reports which showed that BCA treatment showed improvement in serum lipid profile in streptozotocin-induced diabetes in rats (Azizi *et al.* 2014). In a recent report BCA therapeutically showed decrease in plasma triglyceride and very low-density lipoprotein cholesterol (VLDL-C) and increase in HDL level in arsenic-induced toxicity in rats (Jalaludeen *et al.* 2015) which is in agreement with the present findings. Further, it has been reported that hypolipidemic effect of BCA probably due to its free radical scavenging property and membrane protective effect. The free radical scavenging nature of BCA may cause its reaction to directly with various reactive oxygen species (ROS), especially the free radicals which initiates dyslipidemia, which would reduce the oxidative stress (Jalaludeen *et al.* 2015). Another plausible mechanism of antihyperlipidemic effect of BCA may be due to enhanced activity of cholesterol hydroxylase enzyme, which is

responsible for conversion of cholesterol into bile acids and leads to elimination of it from the body (Saini and Sharma, 2013).

Oxidative damage is the main mechanism in isoprenaline-induced myocardial injury. This oxidative damage induces necrosis of heart. Auto-oxidation of catecholamine leads to adrenochrome. All these reactions reduce oxygen supply to heart and produces infarct like lesion in the myocardium. Accumulation of lipids in the heart myocardium results in necrosis. Isoprenaline causes peroxidation of endogenous lipids which is responsible for its toxic action. Various previous studies suggest that free radical generation-mediated oxidative damage plays a significant role in the pathogenesis of cardiac injury (Singh *et al.*, 2013, Dhana and Gurusamy, 2014). Enhanced lipid peroxidation and reduced antioxidant enzymes activity were observed in the isoprenaline-induced myocardial injury. BCA showed significant reduction in the MDA level and increase in enzymatic antioxidants like superoxide dismutase and glutathione reductase in the heart tissue. In an *in vitro* study, biochanin-A showed DPPH free radical scavenging activity as well as improved SOD activity in

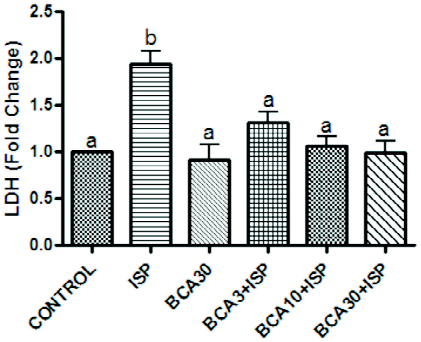
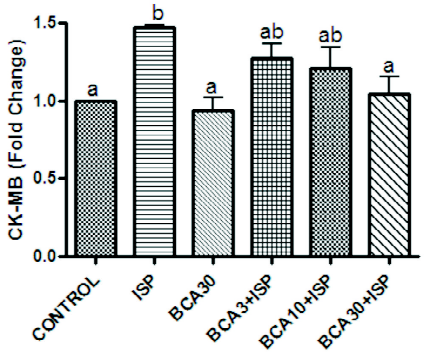


Fig 1.



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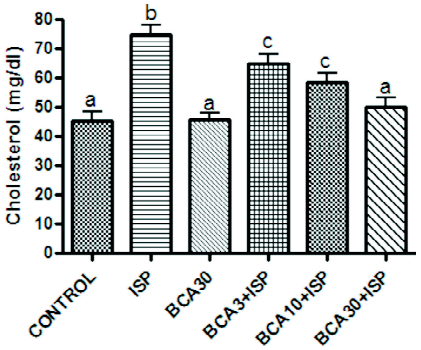

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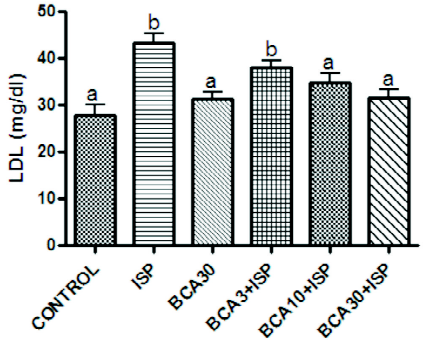

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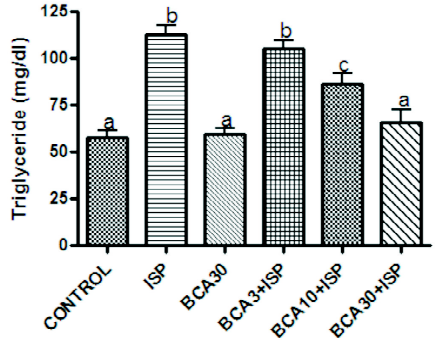

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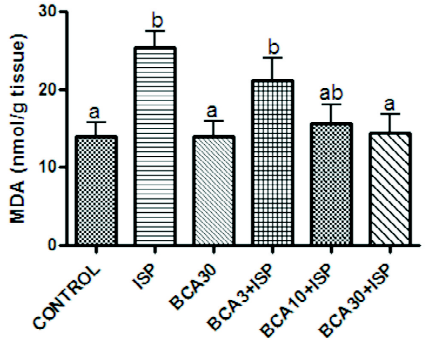

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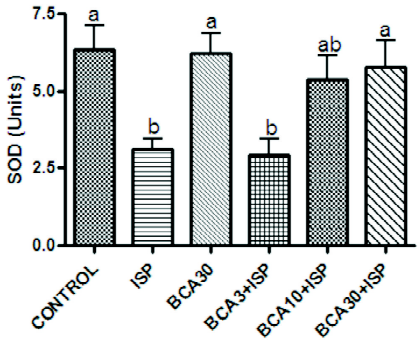

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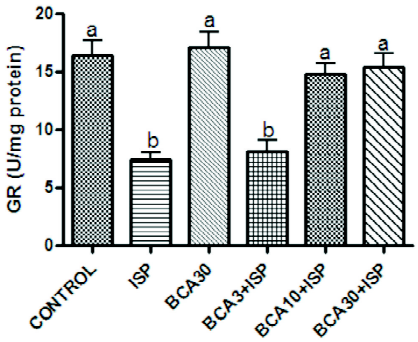

Fig **8.**

Fig 1-8

Shows effect of biochanin A on serum LDH enzyme (1), serum CK-MB enzyme (2), serum cholesterol (3), serum LDL (4), serum triglycerides (5), LPO (6), SOD (7), and tissue glutathione(8) in ISP-induced cardiac injury in rats. Data were analyzed by One way ANOVA followed by Newman-Keuls Post-hoc Test. $^{b}p<0.001$ Vs Control; $^{a}p<0.001$ Vs ISP; n=6.

neonant rat heart myocyte primary cells as well as in H9C2 cells (Bhandary *et al.* 2012). Various *in vivo* studies also in agreement in our findings that BCA showed well defined antioxidant activities in different conditions like arsenic-induced renal, cardiac, hepato and hemato toxicity in rats (Jalaludeen *et al.,* 2015). Apart from this, BCA had shown improvement in lipid peroxidation and antioxidant enzymes capacity in rat brain in a rat model of Parkinson's disease (Wang *et al.,* 2015) and in hypertensive ovariectomized rats (Ovx-HT) (Sachdeva *et al.* 2015). In the present study, BCA may be showing protection in myocardial injury due to its antioxidant capacity. Earlier, it has been shown that various antioxidant enzymes play important role in the protection of tissue damage resulting from free radical formation. Findings of the current study are in complete agreement with various earlier reports in which BCA showed improved antioxidant activity.

In conclusion, results of the present study suggest that isoflavonoid biochanin-A showed improvement in myocardial injury through antihyperlipidemic and antioxidant potential in rats.

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Research Article

AMELIORATING POTENTIAL OF *BACOPA MONNIERI* ON OXIDATIVE STRESS FOLLOWING ACETAMINOPHEN AND ALCOHOL INDUCED TOXICITY IN RATS

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ABSTRACT

The objective of the study was to evaluate the antioxidant efficacy of 50% hydroethanolic extract of *Bacopa monnieri* (HEBM) against alcohol and acetaminophen induced toxicity in rats. The rats were divided into seven groups (n=5). During 28 days study, alcohol @ 1ml of 50% v/v solution, acetaminophen @ 1000 mg/kg p.o., silymarin @ 100 mg/kg body weight and hydroethanolic extract of *Bacopa monnieri* (HEBM) in 2 dose rates (100 and 200 mg/kg body weight) were given as per the experimental design. $HEBM_{100}$ and $HEBM_{200}$ significantly ($p<0.05$) restored the values of LPO, GSH and SOD in tissues in dose dependent manner. Histopathology revealed extensive vacuolization and necrosis in liver of non-treated rats. The lesions were attenuated by treatment with $HEBM_{100}$ and $HEBM_{200}$. It can be concluded that hydroethanolic extract of *Bacopa monnieri* @ 100 and 200 mg/kg po possessed protective efficacy activity against ethanol and acetaminophen induced toxicity and showed better antioxidant efficacy at 200 mg/kg po against ethanol and acetaminophen induced oxidative stress in rats.

Key words: Acetaminophen, Alcohol, Antioxidants, *Bacopa monnieri,* rats.

INTRODUCTION

Acetaminophen, a NSAID is one of the commonly prescribed over the counter drug which is well reported for its hepatotoxic action after chronic use or overdosing. Acetaminophen toxicity is aggravated in alcoholics as ethanol is a potent inducer of CYP2E1 and increases the metabolism of acetaminophen, leading to increased production of its intermediate metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI) causing depletion of glutathione reserves along with increased NAPQI formation which ultimately interferes with protein and nucleic acid functioning leading to oxidative stress (Mitchell *et al.,* 1998).

Bacopa monnieri, Brahmi (Family S*crophulariaceae*) is a profusely branched herb growing in marshy areas, with succulent stem and leaves and small-white flowers. The plant has indistinct odour and a slight bitter taste (Chopra et.al. 1956). *Bacopa monnieri* is reported to be rich in many phytocompounds including alkaloids like brahmine and herpestine, saponins like d-mannitol, hersaponin, acid A and monnierin, flavonoids (luteolin and apigenin) and sterols responsible for its potent antioxidant activity (Hossain et.al. 2012). Various reports reveal its neuroprotective, sedative, tranquillising, anticancer, immunomodulatory, anti-inflammatory and antimicrobial activity. The aim of the study was to evaluate the antioxidant potential of the plant against acetaminophen and alcohol induced toxicity in rats.

MATERIALS AND METHODS

Plant material was identified, collected and authenticated from Medicinal Plant Research and Developmental Centre (MRDC), GBPUAT, Pantnagar, Uttarakhand. Acetaminophen (Paracip, Cipla) and silymarin (Silybon-140, Microlabs) were purchased from a medical store at Pantnagar. Hydroethanolic extract of *Bacopa monnieri* was prepared using 50% ethanol. The cold extracts were prepared by the method described by Singh (2008) with slight modifications. According to this method, powdered material was soaked in 50% hydroethanolic solution (1gm/10ml of 50% hydroethanolic solution) for 24 hours with continuous stirring at 37 ˚C. The mixture was filtered through several layered muslin cloth and centrifuged to separate the supernatant. The final extract was produced after drying the filtrate in fan incubator (JSGW, India) at 35˚C. The percentage yield of extract was calculated. The dried extract was scrapped and kept in air tight bottles in refrigerator at 4˚C.

Qualitative chemical analysis of various extracts of *Bacopa monnieri* was done to detect major phytochemical groups viz., alkaloids, anthraquinones, flavonoids, saponins, tannins, sterols, reducing sugars, glycosides, resins, triterpenes and proteins by standard methods (Harborne, 1973; Sofawara,1982). Thirty five albino Wistar rats weighing between 150-200 gm (1-1.5 months) were procured from Experimental Animal House, Pantnagar for the study. The animals were kept in plastic cages under standard managemental conditions in experimental house of the department. They were acclimatized to animal house conditions for two weeks before the commencement of experiment. The animals were maintained on the standard

rat feed and water *ad libitum*. All the experimental animals were kept under constant observation during entire period of study. All studies were performed in accordance with the guidelines on regulation of scientific experiments as approved by the Institutional Animal Ethics Committee (registration no.IAEC/VPT/CVASc/133)

Experimental design

Thirty five rats were divided randomly and equally into seven groups. During 28 days study,

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

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

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

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

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

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

During 28 day study period, the rats were observed for any toxic signs and symptoms. At the end of study, all the rats were sacrificed humanely, blood and tissue samples were collected to evaluate antioxidant parameters (Sedlak and Lindsay,1968; (Rehman,1984); Prins and Loos,1969; Madesh and Balasubramanian 1998) and histopathological changes.

Calculation of percent protection

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

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

Statistical analysis

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

RESULTS AND DISCUSSION

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

Behavioural changes included drowsiness and dullness in rats of groups III, IV, V, VI and VII after ethanol administration and dull hair coat in groups II, IV, V, VI and VII after acetaminophen administration. Drowsiness and dullness observed in rats of groups III, IV, V, VI and VII after ethanol administration might be due to alcohol intake. Dullness of hair coat after acetaminophen administration in groups II, IV, V, VI and VII might be attributed to its toxicity. Administration of ethanol caused moderate increase in body weight during first 15 days. The appetite stimulant action of ethanol may be considered as the reason behind moderate increase in body weight during first 15 days, which kindled the rats to consume more food. There was a rapid decline in body weight of rats after acetaminophen administration but rats showed progressive increase in body weight after treatment with extract and silymarin in their respective groups. A rapid decline in body weight of rats after acetaminophen administration might be due to hepatic damage which has caused reduction in feed intake. Progressive increase in body weight after treatment with HEBM might be due its hepatoprotective and antioxidant action leading to restoration of normal feeding pattern in rats (Table 1).

Ethanol and acetaminophen toxicity was shown by enhanced lipid peroxidation (measured by increased production of malondialdehyde (MDA)) and reduced levels of GSH and SOD in groups II, III and IV. $HEBM_{200}$ was more effective in reversing these parameters significantly ($p<0.05$) as compared to silymarin. $HEBM_{200}$ significantly

Table 1:

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

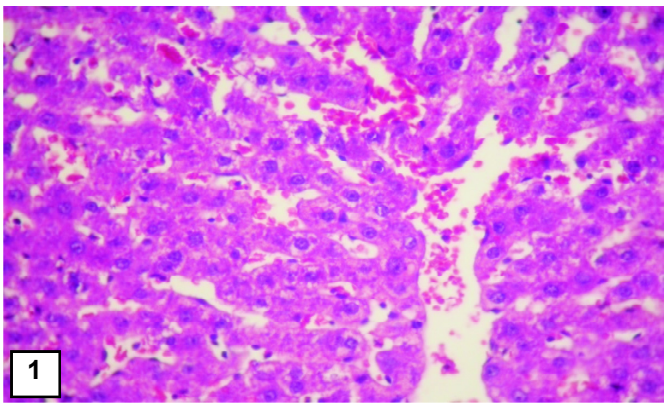
GROUPS	BODY WEIGHT (g)**			
	I WEEK	II WEEK	III WEEK	IV WEEK
I.CONTROL	160.00±4.47	162.00±4.63	164.00±4.58	167.00±4.63
II.APAP *ONLY	152.00±10.67	153.00±10.44	136.00±7.96	138.00±7.84
III.ETHANOL ONLY	168.00±5.83	168.00±5.83	188.00±5.83	204.00±4.00
IV. E*+ APAP	146.00±5.09	150.00±5.24	135.00±4.74	136.00±5.53
V.E+APAP+ $HEBM_{100}$	134.00±5.09	128.00±6.44	117.00±8.88	141.00±5.78
VI. E+APAP+ $HEBM_{200}$	126.00±7.48	132.00±8.74	119.00±8.57	131.00±6.96
VII. E+APAP+S^{*}_{100}	123.00±10.19	145.00±7.41	139.00±6.40	158.00±5.61

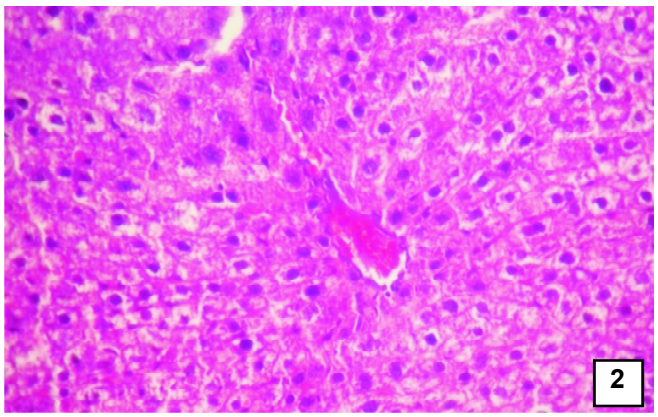
**Changes in body weight were compared a in a row within a group and the weekly changes in body weight were statistically non-significant.

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

Groups	Parameters		
	LPO (nM MDA/ml)	GSH (mM/ ml)	SOD(U/ml prot.)
I.CONTROL	58.36±0.11	1.09±0.02	15.36±1.25
II.APAP *ONLY	107.46±1.33^{a,c}	0.14±0.07^{a}	5.17±0.53^{a}
III.ETHANOL ONLY	76.47±0.64 ^{a}	0.15±0.04 ^{a}	5.29±0.21 ^{a}
IV. E*+ APAP	108.80±1.65^{a,c}	0.16±0.05 ^{a}	5.44±0.30 ^{a}
V.E+APAP+ $HEBM_{100}$	85.82±1.87 ^{a,b,c,d}(45.56%)	0.58±0.21 ^{a,b,c,d}(45.18%)	8.46±0.56 ^{a,b,c,d}(30.40%)
VI. E+APAP+ $HEBM_{200}$	64.86±2.33 ^{a,b,c,d}(87.09%)	0.72±0.03 ^{a,b,c,d}(60.39%)	21.79±0.97 ^{a,b,c,d}(164.76%)
VII. E+APAP+S^{*}_{100}	66.04±0.64 $^{a.b.c.d}$(84.76%)	0.94±0.06 ^{b,c,d}(84.32%)	18.99±0.40 ^{a,b,c,d}(136.60%)

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





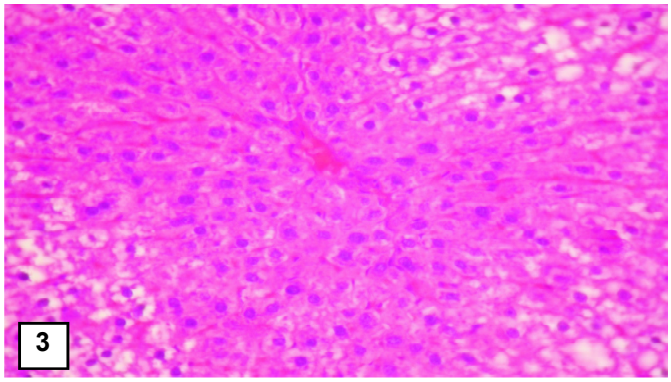


Fig. 1-3:
Rat liver showing distorted central vein, sinusoidal haemorrhages, severe vacuolar degeneration and coagulative necrosis of hepatocytes in group IV (Fig 1); attenuation of centrilobular necrosis after $HEBM_{100}$ administration in group V (Fig 2) and $HEBM_{200}$ in group VI (Fig 3) (HandE,40X).

($p<0.05$) restored the values of lipid peroxidase (LPO), reduced glutathione (GSH) and superoxide dismutase (SOD) in lungs, liver, kidneys and brain which were significantly altered by acetaminophen administration in alcoholic rats. $HEBM_{100}$ also showed protective effect on LPO, GSH and SOD in tissues but LPO levels in kidney was non-significantly lowered as compared to ethanol-acetaminophen intoxicated group (Table 2 and 3). The antioxidant parameters were stabilized after HEBM administration and this can be attributed to the presence of saponins. Saponins are natural products, which have been shown to possess antioxidant property (Singh and Dhawan 1997) and were also found present in phytochemical analysis of the extract. Restoration of tissue

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

Groups	Lungs			Liver			Kidney			Brain		
	LPO (nMMDA/g)	GSH (mM/g)	SOD (U/mg protein)	LPO (nMMDA/g)	GSH (mM/g)	SOD (U/mg protein)	LPO (nMMDA/g)	GSH (mM/g)	SOD (U/mg protein)	LPO (nMMDA/g)	GSH (mM/g)	SOD (U/mg protein)
Control	11.19± 1.74	3.09± 0.15	11.57± 0.33	26.95± 2.6	4.50± 0.31	14.61± 0.12	44.97± 5.54	3.73± 0.2	10.34± 0.06	41.88± 1.58	3.60± 0.12	13.53± 0.49
APAP *only	23.12± 3.19[a]	1.86± 0.45[a]	6.87± 0.14[a]	62.65± 1.31[a,c]	2.65± 0.59[a]	5.44± 0.08[a]	72.36± 1.44[a]	2.20± 0.72	5.25± 0.06[a]	57.18± 2.47[a]	2.08± 0.13[a]	7.33± 0.34[a]
Ethanol only	18.11± 1.01[a]	1.98± 0.33[a]	7.34± 0.13[a]	47.76± 4.27	2.92± 0.03[a]	5.18± 0.06[a,b]	71.14± 3.77[a]	2.72± 0.7	5.75± 0.10[a,c]	55.68± 2.29[a]	2.59± 0.28[a]	8.45± 0.53[a]
APAP + E*	29.95± 1.46[a]	0.21± 0.03[a,b,d]	4.98± 0.22[a,b,d]	63.00± 2.15[a,c]	2.12± 0.64[a,c]	4.65± 0.11[a,b,c]	74.21± 3.96[a]	2.09± 0.29[a]	4.61± 0.17[a,b,c]	63.3± 1.63[a,c]	1.61± 0.32[a,c]	5.33± 0.40[a,b,c]
E+APAP+ HEBM$_{100}$	12.85± 1.88[b,c,d]	2.26± 0.34[d]	10.34± 0.18[a,b,c,d]	39.57± 0.64[a,b,c,d]	3.35± 0.14[a,b,d]	8.43 ±0.04[a,b,c,d]	67.43± 1.17[a]	3.32± 0.04	8.20± 0.04[a,b,c,d]	54.99± 0.46[d]	3.09± 0.04[b,d]	11.59± 0.24[b,c,d]
E+APAP+ HEBM$_{200}$	(91.14%) 12.13±1.57	(71.03%) 3.07±0.53[b,c,d]	(81.39%0 11.23±0.08[b,c,d]	(64.64%) 35.67±1.17[a,b,c,d]	(51.92%) 3.99±0.06[b,d]	(37.99%) 11.32±0.07[a,b,c,d]	(23.20%) 55.17±4.92[b,c,d]	(75.20%) 3.67±0.49[d]	(62.64%) 9.71±0.05[a,b,c,d]	(38.79%) 47.33±3.47[b,c,d]	(74.55%) 3.41±0.04[b,c,d]	(76.24% 13.01± 0.53[b,c,d]
E+APAP+S*$_{100}$	(94.95%) 11.53± 0.19[b,c,d]	(99.17%0 3.19± 0.02[b,c,d]	(94.86%) 11.50± 0.42[b,c,d]	(75.56%) 34.50± 0.93[a,b,c,d]	(78.56%) 3.01± 0.07[a]	(67.02%) 11.71± 0.09[a,b,c,d]	(65.11%) 48.87±0.79 [b,c,d]	(96.53%) 3.69± 0.42[d]	(88.99%) 9.60± 0.07[a,b,c,d]	(74.56%) 42.3± 1.54[b,c,d]	(90.09%) 3.55± 0.29[b,c,d]	(93.60% 13.50± 0.27[b,c,d]
	(98.15%)	(103.35%0	(98.99%)	(78.86%)	(37.37%)	(70.96%)	(86.66%)	(97.75%)	(87.03%)	(98.06%)	(97.40%)	(99.56%)

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

lipid peroxidation and reduced tissue GSH levels on co-administration of HEBM may be attributed to the protective action on lipid peroxidation and enhancing effects of HEBM on cellular antioxidant defense contributing to the protection against oxidative damage in acetaminophen induced oxidative stress. An increase in levels of SOD after HEBM administration to rats suggested that the hydroethanolic extract of *B. monnieri* has an efficient protective mechanism in response to reactive oxygen species (ROS).

HEBM possessed significant quantity of polyphenols like flavonoids and tannins, they might have played a major role in free radical scavenging activity and further these also may contribute to protection against free radicals induced oxidative stress. HEBM has also shown DPPH free radical scavenging activity *in vitro* (Pant *et al.* 2015).

On histopathological examination, liver tissue sections of group IV (ethanol+ acetaminophen) showed extensive vacuolization and necrosis at some places. These microscopic lesions were attenuated in the groups where *Bacopa monnieri* was administered with recuperative changes seen at higher doses.(Figure 1-3).

Therefore, it can be concluded that hydroethanolic extract of *Bacopa monnieri* @ 100 and 200 mg/kg po possessed protective efficacy activity against ethanol and acetaminophen induced toxicity and showed better antioxidant efficacy at 200 mg/kg po against ethanol and acetaminophen induced oxidative stress in rats.

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Research Article

COMPARISON OF DRUGS RESIDUAL CONCENTRATIONS IRRESPECTIVE OF THE SAMPLE TYPES WITHIN DISTRICTS OF KUMAON REGION OF UTTARAKHAND

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ABSTRACT

The present investigation was carried out for comparison of drugs residues of sulfaquinoxaline and oxytetracycline in samples of poultry tissues (muscle, liver, fat and kidney) collected from different districts and respective sub-centers of Kumaon region of Uttarakhand. In the present study, reverse phase HPLC was used to detect and quantify the drugs (sulfaquinoxaline and oxytetracycline) residues in different tissues of poultry birds, eggs, feed and water sample. A wavelength of 254 nm was used for detection of sulfaquinoxaline and 355nm for oxytetracycline as it gave most accurate results. Methanol was used as a mobile phase (methanol: water, 50: 50) for sulfaquinoxaline and acetonitrile, methanol and aqueous oxalic acid solution (10 mM aqueous oxalic acid solution: acetonitrile: methanol, 12.5:87.5:50, v/v/v) for oxytetracycline. A total of 480 meat samples (including muscle, liver, kidney and fat, 120 each) from Kumaon region were analyzed for sulfaquinoxaline residues, out of which 29% had detecTable levels (0.02-0.289µg/g) of sulfaquinoxaline. Mean difference of residual concentrations of sulfaquinoxaline irrespective of the sample type of district US Nagar with the districts Champawat, Bageshwar and Pithoragarh vary significantly at $P<0.05$. However, mean difference concentrations of sulfaquinoxaline were statistically not significant with districts Nainital and Almora. Mean difference of residual concentrations of oxytetracycline irrespective of the sample type of district Udham Singh Nagar with the districts Champawat, Bageshwar and Pithoragarh varied significantly at $P<0.05$. However, mean difference concentrations of oxytetracycline were not significant with districts Almora and Nainital.

Keywords: Oxytetracycline, sulphaquinoxaline, Kumaon region, Uttarakhand, poultry tissues.

INTRODUCTION

About 15% of the therapeutically active substances used in veterinary medicine are administered to birds but there is very limited scientific information available on avian pharmacotherapy. Drugs like sulfonamides, tetracycline, etc., are being used frequently to treat various diseases in poultry. As a result of their widespread use, there is concern about whether the levels used of these drugs can generate serious problems in human health, e.g., allergic or toxic reactions. Sulfonamides are relatively nontoxic when used in normal therapeutic doses (Barragry, 1994). In veterinary practice, sulfonamides were proposed for treatment and in the past, as growth promoters for some decades. Tetracyclines (TCs) are a group of broad-spectrum antibiotics that have been used for more than 50 years for the treatment of bacterial infections in both humans and animals. With the disposal of animal manure as fertilizer into agricultural land, tetracyclines enter the environment. However, tetracycline chelate with multivalent cations and proteins, resulting in low extraction efficiencies from animal manure for tetracycline residue analysis.

MATERIALS AND METHODS

Samples were collected from different places of Kumaon region of Uttarakhand. For sample collection, Kumaon region was divided into six centers (districts) and different samples were collected by multistage cluster sampling technique as described by Das (2000). The centers were selected on the basis of use of drugs in various agricultural operations, public health programmes and animal husbandry practices. From Kumaon region, six centers namely, Udham Singh Nagar (U S Nagar), Nainital, Almora, Champawat, Bageshwar and Pithoragarh were identified. For collection of samples, from each identified center (districts), certain places were selected as sub centers. Twenty local poultry farms were selected randomly from each district's sub-center. A total of 140 samples from each district which comprised of poultry tissues (muscle, liver, kidney and fat) from respective sub centers and analyzed within 3 days of collection by HPLC.

Reverse phase HPLC was used to detect and quantify the sulfaquinoxaline and oxytetracycline residues in different tissues of poultry birds. Methanol was used as a mobile phase (methanol: water, 50: 50) for sulfaquinoxaline and acetonitrile, methanol and aqueous oxalic acid solution (10 mM aqueous oxalic acid solution: acetonitrile: methanol, 12.5:87.5:50, v/v/v) for oxytetracycline.

Extraction of sulfaquinoxaline from tissues

Extraction of sulfaquinoxaline from tissues was carried out by the method as described by Ioerger and

Smith (1993) with modifications. 4 g of frozen tissue was taken and kept for thawing. The thawed tissue was then put in a mortar and triturated well. Double amount of methanol was added to the triturated tissue and subjected to homogenization till it became paste. The paste was subjected to sonication at 10 amplitude microns for 30 sec, with a pause of 5 seconds (a total of 15 cycles) by using ultrasonic tissue disintegrator. The sonicated tissue was centrifuged at 9000 rpm for 15min. The supernatant was collected in petridish and dried at 37^{0}C. Clean up process of sulfaquinoxaline was done by the technique described by Telling and Sissons (1977) with slight modifications carried out using solid- phase extraction C_{18} cartridges. The dried eluate was reconstituted in 2ml of methanol and loaded on to the conditioned C_{18} cartridges (conditioning was done first with 10ml water and then by 10ml methanol) and allowed to pass through vacuum (20mmHg). The cartridges were washed with 10ml of methanol. The eluate obtained after loading of cartridges was filtered through 0.22 µm filter paper. 20 µl of the sample thus obtained was injected into HPLC system for analysis.

Extraction of oxytetracycline from tissues

Oxytetracyline extraction from tissue samples was carried out as per the method of Salehzadeh *et al.* (2006) with slight modifications. Analysis of samples was carried out using 4 g of either kidney, liver or muscle tissues. The samples were allowed to defrost at room temperature. 8 ml of McIlvaine buffer-EDTA solution was added to tube and was blended 30sec with homogenizer. The paste was subjected to sonication at 10 amplitude microns for 30sec, with a pause of 5 seconds (a total of 15 cycles) by using ultrasonic tissue disintegration. The sonicated tissue was centrifuged at 5000 rpm for 15 min. The supernatant was transferred into other tube. 8 ml of McIlvaine buffer-EDTA solution was added to the tube containing supernatant using vortex-mixer, the sedimented tissue resuspended. The suspension was shaked for 10 min, centrifuged 15 min at 5000 g and then again the supernatant was added to first supernatant in the tube. SPE cartridges were conditioned with 10 ml methanol and 10 ml of HPLC grade water. The dried sample was reconstituted in 2 ml of mobile phase and passed through the conditioned cartridge. Oxytetracycline was eluted with 1 ml methanolic oxalic acid solution (0.01M). The eluate was filtered through 0.22 µm filter paper vortexed-mixed for 30sec and 20µl was injected into the HPLC system.

RESULTS AND DISCUSSION

Mean difference of the residual concentrations of sulfaquinoxaline and oxytetracycline in different districts of Kumaon region of Uttarakhand are shown in Tables 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 sulfaquinoxaline irrespective of the sample type of district Udham Singh Nagar with the districts Champawat, Bageshwar and Pithoragarh vary significantly at P<0.05. However, mean difference concentrations of sulfaquinoxaline were statistically not significant with districts Nainital and Almora. Mean difference of residual concentrations of oxytetracycline irrespective of the sample type of district US Nagar with the districts Champawat, Bageshwar and Pithoragarh vary significantly at P<0.05. However, mean difference concentrations of oxytetracycline were not significant with districts Almora and Nainital.

Mean difference of residual concentrations of sulfaquinoxaline irrespective of the sample type of district Nainital with all the other districts of Kumaon region were not significant. Mean difference of residual concentrations of oxytetracycline irrespective of the sample type of district Nainital with all the other districts of Kumaon region varied non-significantly.

In Kumaon region mean residual concentration of sulfaquinoxaline and oxytetracycline irrespective of the

Table 1:
Comparison of mean difference residual concentrations of sulfaquinoxaline and oxytetracycline 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	
		Sulfaquinoxaline	Oxytetracycline
U S Nagar	Nainital	0.0122±0.005	0.012±0.004
	Almora	0.0138±0.005	0.0115±0.004
	Champawat	0.0208±0.005*	0.0177±0.004*
	Bageshwar	0.0249±0.005*	0.0204±0.004*
	Pithoragarh	0.0241±0.005*	0.0216±0.004*
Nainital	US nagar	-0.0122±0.005	-0.012±0.004
	Almora	0.0016±0.005	-0.0005±0.004
	Champawat	0.0086±0.005	0.0056±0.004
	Bageshwar	0.0126±0.005	0.0083±0.004
	Pithoragarh	0.0118±0.005	0.0096±0.004
Almora	US nagar	-0.0139±0.005	-0.0115±0.004
	Nainital	-0.0016±0.005	0.0005±0.004
	Champawat	0.0069±0.005	0.0062±0.004
	Bageshwar	0.0110±0.005	0.0089±0.004
	Pithoragarh	0.0102±0.005	0.0101±0.004
Champawat	US nagar	-0.0208±0.005*	-0.0177±0.004*
	Nainital	-0.0086±0.005	-0.0056±0.004
	Almora	-0.0069±0.005	-0.0062±0.004
	Bageshwar	0.0040±0.005	0.0027±0.004
	Pithoragarh	0.0032±0.005	0.0039±0.004
Bageshwar	US nagar	-0.02497±0.005*	-0.0204±0.004*
	Nainital	-0.0127±0.005	-0.008±0.004
	Almora	-0.0110±0.005	-0.008±0.004
	Champawat	-0.0040±0.005	-0.002±0.004
	Pithoragarh	-0.0008±0.005	0.0012±0.004
Pithoragarh	US nagar	-0.0241±0.005*	-0.0216±0.004*
	Nainital	-0.0118±0.005	-0.0096±0.004
	Almora	-0.0102±0.005	-0.0101±0.004
	Champawat	-0.0032±0.005	-0.0039±0.004
	Bageshwar	0.0008±0.005	-0.0012±0.004

*Mean difference concentrations differ significantly. Values in negative indicates lower residue level in district (J) than district (I).

sample type were detected maximum in district U.S nagar and minimum in district Pithoragarh. Levels of sulfaquinoxaline and oxytetracycline residues were present in poultry tissue samples. These findings are in conformity with the presence of drugs in samples of feed grain of this region. This may be due to contamination with drinking water supplied to poultry for medication. Total 480 meat samples (including muscle, liver, kidney and fat, 120 each) from Kumaon region were analyzed for sulfaquinoxaline residues, out of which 29% has detectible levels (0.02-0.289 µg/g) of sulfaquinoxaline. The results obtained in the present study are in close agreement with Salem (2004) and Shaikh *et al.* (2000) who found sulfonamide residues in chicken meat samples. They also used HPLC for the detection of sulfonamide residues as in this study and found similar results. The present study revealed that the recovery rate of OTC residues in broiler muscles liver and kidney as well as were 68.2%, 61.8 % and 72.6. Such result was in agreement with those recovery rates obtained by Furusawa (2003) and Hussein (2001) in broiler chicken muscles. However, many investigators such as Mulders and Lagemeet (1989), Wu *et al.* (1994), Kawata *et al.* (1996) and Ruycky *et al.* (1999) reported lower recovery rate rather than that of the present results. On the other hand, Fujita *et al.* (1997) and Huang *et al.* (1997) obtained higher recovery rate where they recorded a rate of 90.4 and 92.9 %, respectively.

It can be concluded from the residual study that mean residual concentrations sulfaquinoxaline and oxytetracycline were found more in Udham Singh Nagar and least in Pithoragarh. As we move from plains to high altitude of Kumaon region, less frequent use of drugs has been exercised, this may be one the reasons that in plain areas more concentrations of drugs have been detected than in high altitude areas.

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Research Article

EFFECT OF CURCUMIN ON PERI-OR POSTNATAL EXPOSURE TO LEAD ACETATE INDUCED IMPAIRMENT IN SPONTANEOUS LOCOMOTOR ACTIVITY (SMA) IN SWISS ALBINO MICE

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ABSTRACT

The purpose of the present study was to assess whether curcumin can alter the lead acetate-induced neurotoxicity and oxidative stress in mice. Swiss albino mice whose mothers had been exposed to lead acetate during pregnancy and lactation at the dose rate of 100 ppm in drinking water were continued on the respective regimens for 63 days. The effect of curcumin evaluated in 63-day lead acetate-exposed mice where curcumin was given from 22 days onwards of experiment. The end points included the measurement of change in body weight, weight of vital organ(s), lipid peroxidation (LPO), antioxidant profile and spontaneous motor activity were recorded. Apparently, no signs and symptoms of toxicity were visible. Body weights of mice were significantly different from controls at weekly interval; however, in curcumin treated group body weights were significantly protected. Spleen and liver absolute weights were significantly decreased on day 21 and 63, respectively. On day 21, lead acetate caused increased lipid peroxidation (LPO), decreased reduced glutathione (GSHr) level and the activities of superoxide dismutase (SOD), catalase in liver and brain, however, on day 63 the alteration of LPO and antioxidant more prominent in liver and marginally in brain. Co-administration of *curcumin* decreased lead-mediated toxicity and oxidative stress in liver and brain of mice. Mice were exposed to low level lead acetate resulted into reduction in spontaneous locomotor activity (SMA) on day 21 and 63; however in curcumin and withdrawal group activity remain improved on day 63. The result implied a correlation between SMA and peroxidative damage in brain. Thus, the study revealed that curcumin could be a potential risk modifier for the development of lead acetate-induced toxicity in animals.

Keywords: *Curcumin,* Lead acetate, Lipid peroxidation, Anti-oxidants, mice.

INTRODUCTION

Lead (Pb) is one of the important heavy metal, and due to its widespread use in industry, it has became an important environmental pollutant that exerts toxic effects on human health. Lead poisoning is a potential factor in brain damage, mental impairment and severe behavioral problems, as well as anemia, kidney insufficiency, neuromuscular weakness and coma (Liuji *et al.*, 2002). The main important mechanism at very low concentrations of lead can influence the synthesis of heme by inhibiting *viz:* delta-aminolevulinic acid dehydratase (δ-ALAS) and heme syntheatse (ferrochelatase), which are important sulphydryl containing enzymes, are the most susceptible (Sandhu and Brar, 2009).

Oxidative stress has been suggested as one possible mechanism for lead neurotoxicity. Direct interaction of lead with biological membranes, increase of intracellular levels of calcium, impairing mitochondrial functions; lead induced decrease on free radical scavenging enzymes and glutathione and increase lipid peroxidation; (Sandhir *et al.*, 1994).

Curcumin is a yellow coloured pigment present in the rhizome of *curcuma longa* (turmeric), a perennial herb belonging to *zingiberaceae* family. Curcumin is non-toxic, highly promising natural antioxidant, hepatoprotective, hypocholesteremic, anticarcinogenic and antimutagenic compound having a wide spectrum of biological applications. It is expected that curcumin may become novel agent in the near future to control various diseases, including inflammatory disorders, carcinogenesis and oxidative stress pathogenesis in man and animals (Chattopadhyay *et al.* 2004).

Few studies investigated that alteration neurobehavioral activity and oxidative stress in pups whose dams were exposed to lead acetate during pregnancy and lactation. This study was carried out to investigate the effect on neurobehavioral activity and the effect of developmental lead exposure on the brain antioxidant system.

MATERIALS AND METHODS

Experimental design

The study was conducted in Swiss albino mice. The animals were maintained (28-31ºC temperature, 50-55% relative humidity) in polypropylene cages and provided with feed and water *ad libitum*. Animals were provided with nutritionally adequate standard laboratory diet. Before the start of experiment, animals were kept in laboratory conditions for acclimatization. All the experimental animals were kept under observation during the entire period of study.

Two females and one male per cage were allowed for mating. On day 15 of pregnancy dams were divided into two groups. Group I received 100 ppm lead acetate in

drinking water during rest of the gestation as well as lactation period, whereas group II received water *ad libitum* without lead acetate. At birth, lead acetate exposed pups continued to receive lead acetate during entire lactation period as well as after weaning up to 63 days. On day 21 all pups are weaned, and lead acetate exposed pups were randomly divided into three groups *viz.* (i) lead acetate (ii) lead acetate + curcumin and (iii) withdrawal groups. Control groups weaned pups served as control for further study. The body weights were recorded at weekly interval. The animals in all groups were fasted overnight and scarified on day 21 and 63 by cervical dislocation. Further brain, liver, kidneys, heart and spleen were collected and blotted with tissue paper, then weighed. Brain, liver and kidney were kept at -80°C until biochemical analysis.

A 50 mg of tissue were taken in 0.5 ml of ice-cold PBS [composition: NaCl 8 g; KCl 0.2 g; KH_2PO_4 0.24 g; Na_2HPO_4 1.44 g in 1 L of distilled water; pH 7.4]. Another 20 mg of tissue were taken separately in 0.2 ml of 0.02 M EDTA for estimation of reduced glutathione (GSH). The homogenate (10%) was prepared with a homogenizer under ice-cold condition and centrifuged for 10 min at 3000 rpm. The supernatant was stored at -20 °C until subjected to assay.

Assessment of antioxidant parameters

Peroxidative damage in tissue was assessed by evaluating the lipid peroxidation (LPO) in terms of malondialdehyde (MDA) production as described by Paula *et al.* (2005). Results have been expressed as nanomoles of MDA formed/gram of tissue. Reduced glutathione (GSH) content was measured in the tissue homogenate by the method of Sedlak and Lindsay (1968). Superoxide dismutase (SOD) activity in the tissue was determined by the procedure of Madesh and Balasubramanian, (1998 and the activity was expressed as units/milligram protein. Catalase activity in the tissue homogenate was assayed by the spectrophotometric method of Aebi (1983) and expressed as milli moles of H_2O_2 utilized/minute/mg protein. The protein content in the supernatant of the brain homogenate was determined following the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Measurement of Spontaneous motor activity

The Spontaneous motor activity (SMA) was measured using a photo actophotometer (Inco Instruments, Ambala, India) following the method of Kulkarni (1999). The movement of the animal cuts off a beam of light falling on the photocell and a count was recorded and displayed digitally. Each group mice was placed individually in the actophotometer and the basal activity was measured. To measure locomotor activity, number of squares crossed by the animal every 5 minutes during 30 minutes on day 21 and 63 were recorded.

Data analysis

Data have been expressed as Mean ± S.E. with 'n' equal number of animals. Data of 21 days were analyzed by Student 't' test and all other data were analyzed by one way ANOVA followed by Duncan's multiple comparison post-hoc test using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Difference at $p<0.05$ was considered statistically significant.

RESULTS

On gross observation, no visible signs and symptoms of toxicity were observed in the treated rats. All the treated animals appeared as that of the control animals. Further, there was no mortality.Figure 1(A, a) present the effects of curcumin on body weight in the lead acetate exposed mice. Curcumin did not have any influence on body weight. Lead acetate decreased the body weights of rats from the 7th day onwards (Fig. 1). Effects of curcumin on organ weight in the lead acetate exposed mice shown in Table 1. In 21-day study, lead acetate did not affect the absolute weights of brain, liver, kidneys, heart, but decreased the absolute weight of spleen. The absolute weights (63-day study) of brain, kidney and heart were not altered by any of the treatments. The absolute weight of liver was significantly and comparably decreased in the mice given lead acetate or

Table 1:
Effects of *curcumin* on the organ weight (gm) in the lead acetate-exposed mice

	Organ weight (g) on 21 day				
Groups	Brain	Liver	Kidney	Heart	Spleen
Control	0.32±0.01 ^{a}	0.47±0.04^{a}	0.07±0.01^{a}	0.07±0.01^{a}	0.09±0.01^{a}
Lead acetate	0.32±0.02^{a}	0.36±0.04^{a}	0.07±0.01^{a}	0.06±0.01^{a}	0.06±0.01^{b}
	Organ weight (gm) on 63 day				
	Brain	Liver	Kidney	Heart	Spleen
Control	0.35±0.01^{a}	1.07±0.06^{a}	0.19±0.01^{a}	0.16±0.01 ^{a}	0.11±0.01^{a}
Lead acetate	0.31±0.02^{ab}	0.95±0.04^{b}	0.15±0.01^{ab}	0.13±0.01^{a}	0.09±0.01^{a}
Lead acetate + Curcumin	0.35±0.01^{a}	0.73±0.02^{b}	0.12±0.01^{b}	0.14±0.01^{a}	0.08±0.01^{a}
Withdrawl	0.28±0.02^{b}	0.74±0.02^{b}	0.16±0.01^{a}	0.14±0.01^{a}	0.09±0.01^{a}

Values (mean±SE; n=6) bearing no superscripts common vary significantly ($p<0.05$) in Student 't' test and Duncan's multiple comparison post-hoc test.

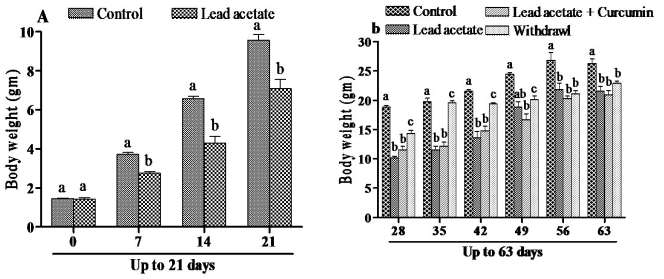

Fig. 1

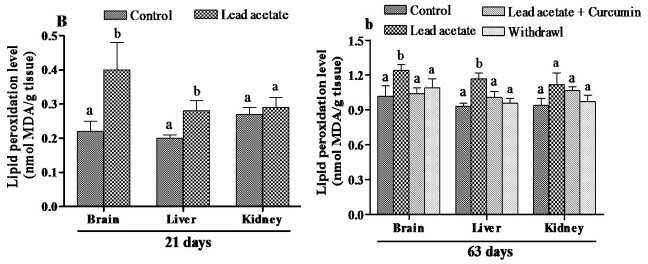

Fig. 2

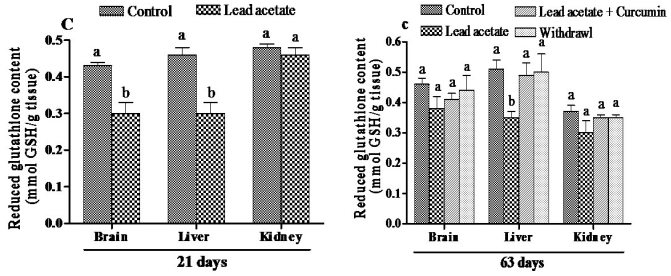

Fig. 3

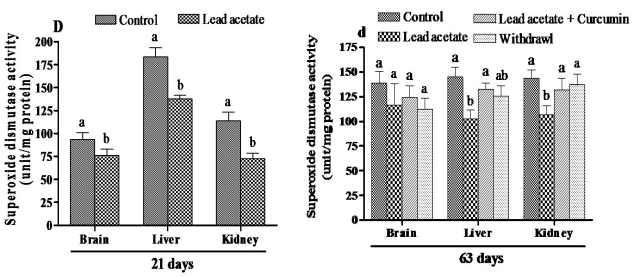

Fig. 4

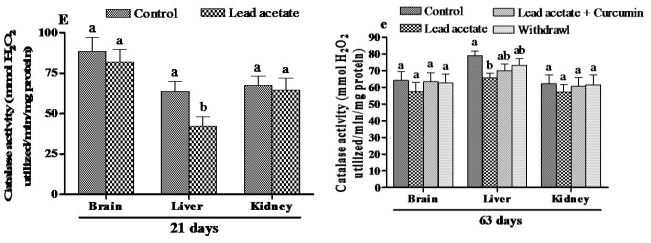

Fig. 5

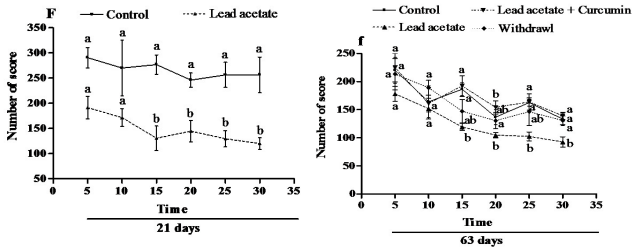

Fig. 6

Fig. 1-6:
Effects of curcumin on the on the body weight (1); lipid peroxidation level (2); reduced glutathione content (3); superoxide dismutase activity (4) catalase activity(5); spontaneous motor activity (6) in the lead-exposed mice. Each bar represents mean ± SE (n=6). Bars bearing different superscripts vary significantly ($p<0.05$) in (A,B,C,D,E & F) Student 't' test and (a,b,c,d,e & f) Duncan's multiple comparison post-hoc test.

lead acetate with curcumin or withdrawal group.

The effects of curcumin on LPO in brain, liver and kidney in the lead acetate exposed mice are presented in Figure 2 (B, b). In 21-day study, LPO measured in terms of MDA production was increased significantly in brain and liver of mice given lead acetate. Similar peroxidative changes were observed in brain and liver in case of 63-day study, where the magnitude of LPO was more than that occurred in 21-day study. However, in curcumin and withdrawal group significantly improved the LPO of the lead acetate-exposed mice.

Effects of curcumin on GSH content in brain, liver and kidney are presented in figure 3 (C, c) in the lead acetate exposed mice. In case of 21-day study, lead acetate caused significant depletion of GSH in brain and liver. In case of 63-day study, also a similar pattern of GSH depletion occurred in liver but not in brain and kidney. However, lead mediated decrease was significantly improved in liver with curcumin and withdrawl group.

Effects of curcumin on SOD activity in brain, liver and kidney in the lead acetate exposed mice are shown in Figure 4 (D, d). In 21-day study, the activity of SOD was significantly decreased with lead acetate in brain, liver and kidney. In 63-day study, the activity of SOD was significantly decreased with lead acetate in liver and kidney. These lead-mediated decreases in SOD activity was significantly enhanced with curcumin and withdrawl group.

Effects of curcumin on the activities of catalase in liver, kidney and brain in the lead acetate exposed mice are deficits in Figure 5 (E, e). In 21-day study, catalase activity was significantly decreased with lead acetate in liver. In 63-day study, lead acetate significantly decreased the catalase activity in liver. However, this activity was significantly improved with curcumin and withdrawal group. In brain and kidney catalase activity was marginally altered.

The locomotor activity results shows that exposure mice to lead acetate cause decreased SMA compared to control Fig. 6 (F, f). Lead acetate induced hypoactivity is protected in curcumin as well as withdrawal group.

DISCUSSION

With regard to physical assessment, absence of overt clinical signs and symptoms of toxicity suggests that the dose and duration of lead acetate treatment were not optimum for inducing symptomatic manifestation. Reduction in body weight and organ weight is the classical indicator of systemic toxicity and deterioration of general health. The body weights of the lead acetate treated mice were significantly decreased compare to the control group, concomitant treatment with curcumin in lead acetate group has not protected significantly. The present study is tune with Maldonado-Cedillo *et al.* (2015). Alteration in organ weight is another index for development of toxicity. In present study, lead acetate decreases the absolute weight of spleen and liver. These effects of lead acetate on liver weight suggest that the decrease in body weight was disproportionately associated with the organ weight. When other vital organs were not affected, our results imply that the liver could be the one of the important target organs of lead acetate toxicity (Haouas *et al.*, 2014). Further, oxidative stress was found to be associated with decrease in the organosomatic index of these two organs along with decrease in body weight in mice given lead acetate through drinking water at 100 ppm for 63 days.

The tissue injury observed following treatment of mice with lead acetate was brought about due to oxidative stress is evident from a marked increase in the level of LPO which could be attributed to the generation of free radicals (Ademuyiwa *et al.,* 2009). The increase in of lipid peroxidation indicates production of oxidative stress, which is an imbalance between the production of free radicals capable of causing peroxidation of lipid layer of cells or loss of membrane integrity or oxidation and the body defense system (Abdel-Wahhab and Aly, 2005).

GSH thiol containing enzyme plays a major role on membrane protection. In the assessment of extent of oxidative stress, the level of GSH is accepted as a good indicator (Asensi *et al.* 1999). However, cell damage can be prevented by detoxification of free radicals, means by preventing the progress of LPO. In the present study, we observed an elevated level of LPO followed by a depleted reduced GSH level in different tissues, which was in agreement with previous observations (Attia *et al.* 2013). These two parameters are considered as the primary bio-markers of oxidative stress. Curcumin, which is powerful antioxidant and free radical scavenger, results in the present study curcumin co-administered with lead acetate are in agreement with Badary *et al.* (1999).

The detoxification of reactive oxygen species (ROS) involves the co-operative action of all intracellular antioxidant enzymes; hence disruption of one enzyme would disrupt the other enzymes. Since, SOD is the first line of antioxidant enzymatic defense catalyzes the conversion of superoxide radicals to less toxic H_2O_2. Then catalase metabolizes H_2O_2 to water (Mates *et al.* 1999). The effects of lead acetate exposure on antioxidant function of brain, liver and kidney were different, which was in agreement with Sandhir *et al.*, (1994). In the present study, the effect of lead acetate exposure induced decrease in activities of SOD, catalase and protective effect of curcumin against lead-induced toxicity in brain, liver and kidney, which was in agreement with previous observations (Shukla *et al.*, 2003).

In the present study, administration of lead acetate during peri and postnatal in mice resulted into decreased spontaneous motor activity on day 21 and 63. Additionally, Rocha *et al.* (2001) reported that behavioral response was affected by metal treatment indicating an alteration in the dopaminergic system. This observations can be explained by lead action on catecholamine and glutamatergic systems either inhibition of dopamine synthesis and release in synapse or by inhibition of dopamine receptors (Cory-Slechta *et al.,* 1997). In another study reported that, catecholaminergic and serotoninergic transmission in brain may be altered by heavy metals (Antonio and Leret, 2000). Cadmium and lead are also able to affect cholinergic transmission (Schneider *et al.,* 2001). In the present study, repeated administration of low concentration of lead acetate in mice results in hypoactivity.This may be due to reducd synaptic transmission or neurological disturbances due to increased concentration of lead. Antonio *et al.,* (1998) reported that exposure of lead causes decrease in dopamine synthesis in nigrostriatal and mesolimbic pathways and imbalance in dopamine/glutamate rapport in limbic system. To conclude, lead acetate exposure during pre-and postnatal period induced oxidative stress has lead to hypoactivity in offspring's when measured at weaning (day 21) or adult life (day 63). Further, the oxidative stress and the reduced spontaneous motor activity in mice were effectively ameliorated by curcumin during adult life

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Research Article

AMELIORATIVE EFFICACY OF *HEDYCHIUM SPICATUM* ROOT POWDER IN INDOXACARB INTOXICATED WLH COCKERELS

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ABSTRACT

The present study was carried out to know the ameliorative property of *Hedychium spicatum* in forty nine six weeks age WLH cockerels. The bird was randomly divided into seven groups. Group I was considered as control and fed grower ration, in group II were given indoxacarb @ 250ppm, III (silymarin@250ppm), IV (indoxacarb@250ppm+silymarin@250ppm), V (HSRP@4000ppm), VI (indoxacarb@250ppm+HSRP@2000ppm) and VII (indoxacarb@250ppm+HSRP@4000ppm) for 16 weeks. The blood was collected from the wing vein for haematological study. There was significant ($P<0.05$) reduction in TEC, TLC, Hb, PCV, ALC, AHC and lymphocyte in indoxacarb treated groups whereas significant ($P<0.05$) elevation of DLC (%) like heterophil(%), eosinophil(%), basophil(%) and monocyte (%) in indoxacarb treated group in comparison to control. In group VI and VII treated with HSRP ameliorate these values and normalize toward the control. Thus the *Hydechiumspicatum* have the antioxidant property which is responsible for amelioration of haematological parameters.

Key words : Hedychium spicatum, indoxacarb, cockerels

INTRODUCTION

Hedychium spicatum (Zingiberaceae) is an Ayurvedic traditional medicinal plant known as Spiked Ginger Lily (English) (Anonymous, 1994). It is a perennial rhizomatous herb, commonly found in subtropical Himalayan region. The plant is about 1 meter tall with fleshy aromatic rhizomes and elongated stem with broadly lanceolate leaves and has different developmental stages of flowering and fruiting (Kirtikar and Basu, 1999).

The rhizome of the plant contains medicinal properties like antioxidant, antimicrobial, anthelmintic, antidiabetic, hepatoprotective, anti-inflammatory, antiemetic, antidiarrhoeal, analgesic, expectorant, antiasthmatic, emmenagogue, hypoglycaemic, hypotensive, insect repellent, antifungal, pediculicidal and cytotoxic activities (Asolkar *et al.,* 1992).

MATERIALS AND METHODS

Experimental design

Eight weeks old, white leghorn male chicks procured from Instructional Poultry Farm, G. B. Pant University of Agriculture and Technology, Pantnagar were randomly divided into seven groups of seven birds each.The feed and water was given ad *libitum* during the study. After acclimatization for two weeks,group I was considered as control and fed grower ration, in group II were given indoxacarb @ 250ppm, III (silymarin@250ppm), IV (indoxacarb @250ppm+silymarin@ 250ppm), V (HSRP@ 4000ppm), VI (indoxacarb @250ppm+HSRP @2000ppm) and VII (indoxacarb @250ppm+HSRP @4000ppm) for 16 weeks. The dose of indoxacarb had been selected bases on LD_{50}value. The research proposal was approved by IAEC.

Plant materials

The rhizome of *Hedychium spicatum*L. was collected from the Medicinal Research Development Center, G. B. Pant University of Agriculture and Technology,Pantnagar. The rhizome was gathered from the plants, chopped like slice, shade dried for 15-20 days. Later the chopped rhizomes were placed in fan equipped incubator at 37°C for 2-4 hr and grinded in electric grinder to fine homogenous powder to get rhizome powders of *Hedychium spicatum* (HSRP). The powder was stored in sealed plastic container in dry place at room temperature till further use. The powder was used to feed the animals under study with the steamed standard computed ration.

Preparation of medicated ration

Medicated feed was prepared for about a day before the start of experiment and kept in close container. Dried rhizome powder of *H. spicatum* was pulverized to prepare 2000 ppm and 4000 ppm medicated feed by mixing 20g and 40g of powder of rhizomein 10 kg feed. To prepare homogeneously mixed ration, first mix the required amount of rhizome of *H. spicatum* powder in about 500 g of ration and then more feed was added gradually in fractions to make 10 kg feed. Similarly, the indoxacarb (250 ppm) medicated ration was prepared by adding 16 ml (2.5gm) of indoxacarb (DuPont™ Avaunt-15.8% EC) to a fraction of 10 kg feed and further mixing it thoroughly to whole feed. Similarly, silymarin tablets (Silybon-140)™ from Microlab® was purchased from local market and prepare the medicated ration by mixing the powder of 18 silybon-140 tablet and added and mixed thoroughly in 10 kg feed prepare the required quantity of ration (250 ppm).

Blood collection

Blood (1 ml) was collected from each bird in clean EDTA coated tubes and haematological parameters such as total erythrocyte count (TEC, x 10^6/µl blood), total leukocytes count (TLC, x 10^3/µl blood) Natt and Herric (1952) and Packed Cell Volume (PCV %) was estimated by capillary/ microhematocrit method and hemoglobin (gm/dl) was estimated by Drabkin's (cyanmet-hemoglobin) method. Differential leukocytes count (DLC %) (Lucas and Jamroz, 1961) and others haematological parameters were estimated by the methods of Benjamin (2004).

Statistical analysis

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

RESULTS AND DISCUSSION

There was significant (P<0.05) reduction in TEC (X10^3/µL), TLC (X10^3/µL),Hb (gm/dL) and PCV (%) was observed in indoxacarb treated group IIin comparison to control group I at 8 and 16 weeks intervals. Silymarin and HSRP treatment in groups IV, V, VI, VII improved TEC (X10^3/µL), TLC (X10^3/µL),Hb (gm/dL) and PCV (%) level significantly (P<0.05) and was at par with control at both 8 and 16 weeks intervals.As reported earlier, indoxacarb produced haemotoxic effects in human poisoned with indoxacarb (Viswanathan *et al.,* 2015). Indoxacarb induced haemoglobinemia in rat might be due to oxidative denaturation of Hb leading to haemolysis. The impaired Hb synthesis might haveoccurred due to bone marrow suppression and delayed RBC maturation (Mertens, 1997a; Frame, 1997b and Shit *et al.,* 2008). Reduction in TLC might have been due to direct toxic effect indoxacarb on bone marrow and lysis of blood cells. Finding lymphocytopenia were also reported in chronic toxicity of indoxacarb in rats and dogs and mice (Frame, 1997b; Kaur *et al.,* 2016). Thus, the finding of this study suggested that indoxacarb produced stress leading to release of glucocorticoids which further caused lymphocytopenia and heterophilia. The chemical xenobiotics have been reported to enhance production of oxidant radicals which release ACTH that produces lymphocytopenia and neutrophilia (Hyenes and Murad, 1980). The TLC was restored in groups VI and VII treated with low and high doses HSRP due to the antioxidant properties which might be have neutralised the toxic effects of various oxidants species produced during toxicity of indoxacarb and normalised the functions of lymphoid and haemopoitic system effectively(Thapliyal *et al.*, 2014).*Hedychium spicatum* have potent antioxidant property as reported earlier which could be attributed for its antianaemic efficacy(Sravani and Paarakh, 2012; Rawat *et al*., 2011).

The decrease in TEC and PCV value might be due to indoxacarb induced oxidative denaturation of haemoglobin leading to haemolysisand delayed maturation following possible depletion of haemopoeitic system including spleen, bone marrow and thymus (Frame, 1997a; Mertens 1997b).The indoxacarb produced the thymic necrosis, spleenic lymphoid depletion and bone marrow atrophy in rats (Frame, 1997b). As the rhizome powder of *Hedychium spicatum* have good antioxidant attributes which might have helped in normal functioning of haemopoitic system by scavenging the oxidant radicals and preventing lipid peroxidation, the prime causes of RBC fragility and oxidative denaturation of haemoglobin (Thapliyal *et al*., 2014).

A significant (P<0.05) reduction in absolute lymphocyte count and absolute heterophil count X10^3/µl was observed indoxacarb treated group II in comparison to control

Table 1.
Effect on haematological parameters following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels (Mean±SE, n=7).

Groups	Treatment	TEC(X10^6/µL)		TLC(X10^3/µL)		PCV (%)		Hb(gm/dl)		ALCx10^3/µl		AHCx10^3/µl	
		8	16	8	16	8	16	8	16	8	16	8	16
I	Control	3.14± 0.086^{a}	3.01± 0.063^{abc}	24.28± 0.61^{ab}	10.79± 0.47^{ab}	10.79± 0.47^{ab}	33.14± 0.41^{b}	10.9± 0.23^{ab}	10.72± 0.22^{ab}	10.79± 0.47^{ab}	11.02± 0.09^{a}	10.59± 0.49^{abc}	10.95± 0.15^{ab}
II	Indoxacarb (250ppm)	2.71± 0.079^{b}	2.68± 0.07^{d}	19.71± 0.86^{d}	7.81± 0.31^{d}	7.81± 0.31^{d}	26.92± 0.29^{d}	9.14± 0.35^{c}	9.07± 0.31^{d}	7.81± 0.31^{d}	7.66± 0.38^{c}	8.92± 0.48^{d}	9.04± 0.48^{d}
III	Silymarin (250ppm)	3.2± 0.092^{a}	3.15± 0.04^{a}	25.071± 0.41^{a}	11.17±0 0.28^{a}	11.17± 0.28^{a}	34.50± 0.48^{a}	11.07± 0.31^{a}	10.92± 0.25^{a}	11.17± 0.28^{a}	11.11± 0.46^{a}	11.02± 0.12^{a}	11.33± 0.28^{a}
IV	Indoxacarb (250ppm)+ Silymarin (250ppm)	2.97± 0.08^{a}	2.91± 0.08^{c}	22.71± 0.71^{abc}	9.81± 0.42^{bc}	9.81± 0.42^{bc}	30.28± 0.31^{c}	10.21± 0.21^{b}	9.92± 0.17^{c}	9.81± 0.42^{bc}	10.08± 0.48^{ab}	9.94± 0.26^{abcd}	10.23± 0.40^{bc}
V	*H. spicatum*(4000ppm)	3.12± 0.042^{a}	3.11± 0.059^{ab}	24.42± 0.57^{ab}	10.74±0 .27^{ab}	10.74± 0.27^{ab}	33.07± 0.54^{b}	10.93± 0.16^{ab}	10.78± 0.14^{ab}	10.74± 0.27^{ab}	10.77± 0.32^{a}	10.88± 0.22^{ab}	11.07± 0.15^{ab}
VI	Indoxacarb (250ppm)+ *H. spicatum*(2000ppm)	2.98± 0.055^{a}	2.94± 0.042^{bc}	21.42± 1.25^{dc}	9.19± 0.60^{c}	9.19± 0.60^{c}	30.14± 0.34^{c}	10.28± 0.18^{ab}	10.07± 0.27^{bc}	9.19± 0.60^{c}	9.22± 0.52^{b}	9.36± 0.58^{cd}	9.68± 0.52^{d}
VII	Indoxacarb (250ppm)+ *H. spicatum*(4000ppm)	3.08± 0.05^{a}	3.007± 0.058^{abc}	22.07± 0.83^{bcd}	9.49± 0.31^{c}	9.49± 0.31^{c}	31.42± 0.75^{c}	10.71± 0.31^{ab}	10.14± 0.28^{bc}	9.49± 0.31^{c}	9.94± 0.25^{ab}	9.68± 0.47^{bcd}	10.33± 0.18^{abc}

HSRP –*Hedychium spicatum* root powder, TLC- Total leukocyte count, TEC- Total erythrocyte count, ALC-Absolute lymphocyte count and AHC-Asoluteheterophil count; Mean values in column bearing different alphabetic superscript a and b differ significantly (p<0.05) when compared vertically

Table 2.
Effect on deferential leukocyte count (%) following administration of HSRP in feed for 16 weeks in indoxacarb treated WLH cockerels (Mean±SE, n=7)

Groups	Treatment	Heterophil (%)		Lymphocyte (%)		Monocyte (%)		Eosinophil (%)		Basophil (%)	
		8	16	8	16	8	8			8	16
I	Control	43.57± 0.202^{bc}	43.57± 0.297^{bc}	44.42± 1.60^{a}	43.85± 0.40^{a}	2.00± 0.218^{ab}	2.00± 0.218^{ab}	4.42± 0.202^{b}	4.85± 0.143^{b}	2.00± 0.218^{ab}	2.28± 0.184^{b}
II	Indoxacarb(250ppm)	45.14± 0.340^{aB}	46.14± 0.340^{aA}	39.71± 0.74^{b}	39.14± 0.59^{b}	3.00± 0.218^{a}	3.00± 0.218^{a}	5.42± 0.202^{a}	5.71± 0.184^{a}	3.00± 0.218^{a}	3.28± 0.184^{a}
III	Silymarin(250ppm)	44.00± 0.309^{ab}	44.85± 0.340^{b}	44.57± 0.81^{a}	43.85± 1.28^{a}	2.00± 0.218^{ab}	2.00± 0.218^{ab}	4.14± 0.143^{bc}	4.00± 0.103^{c}	2.00± 0.218^{ab}	2.14± 0.143^{b}
IV	Indoxacarb(250ppm)+ Silymarin(250ppm)	43.85± 0.340^{b}	43.85± 0.261^{bc}	43.14± 0.95^{a}	43.14± 0.39^{a}	2.42± 0.202^{a}	2.42± 0.202^{a}	4.85± 0.143^{ab}	4.71± 0.184^{b}	2.42± 0.202^{a}	2.71± 0.184^{a}
V	*H. spicatum*(4000ppm)	44.57± 0.297^{a}	45.00± 0.309^{b}	44.00± 0.43^{a}	43.71± 0.94^{a}	2.00± 0.218^{ab}	2.00± 0.218^{ab}	4.28± 0.184^{bc}	4.14± 0.143^{c}	2.00± 0.218^{ab}	2.00± 0.218^{b}
VI	Indoxacarb(250ppm)+ *H. spicatum*(2000ppm)	43.71± 0.360^{b}	44.42± 0.297^{b}	42.85± 0.67^{a}	42.28± 0.80^{a}	2.57± 0.202^{a}	2.57± 0.202^{a}	5.00± 0.218^{a}	4.85± 0.143^{b}	2.57± 0.202^{a}	2.85± 0.261^{a}
VII	Indoxacarb(250ppm)+ *H. spicatum*(4000ppm)	43.85± 0.553^{b}	44.28± 0.360^{b}	43.14± 0.98^{a}	42.57± 0.64^{a}	2.57± 0.202^{a}	2.57± 0.202^{a}	4.71± 0.184^{ab}	4.85± 0.143^{b}	2.57± 0.202^{a}	2.71± 0.286^{a}

HSRP –*Hedychiumspicatum* root powder; Mean values in column bearing different alphabetic superscript a and b differ significantly ($p<0.05$) when compared vertically

group I at 8 and 16 weeks intervals, respectively (Table 1). The reduction ALC was about 27% and 30% in indoxacarb treated group II in comparison to control at 8 and 16 weeks intervals. In groups VI and VII treated with indoxacarb+HSRP showed a significant ($P<0.05$) elevation which was near to value ofgroup IV and control at 8 and 16 weeks intervals. Reduction in ALC in present investigation may be due to cytotoxic effects of indoxacarb.

Indoxacarb produces leucopenia (Kaur*et al*., 2016)since the lymphocytes are the main cells to play vital role in defense mechanism and reduction in the number of absolute lymphocyte counts indicates that the immunosuppression (Mandal *et al*., 1986).

This amelioration of HSRP might be due to the protective efficacy of *Hedychium spicatum* mainly by its antioxidant property (Puri *et al.,* 2013).

There was significant ($P<0.05$) increased in heterophil (%), eosinophil (%), monocyte (%) and basophil (%) in group II treated with indoxacarb at after 16 weeks interval in comparison to control (Table 2). In groups VI and VII however, these values were at par with the control at 8 and 16 weeks interval. Indoxacarb was reported to cause lymphocytopenia and neutrophilia in rats and mice (Shit *et al.,* 2008; Kaur *et al.,* 2016). Indoxacarb damages blood cells directly by inducing the release of free radicals which may provoke the defence system to releases the heterophil in circulation. The enhanced oxidative stress might be the direct cause for increase in heterophil count in the present study (Shit *et al.,* 2008; Kaur *et al.,* 2016).

The basophil have role in immunomodulation and in allergy(Falcone *et al.,* 2000). The lymphocytopenia produced by indoxacarb might be due to atrophy of bone marrow cells, depletion of spleenic lymphoid and thymic necrosis in rats (Frame, 1997b). Leucopenia associated with lymphopenia was reported in early response to corticosteroids in some species of birds (Campbell, 2007; Davison *et al.*, 1981). *Hedychium spicatum* has been reported to have immunostimulant (Joshi and Mishra, 2009) and antioxidant (Sravani and Paarakh, 2012) properties which might have been responsible for its ameliorative effect against indoxacarb induced haemotoxicity in cockerels.

Thus, it is concluded that the *Hedychium spicatum* ameliorated the chronic toxicity produced by indoxacarb in WLH cockerels.

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Research Article

HEMATOBIOCHEMICAL PROFILE FOLLOWING IMIDACLOPRID TOXICITY AND ITS AMELIORATION BY COW URINE DISTILLATE IN WLH BROILERS

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ABSTRACT

The present study was aimed towards the evaluation of ameliorative effect of cow urine distillate by recording hematobiochemical following imidacloprid toxicity in white leghorn cockerels in continuous exposure for 60 days. Initially 48 birds were divided into six different groups randomly and equally. Group I served as control, whereas, group II and III were provided with 1/10th and 1/20th doses of LD_{50} and group V and VI were given 1/10th and 1/20th doses of LD_{50} with cow urine distillate @ 1ml/bird/day for 60 days. Group IV was provided with cow urine distillate only @ 1ml/bird/day for 60 days. There was significant changes in the hematobiochemical values in imidacloprid treated groups as compared to control group. The study is the indicative of the beneficial role of cow urine supplement on daily basis as it protects the birds from being severely intoxicated. Cow urine has hematinic and immune boosting potential that may help the birds to counteract the undergoing stress following imidacloprid mediated toxicity. It is concluded that imidacloprid produced the dose dependent toxicity in and the cow urine distillate ameliorated imidacloprid induced immunological response after 60 days trial in white leghorn (WLH) cockerels.

Keywords: Imidacloprid, cow urine distillate, immune booster, white leghorn cockerels

INTRODUCTION

India, the second largest populated country in the world, is by large infested with the Uncontrolled and indiscriminate use of insecticides and other fertilizers to fulfil the desired need of food and other supplement to the society. Pesticides are used to control or kill the pests but they may also cause adverse effects on well being of organism and degradation of crops and other fauna. According to the report of Buckley *et al.,* (2004), suggested that there was three million cases of severe poisoning and 2, 20, 000 deaths Indian subcontinents especially from the Asian countries. The report was an indication of the unethical use of pesticides in the agricultural fields. India is one of the largest producers of pesticides in Asia and ranks twelfth in the world for the use of pesticides (Gunnell and Eddleston, 2003). In 1991, Bayer Crop Science introduced a new type of insecticide into the USA; imidacloprid, the first member of a group now known as the neonicotinoids. Imidacloprid (IMC) 1[(6-chloro-3-pyridinyl) methyl]-N-nitro-2-imidazolidinimine, a chloronicotyl was the first representative of neonicotinoids insecticides to be registered for use and presently the most important commercial product because of its high efficacy against insects and low soil persistence (Chao and Casida, 1997). Imidacloprid produces thinning of egg shell, and reduced egg hatchability which are indicators of the endocrine disruption potential of imidacloprid (Berny *et al.*, 1999 and Matsuda *et al.,* 2001). Thus in this context imidacloprid induced endocrine disruption may change the normal physiology of the birds and to study the impact over the hematological and biochemical parameters will show some mechanistic pathway to elucidate the toxicity of imidacloprid in dose dependent manner. Immune system forming a major pillar in animal body system to fight against any of the alien agent is also being targeted by the pesticides. It had been proven that cow urine distillate had immunomodulatory action. In Sushrita Samhita the cow urine has been described as the most effective substance of animal origin with innumerable therapeutic values (Dhama *et al.*, 2005).The aim of the present study was to evaluate the ameliorative potential of cow urine distillate in imidacloprid mediated toxicity in white leghorn cockerels.

MATERIALS AND METHODS

The present study was conducted in 48 white leghorn cockerels, procured from IPF, GBPUAT, Pantnagar. These birds were acclimatized for two weeks in the experimental animal shed of Instructional poultry farm, Nagla, Pantnagar under standard managemental conditions. Poultry feed and water were provided *ad libitum* and kept under constant observation throughout study. All the chemicals required for this study were procured from Hi Media. ERBA diagnostics kits were used for biochemical analysis of glucose, serum total proteins, albumin, serum creatinine, urea, and cholesterol. Initially 48 birds were divided randomly and equally in six different groups having eight (n=8) in each group. Group I was kept as control, group II and III were provided with imidacloprid @ 1/10th and 1/20th of LD_{50} respectively and Group IV

was given only cow urine distillate @1 ml/bird/day, whereas group V and VI were given imidacloprid @1/10th and 1/20th of LD_{50} dose with cow urine distillate @1 ml/bird/day, po,, respectively, for a period of sixty days continuously by oral dosing. After every fifteen days interval, blood samples were collected for hematobiochemical analysis. Experiment was conducted after the permission of the Institute animal ethics committee (IAEC) and adequate measures were taken to minimize pain or discomfort to animals.

Hematological examination

1 ml of blood was collected from each bird in clean heparin coated tubes and hematological Parameters such as total erythrocytes count (×10^6 per ìl) and total leucocyte count (×10^3 per ìl) were determined with the method of Natt and Herrick (1952) using poultry blood and diluting fluid. Haemoglobin and PCV were estimated by standard method of Jain (1986).DLC was done at the end of 60 days by preparing thin blood smear from a drop of blood without anticoagulant. The smear was air dried and stained with Leishman stain for 20 minutes. The leucocytes were counted by zig-zag method and recorded on % basis.

Biochemical examination

The serum total protein**,** albumin**,** globulin, total cholesterol**,** glucose**,** creatinine and blood

Urea nitrogen was estimated by using ERBA diagnostic kits.

Statistical analysis

Statistical analysis of data was done by using ANOVA technique. Comparisons among treated and untreated groups were made with help of student't' test. Statistically significant difference was considered at 5 and 1 percent level (Snedecor and Cochran, 1989).

RESULTS

All the birds were observed continuously for any clinical abnormality in demeanour. The clinical signs following sub chronic exposure to white leghorn cockerels encompass dullness and depression were noticed in group II and III after 15 days exposure to imidacloprid whereas birds of groups V and VI have shown reduced appetite after 30 days of exposure to imidacloprid. There was no mortality throughout the course of the study. More severe signs as ataxia, tremors episode were appeared after 45th day of exposure to the toxicant in group II and III.Group V and VI have shown only mild reduction in feed and water intake. Birds of group I and IV were remain healthy throughout the study.

Hematological changes

The hematological parameters, including haemoglobin, PCV, TEC and TLC have shown significant decline in groups II, III, V and VI as compared to groups I and IV after 60 days exposure to imidacloprid (Table 1 and 2). Group I and IV revealed the significantly (P<0.05) higher value of the above mentioned parameters as compared to group II, III, V and VI. The ameliorative effects of cow urine distillate is justified by the less significant change in hematological values in treatment groups V and VI as compared to group II and III.

Biochemical changes

A significant (P<0.05) decrease in the total protein and albumin were observed in the groups II, III, V and VI as compared to the control group I and IV in dose dependent manner after 15th days onwards till end of study (Table 3). The treatment group IV, V and VI showed marked

Table 1:
Effect on Hb (g %) , PCV (%), TEC ($10^{6/l}$) following daily oral administration of imidacloprid with and without treatment for 60 days in white leghorn cockerels (Mean value± S.E., n=8).

	Hemoglobin					PCV					TEC				
Groups /Days	DAY O	DAY 15	DAY 30	DAY 45	DAY60	DAY O	DAY 15	DAY 30	DAY 45	DAY60	DAY O	DAY 15	DAY 30	DAY 45	DAY60
Group I	8.51± 0.151^{aA}	8.95± 0.128^{abB}	9.10± 0.193^{bC}	9.00± 0.146^{bC}	8.88± 0.101^{abC}	32.5± 0.957aA	32.67± 0.715aA	31.17± 0.946aAB	32.83± 1.447aB	31.83± 0.601aCD	3.34± 0.012aA	3.34± 0.027aBC	3.33± 0.022aD	3.36± 0.011aB	3.36± 0.009aD
Group II	8.46± 0.142^{dA}	8.06± 0.095^{cA}	7.61± 0.127^{bA}	7.53± 0.117^{bA}	6.80± 0.085^{aA}	31.17± 0.601^{bA}	31.33± 0.494^{bA}	29.3 3±0.760^{aA}	290± 0.577^{aA}	27.83± 0.477^{aA}	3.33± 0.012^{eA}	3.27± 0.015^{dA}	3.09± 0.015^{cA}	3.01± 0.030^{bA}	2.83± 0.020^{aA}
Group III	8.60± 0.085^{dA}	8.26± 0.122^{cA}	7.90± 0.051^{bAB}	7.75± 0.067^{bAB}	7.11± 0.119^{aB}	310± 0.577^{aA}	31.17± 1.014^{aA}	30.5 0±0.764^{aAB}	300± 0.577^{aA}	29.17± 0.792^{aAB}	3.35± 0.009^{cA}	3.28± 0.010^{cA}	3.12± 0.022^{bAB}	3.07± 0.041^{bA}	2.96± 0.032^{aB}
Group IV	8.68± 0.098^{aA}	8.78± 0.079^{abB}	8.98± 0.060^{bcC}	9.03± 0.088^{cC}	9.00± 0.057^{bcC}	31.50± 0.428^{aA}	33.0± 0.365^{aA}	32.1 7±0.477^{aB}	32.67± 0.715^{aB}	32.50± 0.428^{dD}	3.32± 0.042^{aA}	3.36± 0.015^{abC}	3.38± 0.008^{abE}	3.39± 0.009^{bB}	3.39± 0.007^{bD}
Group V	8.50± 0.057^{dA}	8.11± 0.060^{cA}	8.00± 0.077^{bcB}	7.83± 0.055^{bB}	7.38± 0.119^{aB}	31.33± 0.422^{bA}	310± 0.447^{abA}	30 0±0.516^{abAB}	29.67± 0.715^{aA}	29.67± 0.422^{aAB}	3.33± 0.006^{dA}	3.3± 0.021^{dAB}	3.17± 0.013^{cC}	3.08± 0.017^{bA}	2.96± 0.019^{aB}
Group VI	8.46± 0.071^{dA}	8.10± 0.073^{cA}	7.91± 0.030^{bcAB}	7.78± 0.040^{bAB}	7.41± 0.110^{aB}	31.17± 0.477^{aA}	31.33± 0.494^{aA}	30.6 7±0.667^{aAB}	30.50± 0.563^{aAB}	30.33± 0.803^{aBC}	3.35± 0.006^{dA}	3.28± 0.012^{cA}	3.14± 0.017^{bBC}	3.08± 0.028^{aA}±	3.04 0.026^{aC}

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

Table 2:
Effect on TLC following daily oral administration of *imidacloprid* with and without treatment for 60 days in white leghorn cockerels (Mean value± S.E. n=8).

Groups /Days	DAY O	DAY 15	DAY 30	DAY 45	DAY60
Group I	11.13±0.211^{aA}	11.12±0.200^{aA}	10.96±0.124^{aB}	11.11±0.202^{aC}	10.96±0.221^{aB}
Group II	11.23±0.244^{cA}	10.87±0.219^{cA}	9.90±0.057^{bA}	9.34±0.219^{aA}	8.93±0.041^{aA}
Group III	11.12±0.244^{cA}	10.80±0.254^{cA}	9.99±0.031^{bA}	9.83±0.032^{bB}	9.13±0.062^{aA}
Group IV	11.06±0.130^{aA}	11.11±0.128^{aA}	11.16±0.146^{aB}	11.46±0.158^{abC}	11.84±0.201^{bC}
Group V	11.16±0.254^{cA}	10.84±0.082^{cA}	9.99±0.029^{bA}	9.93±0.018^{bB}	9.01±0.027^{aA}
Group VI	10.95±0.176^{cA}	10.87±0.205^{cA}	10.17±0.167^{bA}	10.08±0.182^{bB}	9.33±0.144^{aA}

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

Table 3:
Effect on total protein and albumin following daily oral administration of *Imidacloprid* with and without treatment for 60 days in white leghorn cockerels (Mean value± S.E., n=8).

	TOTAL PROTEIN					ALBUMIN				
Groups /Days	DAY O	DAY 15	DAY 30	DAY 45	DAY60	DAY O	DAY 15	DAY 30	DAY 45	DAY60
Group I	3.92±0.013^{aA}	3.92±0.018^{aC}	3.90±0.016^{aC}	3.92±0.023^{aC}	3.91±0.027^{aC}	1.55±0.031^{aA}	1.53±0.016^{aC}	1.53±0.026^{aD}	1.53±0.013^{aC}	1.53±0.015^{aD}
Group II	3.92±0.010^{dA}	3.84±0.033^{cAB}	3.32±0.018^{bA}	3.24±0.036^{bA}	3.06±0.017^{aA}	1.54±0.016^{eA}	1.39±0.018^{dA}	1.34±0.017^{cA}	1.26±0.011^{bA}	1.16±0.009^{aA}
Group III	3.92±0.016^{eA}	3.83±0.020^{dAB}	3.45±0.033^{cB}	3.27±0.034^{bA}	3.18±0.018^{aB}	1.55±0.016^{dA}	1.51±0.015^{dC}	1.43±0.015^{cBC}	1.29±0.020^{bA}	1.22±0.013^{aB}
Group IV	3.91±0.018^{aA}	3.89±0.04^{aABC}	3.91±0.031^{aC}	3.92±0.011^{aC}	3.93±0.033^{aC}	1.56±0.013^{aA}	1.59±0.019^{aD}	1.58±0.013^{aD}	1.60±0.023^{aD}	1.61±0.016^{aE}
Group V	3.92±0.015^{dA}	3.90±0.018^{dBC}	3.53±0.039^{cB}	3.40±0.018^{bB}	3.23±0.021^{aB}	1.56±0.007^{eA}	1.45±0.020^{dB}	1.38±0.015^{cAB}	1.30±0.016^{bA}	1.21±0.019^{aB}
Group VI	3.94±0.018^{eA}	3.82±0.037^{dA}	3.48±0.014^{cB}	3.38±0.021^{bB}	3.25±0.022^{aB}	1.59±0.013^{dA}	1.49±0.006^{cBC}	1.46±0.020^{cC}	1.36±0.022^{bB}	1.27±0.013^{aC}

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

increase in comparison to without treatment group i.e. group II and III. A significant (P<0.05) decrease in blood glucose level was recorded in group II, III, V and VI as compared to the group I and group IV (Table 5). There was significant (P<0.05) increase in serum cholesterol and serum creatinine level levels in all intoxicated groups II, III, V and VI in comparison with control group I and IV after 60 days post exposure of imidacloprid in white leghorn cockerels (Table 5).

DISCUSSION

Higher intensity of clinicopathological manifestation of toxic signs and symptoms in groups treated with imidacloprid alone revealed the toxic effects of the neonicotinoids. Low level of

Clinical pathological manifestation in groups treated with cow urine distillate showed the medicinal and detoxifying potential of cow urine distillate. It was also reported by Chauhan *et al.* (2009) that cow urine serve as a potential booster of immunity if taken on daily basis.

Thus, the amelioration with cow urine distillate had a beneficial effect on the health of the birds.The reduction in Hb, PCV, TEC and TLC might be due to transferring of fluid from extra vascular compartment to the intravascular compartment in order to maintain the normal cardiac output and stroke volume and also pooling of the blood cells in the blood reservoirs such as spleen etc. Group I and IV revealed the significantly (P<0.05) higher value of the above mentioned parameters as compared to group II, III, V and VI. The ameliorative effects of cow urine distillate is evident by the less significant change in hematological values in treatment groups V and VI as compared to group II and III. This is an indicator of immunomodulatory activity of cow urine distillate that stimulate the bone marrow and reticulo endothelial system to generate the more number of erythrocytes and other blood cells to fight against the intoxication with imidacloprid. Reduction in hematological values could be due to impaired biosynthesis of heme in bone marrow (Shakoori *et al.,* 1990). The clinical signs seen in IMC intoxicated birds were similar to those reported in rats (Sheets, 2001). The anaemia might have been occurred due to the hemolytic action of imidacloprid as evidenced in this study. Furthermore, damage to the GIT and liver could also have resulted in malabsorption and maldigestion of nutrients required for erythropoiesis. It has been suggested that compounds having benzene ring or other ring structure acts as a hapten that combines with protein components of white blood cells to develop an antigen that triggers the generation of antibodies which cause either

Table 4:
Effect on glucose, cholesterol and serum creatinine following daily oral administration of *IMIDACLOPRID* with and without treatment for 60 days in white leghorn cockerels (Mean value± S.E., n=8).

	Glucose					Cholesterol					Serum creatinine				
Groups/Days	DAY O	DAY 15	DAY 30	DAY 45	DAY60	DAY O	DAY 15	DAY 30	DAY 45	DAY60	DAY O	DAY 15	DAY 30	DAY 45	DAY60
Group I	302.18± 1.695^{aA}	299.11± 1.906^{aC}	302.25± 1.862^{aD}	299.50± 1.258^{aC}	298.48± 0.834^{aD}	132.76± 0.424^{aA}	133.87± 0.385^{aA}	133.39± 0.497^{aA}	133.50± 0.590^{aA}	133.95± 0.341^{aA}	0.33±0. 011^{aA}	0.33±0. 007^{aA}	0.33±0. 004^{aA}	0.32±0. 008^{aA}	0.33±0. 004^{aA}
Group II	299.68± 2.895^{Ea}	282.33± 3.303^{dA}	260.16± 1.701^{cA}	227.33± 2.538^{bA}	209.22± 1.234^{aA}	132.99± 0.513^{aA}	137.76± 1.005^{bB}	145.63± 0.776^{cC}	156.06± 0.787^{dD}	162.41± 0.900^{eD}	0.32±0. 007^{aA}	0.41±0. 011^{bB}	0.51±0. 013^{cD}	0.61±0. 015^{dC}	0.82±0. 015^{eE}
Group III	300.20± 2.210^{eA}	283.96± 2.325^{cAB}	269.69± 3.161^{cBC}	246.47± 3.011^{bB}	228.62± 2.081^{aBC}	133.19± 0.58^{aA}	135.17± 0.468^{aA}	141.50± 0.680^{bB}	147.81± 0.868^{cB}	156.74± 1.171^{dC}	0.34±0. 3^{aA}	0.40±0. 8^{bB}	0.43±0. 8^{cB}	0.54±0. 12^{dB}	0.74±0. 11^{eD}
Group IV	301.22± 1.842^{aA}	300.46± 0.742^{aC}	299.43± 0.636^{aD}	298.27± 1.982^{aC}	301.32± 0.353^{aD}	132.61± 0.430^{aA}	133.63± 0.646^{aA}	133.76± 0.589^{aA}	133.50± 0.834^{aA}	133.18± 0.333^{aA}	0.33±0. 006^{aA}	0.33±0. 003^{aA}	0.32±0. 004^{aA}	0.32±0. 004^{aA}	0.32±0. 004^{aA}
Group V	301.22± 1.842^{eA}	289.11± 0.997^{dB}	266.39± 2.125^{cB}	246.45± 2.57^{bB}	224.80± 3.698^{aB}	133.68± 0.443^{aA}	135.31± 0.874^{aA}	140.40± 0.858^{bB}	151.63± 0.89^{cC}	157.55± 1.136^{dC}	0.33±0. 3^{aA}	0.40±0. 12^{bB}	0.48±0. 11^{cC}	0.53±0. 11^{dB}	0.69±0. 8^{eC}
Group VI	300.47± 0.739^{eA}	288.36± 2.388^{dAB}	272.86± 2.043^{cC}	248.92± 2.884^{bB}	234.12± 1.564^{aC}	133.92± 0.433^{aA}	134.46± 0.302^{aA}	139.53± 0.543^{bB}	146.27± 0.810^{cB}	152.21± 1.157^{dB}	0.32±0. 009^{aA}	0.39±0. 006^{bB}	0.42±0. 009^{cB}	0.51±0. 018^{dB}	0.64±0. 014^{eB}

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

the lysis or agglutination of leucocytes**.** Imidacloprid is also having a ring structure thus may have caused the leucopoenia as evident from the present study on white leghorn cockerels. Bapu (2001) proved that due to the presence of traces of iron in cow urine, it has been found to posses the haematinic properties. Continuous exposure to insecticide may then leads to lymphopenia which may have an immunosuppressive effect. The adverse effect on differential leucocyte count may be due to altered functioning of bone marrow and stress (Jain, 1986). Marked decrease in total protein, albumin and globulin was also observed in imidacloprid treated groups. According to Kori-Siakpere (1995) decrease in plasma protein, albumin and globulin could be attributed to renal excretion or impaired protein synthesis or due to liver disorders. The decrease in serum total protein also may be attributed to loss of protein either by decrease protein synthesis or increased proteolysis or degradation (Shakoori *et al.*, 1990). The persistent hypoglycaemia might have occurred due to hepatotoxic effect caused by the imidacloprid. Being the primary metabolising site, imidacloprid induced liver toxicity might hamper normal glycolysis, gluconeogenesis and glycogenolysis processes. The present findings were in agreement with the findings of Balani *et al.*, (2011). Significant increase in cholesterol, serum creatinine and blood urea nitrogen was noticed in intoxicated groups. However, group V and VI showed less significant alteration in the clinical values in comparison with group II and III cementing the beneficial and protective role of cow urine distillate against the toxicant. Garg (2004) also reported that supplementation of cow urine to white leghorn layer showed significant amelioration in serum total cholesterol. 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 (Jain, 1986). The injury to renal epithelial cells has been associated with the increase in such markers as serum creatinine and blood urea nitrogen, of renal injury. In addition; increased hepatic urea production from amino acid metabolism could also be responsible for an increase in urea concentration in the serum .Treatment significantly (P<0.05) reduced the level of urea and creatinine in groups IV, V and VI as compared to group II and III. Cow urine distillate has the potent germicidal and antimicrobial activity in association with immune modulating ability (Chauhan *et al.,* 2009).

It is concluded from the study that imidacloprid causing toxicity in a dose dependent manner following sub chronic exposure to white leghorn cockerels and the cow urine distillate has produced significant ameliorative action against the imidacloprid toxicity by keeping the values on nearly normal side by continuous administration @1 ml/bird/day for 60 days.

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Research Article

SCIENTIFIC VALIDATION OF POLYHERBAL FORMULAITON FOR PYREXIA IN ANIMALS USED BY LOCAL HEALERS OF WAYANAD DISTRICT, KERALA

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ABSTRACT

The present study aims at standardizing and evaluating the anti-pyretic activity of polyherbal formulation against lipopolysaccharide (LPS) induced pyrexia model in rats. The herbal formulation consists of *Zingiber officinalis* (Rhizome), *Ocimum tenuiflorum* (Leaves), *Myristicafragrans* (Seeds), *Allium sativum* (Clove), *Elettaria cardamomum* (Seeds) and *Piper nigrum* (Seeds). The prepared polyherbal formulation has been standardized according to WHO guidelines and evaluated for in-vivo anti-pyretic study of crude and alcoholic extract. The content of the formulations were present within the permissible limits. No untoward adverse effects was noticed in rats upon subjecting to acute oral toxicity. The extracts at the dose rate of 250 and 500 mg/kg body wt. showed significant (P<0.001) anti-pyretic activity comparable to paracetamol.

Key words: Polyherbal formulation, lipopolysaccharide, WHO, anti-pyretic.

INTRODUCTION

Pyrexia is a complex physiological response triggered by infectious / aseptic stimuli, is an sequel of tissue damage, trauma, inflammation, graft rejection or pathological conditions arising due to virus, bacteria, fungi and protozoans (Akapa *et al.*, 2014). Elevation of body temperature occur when concentration of interleukins, interferons, cytokines and TNF-α formed are in huge quantity leading to increased synthesis of PGE2 in certain areas of brain. Conventional drugs used for the management of fever are either NSAID's or related salicylates. Most antipyretic drugs act by inhibiting the expression of COX-2 enzyme to reduce the elevated body temperature by inhibiting the bio-synthesis of PGE2. However, these synthetic drugs acts irreversibly by inhibiting COX-2 enzyme with high selectivity, but are toxic to vital organs of the host (Elumalai *et al.,* 2012). The quest for novel antipyretic drugs is perpetual as the Ayurvedic system of treatment approaches the ailmentsby correcting the imbalance through a combination of diet, exercise and herbal medication. The impressive demonstration of efficacy of polyherbals demanded this investigation whose objective is to prepare, standardize and evaluatethe pharmacological activity in LPS induced pyrexia model.

MATERIALS AND METHODS

Ingredients of polyherbal formulation were collected from their natural stance and authenticated by MS Swaminathan Research foundation, Wayanad, Kerala.

Preparation of formulation

Polyherbal formulation compresed of *Zingiber officinalis* dried rhizome 50 gms, *Ocimum tenuiflorum* leaf 50 g, *Myristicafragrans* seed (one), *Allium sativum* 100g, *Eletariacardamomum* 10 seeds were pulverized to a mesh size of 40-50 in an electrically driven pulverizer and sieved to remove the coarse substances. Hand crushed 100 g of *Piper nigrum* seeds were boiled in 500 ml of water and reduced the volume to one-fourth and filtered. The filtrate was added to the above mixture and made to paste. The prepared crude formulation was kept for shade dryingand stored in dry container for further use. A part of the crude formulation were made into thimbles and weighed.Extraction was done using methanol as a solvent in hot continuous soxhlet extract assembly until the solvent turned clear in the Soxhlet apparatus. The liquid extract obtained was collected in round bottom flask and solvent was evaporated by using Rotary Vacuum Evaporator (M/s Buchi, Switzerland) under reduced pressure. The semisolid extract obtained was air dried and stored in the refrigerator at 4 C for further use. The crude formulation and methanolic extract were tested simultaneously for standardization and anti-pyretic activity evaluation.

Physico-chemical evaluation

Various physicochemical parameters like pH, moisture content, total ash value, foreign matter, total fat and crude protein (AOAC, 2005), UV fluorescence analysis (Pimenta *et al.,* 2006) and heavy metal test were estimated according to standard protocols. The formulations was analyzed qualitatively for phytochemical constituents as per the procedure described by Harborne, 2005.

Acute toxicity study

Acute oral toxicity study of the crude formulation and methanolic extract was carried out using healthy albino Wistar rats as per OECD TG-423 (OECD, 2001).

Experimental design

Forty two adult albino Wistar rats were selected for the present study and were randomly grouped. The experimental animals were acclimatized to the laboratory conditions and fasted overnight with ad-libitum water. Initial

Table 1:
Value of proximate analysis of PHF

Sl. No.	Parameters	Crude formulation	Methanolic extract
1	pH	6.25	5.89
2	Moisture	78.27±1.13%	52.08±1/13%
3	Dry matter	21.73±1.13%	47.92±1.13
4	Total ash	5.49±0.31%	4.98±0.31%
5	Acid insoluble ash	0.65±0.25%	0.61±0.25%
6	Crude fibre	1.34±0.01%	1.27±0.01%
7	Crude lipid	6.12±0.75%	6.19±0.75%
8	Crude protein	17.03±0.75%	19.25±0.75%

rectal temperature was noted. Pyrexia was induced by intra muscular injection of lipopolysaccharide (LPS from *E.Coli* serovar O127B8) at the dose of 50 µg/ml/kg. After 18 hrs, those animal showing a raise in body temperature more than 1ºF were selected and divided in to sevengroups of six animals each. G-I served as normal control, G-II as negative control, G-III as positive control (paracetamol 100 mg/kg body wt. p.o.) followed by G-IV, G-V, G-VI and G-VII at the dose rate of 250 and 500 mg/kg, p.o. of crude and methanolic polyherbal extract respectively. The record of increase in temperature after 18th hour has been taken as 0 hour reading followed by administration of test substance and paracetamol. Rectal temperature of animals were recorded every hour after the oral treatment till 5 hours (Annan *et al.,* 2013).

Statistical analysis

Statistical analysis was done by using One way ANOVA followed by Duncan Multiple Range Test (DMRT) was used for comparing the temperature of the five treatment groups in each hour. Repeated measures ANOVA followed by LSD test was done for comparing the temperature in different hours within each group using IBM® SPSS® version 21 software. Values are expressed in Mean ± SEM were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Standardization of biologically active material and development of sTable polyherbal formulation is a challenging task because of varied chemical compound present in the different medicinal plants (Dileepkumar *et al.,* 2015). World health organization has evolved guidelines to formulate national policies on traditional medicine and to study their potential usefulness including evaluation, safety and efficacy (WHO, 1992). So the present study is carried out to standardize the polyherbal formulation and to evaluate its antipyretic activity.

The polyherbal formulation compresed of *Zingiber officinalis, Ocimum tenuiflorum, Myristica fragrans, Allium sativum, Elettaria cardamomum* and *Piper nigrum* showed a high content of moisture content in crude formulation than methanol extract (Table 1). Moisture content indicates the shelf life of a formulation and long storage would lead to spoilage due to susceptibility to microbial attack. Moisture content is the most vital measurement in processing, preservation and storage of food. Ash in the formulation represents the residue remaining after removal of all moisture and organic materials, incinerated at temperature of about 500^0 C represents the mineral content of the herbals (Onwuka, 2005). Crude fibre indicates the level of non-digestible carbohydrate and lignin, low level is considered appropriate for absorption of glucose and fat (Oladiji *et al.,* 2005).Crude lipid content aids in transport of fat soluble vitamins, insulates and protects internal tissues and contribute to important cell processes (Pamela *et al.,* 2005).The polyherbal formulation contains adequate nutritive value. Fluorescence is a phenomenon exhibited by various chemical constituents in plant materials at different range of wavelength say high (365 nm), low (254 nm) and visible light, often crude drugs are assessed qualitatively for fluorescence characters and is an important parameter of pharmacognostical evaluation (Kamil *et al.,* 2010).The result of preliminary phytochemical analysis revealed presence of secondary plant metabolites. Polyherbal formulation shown the presence of all major phytoconstituents like alkaloids, flavonoids, glycosides, steroids, tannins, diterpenes and saponins and absence of heavy metals in both crude and methanolic extracts. The results of the acute oral toxicity confirms that the crude and methanolic extracts were safe up to 2000 mg/kg. The animals were healthy, feed and water intake was

Table 2:
Anti-pyretic activity of polyherbal formulation and paracetamol in LPS induced pyrexia in rats.

Gps	R0	R1	R2	R3	R4	R5	P-value
1	98.38 ± 0.05^{c}	98.38 ± 0.08^{d}	98.38 ± 0.08^{c}	98.42 ± 0.06^{c}	98.38 ± 0.08^{b}	98.35 ± 0.05^{c}	0.283
2	101.62 ± 0.07^{ab}	101.7 ± 0.05^{a}	101.68 ± 0.05^{a}	101.62 ± 0.05^{a}	101.62 ± 0.06^{a}	101.62 ± 0.04^{a}	0.073
3	101.98 ± 0.18^{aA}	98.57 ± 0.11^{cdB}	98.35 ± 0.08^{cC}	98.32 ± 0.08^{cC}	98.33 ± 0.07^{bC}	98.33 ± 0.07^{bcC}	< 0.001
4	101.57 ± 0.13^{bA}	99.7 ± 0.07^{bB}	98.8 ± 0.05^{bC}	98.6 ± 0.05^{bD}	98.52 ± 0.05^{bE}	98.5 ± 0.04^{bE}	< 0.001
5	101.72 ± 0.14^{abA}	98.75 ± 0.04^{cB}	98.45 ± 0.04^{cC}	98.45 ± 0.04^{bcC}	98.38 ± 0.05^{bD}	98.37 ± 0.05^{bcD}	< 0.001
6	101.57 ± 0.13^{bA}	99.7 ± 0.07^{bB}	98.8 ± 0.05^{bC}	98.6 ± 0.05^{bD}	98.52 ± 0.05^{bE}	98.5 ± 0.04^{bE}	< 0.001
7	101.72 ± 0.14^{abA}	98.75 ± 0.04^{cB}	98.45 ± 0.04^{cC}	98.45 ± 0.04^{bcD}	98.38 ± 0.05^{bE}	98.37 ± 0.05^{bcE}	< 0.001
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

Means having same small letter as superscript are homogenous within a column.
Means having same capital letter as superscript are homogenous within a row.

normal. There was no behavioural changes during the entire period of study.

Antipyretic activity of both crude formulation and its extract (Table 2) exhibited significant dose dependent activity. However, there was no significant difference in antipyretic activity exhibited by both crude and methanolic fractions of the polyherbal formulations at the dose of 500 mg/kg bwt and showed significant ($P<0.001$) reduction in body temperature at second hour itself and were comparable with standard drug, paracetamol. The presence of secondary metabolite might be attributed to the underlying pharmacological activity (Agarwal *et al.,* 2011, Annan *et al.,* 2013). Therefore, the current study provides substantial scientific validation for the use of the formulation in pyrexia cases by the healer of Wayanad district, Kerala. Further, plant specific extraction and characterization of individual phytochemicals is required to assess the exact mechanism of action of the polyherbal formulation.

The study showed that the content of the formulation were present within permissible limits as per the WHO guidelines. The crude formulation and methanolic extract showed significant antipyretic activity comparable to standard drug, paracetamol. The result of the present study serves as reference monograph in the preparation of drug formulation.

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Research Article

PROTECTIVE EFFECT OF *MORINGA OLEIFERA* AGAINST SODIUM FLOURIDE INDUCED TOXICITY IN WISTAR RATS

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ABSRACT

The present investigation was undertaken to study the effect of aqueous extract of *Moringa oleifera* (AEMO) on some antioxidant and biochemical parameters in Wistar rats induced with sodium fluoride toxicity and Vitamin C. Thirty six rats were divided randomly and equally into six groups viz. T_1, T_2, T_3, T_4, T_5 and T_6. T_1 served as control which received normal saline only. T_2 served as positive control received sodium fluoride @ 20 mg/kg b wt, T_3 received ascorbic acid @ 200 mg/kg b wt and T_4, T_5 and T_6 were treated with different doses viz. 250, 500 and 750 mg/kg b. w of AEMO, respectively, along with sodium fluoride @ 20 mg/kg b wt. After 28 days, biochemical from serum and antioxidant parameters in organs collected after sacrificing rats were recorded. Lipid peroxidation in kidney and liver was assessed by increased level of TBARS, lipid hydro peroxidase and decreased activities of SOD, catalase and glutathione peroxidase. The content of glutathione also decreased in both organs. Aqueous extract at all three doses viz. 250, 500 and 750 mg/kg b wt decreased lipid peroxidation in both organs by improving the activities of antioxidant enzymes and decreasing the level of TBARS and lipid hydro peroxidase. The glutathione content also improved in both organs. The activities of antioxidant enzymes glutathione peroxidase, superoxide dismutase and catalase also improved in all treatment groups ($P<0.05$). Fluoride elevated the enzymes like SGOT, SGPT and SALP indicating liver injury which were decreased treatments with AEMO in T_4, T_5 and T_6. The amount of total protein, albumin and globulin also decreased in T_2 group which was improved by AEMO treatment indicating its protective effect against sodium fluoride toxicity in rats.

Key Words: Antioxidant enzymes, biochemical, *Moringa oleifera* sodium fluoride toxicity, Vitamin C,. Wistar rats

INTRODUCTION

Fluorides are naturally occurring harmful contaminant in an environment. It is a cumulative poison and thus leads to fluorosis; a serious public health problem. Fluoride causes damage not only to hard tissues of teeth and skeleton, but also to soft tissues, such as brain, liver, kidney, spleen and endocrine glands. Reactive oxygen species (ROS) are produced naturally as a part of intracellular metabolic processes and induce oxidative damage to cell membranes, lipids, proteins and nucleic acids. Many reports have revealed enhanced ROS/free radical formation by fluoride exposure (Chauhan *et al.,* 2013). Ascorbic acid is well known antioxidant vitamin involved in several biochemical processes in biological systems (Carr and Frei,1999). Recent studies on certain herbal plants with antioxidants potential indicated reduction in oxidative stress caused by sodium fluoride such as *Moringa oleifera, Tamarindus indica, Limonia acidissima etc.* (Vasant and Narasimhacharya, 2013).

Moringa oleifera Lam is a tropical plant belonging to family Moringaceae, native of India which was introduced in Brazil around 1950. Moringaceae is a single genus family with 13 known species. Among these *oleifera* is most widely used and utilized species (Morton, 1991), commonly known as Drumstick or Horseradish (English), Shevga (Marathi), Muringa (Malayalam) and Sahjan (Hindi). It is a versatile tree useful for human beings and animals and also has industrial values. *Moringa* pod is an important commercial vegeTable crop throughout India. Hence the present study was undertaken to evaluate the Lipid peroxidation, antioxidant and biochemical parameters in Wistar rats induced with or without the sodium fluoride toxicity and supplementation of Vitamin C.

MATERIALS AND METHODS

Plant material

The plant material i.e. *Moringa oleifera* seeds were procured from local market and aqueous extract of dried *Moringa oleifera* seeds was prepared and was used as herbal medicine. The *Moringa oleifera* seeds were procured from Nagpur region and were authenticated from Department of Botany, Institute of Science, RTM Nagpur University, Nagpur. The seeds were dried at room temperature and powdered. The powder was stored in glass bottle in a cool and dry place away from direct sunlight and used for preparation of aqueous extract. The Aqueous extract of *Moringa oleifera* (AEMO) was prepared by the method described by Rosenthaler (1930) and was stored in a desicator in cool and dry place until further used in this study.

Experimental design

A total of thirty six 150-200 g female Wistar rats from Animal Breeding Centre were divided randomly and equally into six groups T_1, T_2, T_3, T_4, T_5 and T_6. The rats were acclimatized for 15 days to the environment, before the start of this experiment. Six groups formed were

labeled as. Group 'T_1' served as negative control and was treated with normal saline. Group 'T_2' served, as positive control, which received Sodium fluoride only @20mg/kg b.wt. Group 'T_3' received Sodium fluoride only @20mg/kg b.wt and Vit C@ 200mg/kg b.wt. as a referral standard and Group T_4, T_5 and T_6 were treated with *Moringa oleifera* @ 250, 500, 750 mg/Kg b.wt, respectively, along with sodium fluoride only @20mg/kg b.wt. For induction of toxicity, sodium fluoride was used @20 mg / kg bwt dissolved in distilled water. The present research work was approved by IAEC.

Antioxidant and biochemical parameters

Blood samples were collected in non-heparinized tubes and were kept undisturbed. The serum was separated and stored at -20^0 C for subsequent analysis. Tissues such as liver and kidney were also collected in ice-cold containers for estimations of various oxidative stress and lipid peroxidation parameters i.e., TBARS (Niehaus and Samuelson, 1968), lipid hydroperoxides (Jiang *et al.*,1992), reduced glutathione (Ellman, 1959), glutathione peroxidase (Rotruck *et al.,* 1973), superoxide dismutase (SOD) (Kakkar *et al.,* 1984) and catalase (Sinha, 1972) were assayed by the method of biochemical estimations of serum total proteins were done by Biuret and BCG dye binding method, respectively (Anino, 1976), Serum alkaline phosphatase was estimated by the method given by Kind and King (1954) and serum SGOT and SGPT was done by Reitman and Frankels method (Tietz, 1987) on the spectrophotometer by using commercial reagent kits (RFCL).

RESULTS AND DICUSSION

Fluoride induced oxidative stress in liver and kidney of rats is shownreveals in Table 1. The present study confirmed this by estimating the levels of TBARS and lipid hydroperoxidase in liver and kidney. Both these values were the indicators of lipid peroxidation which are increased in liver and kidney with fluoride toxicity. Both were the end products of LPO. The observation strongly suggested that these parameters play an important role in fluoride toxicity. The review collected also showed that fluoride increases the levels of TBARS in tissue of experimental animals.

The aqueous seed extract was studied on TBARS and lipid hydroperoxides of liver and kidney. The liver and kidney TBARS and lipid hydroperoxides levels were elevated by sodium fluoride toxicity. These elevated levels were significantly reduced by *Moringa oleifera* treatment at high dose levels in present study. Gupta *et al.* (2013) reported significant decrease in LPO in liver and kidney tissues by *T. indica* extract. Ekambaram *et al.* (2010) also reported dose dependant decline in levels of TBARS in liver of extract treated rats. These elevated levels were decreased by treatment of *Moringa oleifera* in present study. These finding are also in agreement to Gupta *et al.* (2013) and Ekambaram *et al.* (2010). The levels of glutathione and glutathione peroxidase were decreased by sodium fluoride. The decreased levels of glutathione and glutathione peroxidase were elevated by extract. Gupta *et al.* (2013) also reported tissue specific depletion of GSH from hepatic and renal tissues. In the present study decreased levels of glutathione and glutathione peroxidase were restored back nearly to normal. The depleted levels of SOD and catalase in liver and kidney by sodium fluoride were increased significantly. Gupta *et al.* (2013) reported the decrease in the activity of SOD, CAT, and GSH in liver and kidney of rats in sodium fluoride were restored back nearly to normal.

Serum biochemical values are shown in Table 2 which are the indicators of enzymic levels and were analyzed to assess the fluoride induced toxicity. Due to functional diversity of hepatocytes, alterations in the levels of specific enzymes are used as an index for the intoxication of cell population. The effect of *Moringa oleifera* was observed on SGPT, SGOT, alkaline phosphatase, Total protein against sodium fluoride toxicity. In SGPT amelioration was not observed

Table 1:

Effect of aqueous extract of *Moringa oleifera (AEMO)* on antioxidant parameters (Mean ± S.E; n=6) following sodium fluorid induced toxicity in rats.

Treatment Groups	TBARS (mmoles/ 100g tissue)		Lipid Hydroperoxides (mmoles/100g tissue)		Glutathione (mmoles/g tissue)		Glutathione peroxidase (g of GSH consumed/min/ mg protein)		SOD (Units/mg protein)		Catalase (Units/mg protein)	
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
T_1	0.78±0.004^e	1.44±0.007^d	24.59±0.187^a	20.625±0.186^a	7.26±0.01^a	8.47±0.019^a	70.71±0.173^a	37.46±0.124^a	10.70±0.14^a	8.13±0.02^a	72.43±0.007^f	66.31±0.227^e
T_2	1.73±0^a	2.33±0.017^a	16.003±0.89^f	12.01±0.119^f	3.04±0.033^e	4.79±0.012^f	50.59±0.17^f	23.79±0.048^f	5.90±0.064^f	5.78±0.014^f	130.86±0.763^a	101.42±0.183^a
T_3	0.85±0.014^d	1.48±0.003^d	21.52±0.166^c	18.30±0.083^c	6.01±0.036^b	7.54±0.027^b	69.66±0.158^b	35.35±0.031^b	9.27±0.028^c	7.87±0.022^b	78.74±0.247^e	76.81±0.275^d
T_4	1.24±0.014^b	1.80±0.001^b	18.41±0.13^e	14.58±0.193^e	4.85±0.115^d	5.91±0.047^e	58.52±0.193^e	28.53±0.14^e	7.40±0.032^e	6.26±0.014^e	99.06±0.094^b	94.59±0.185^b
T_5	1.05±0.016^c	1.63±0.009^c	20.52±0.153^d	17.80±0.131^d	5.06±0.024^c	6.23±0.018^d	61.27±0.076^d	30.84±0.122^d	8.19±0.022^d	6.87±0.012^d	89.40±0.175^c	86.78±0.134^c
T_6	0.88±0.026^d	1.50±0.025^d	22.36±0.219^b	19.81±0.079^b	5.97±0.05^b	6.96±0.013^c	68.84±0.123^c	34.57±0.146^c	9.79±0.014^b	7.79±0.032^c	81.66±0.218^d	76.95±0.254^d

T_1=Control ; T_2 =Sodium fluoride (SF) only @20mg/kg b.w; T_3= SF + L-Ascorbic acid @200 mg/kg b.w.; T4= SF +AEMO @250 mg/kg b.w; T5= SF +AEMO @500 mg/kg b.w.; T6= SF + AEMO@750 mg/kg b.w., Values not sharing a common superscript in a column differ significantly (P<0.05)

Table 2:
Effect of AEMO extract of *Moringa oleifera* on serum enzymes (Mean ± S.E; n=6) on sodium fluoride induced toxicity in rats

Treatment Groups	SGPT(IU/L)	SGOT (IU/L)	Alkaline phosphatase (IU/L)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
T_1	40.365±0.471^{e}	60.17±0.59^{d}	25.35±0.303^{d}	6.22±0.07^{a}	4.81±0.049^{a}	1.41±0.035^{a}
T_2	57.73±0.74^{a}	79.23±0.324^{a}	35.86±0.696^{a}	5.77±0.189^{c}	4.35±0.097^{d}	1.25±0.039^{b}
T_3	44.53±0.809^{d}	64.85±0.541^{c}	29.32±1.016^{c}	6.21±0.072^{a}	4.78±0.047^{a}	1.42±0.024^{a}
T_4	53.35±0.674^{b}	70.98±1.116^{b}	33.10±0.808^{b}	5.91±0.064^{bc}	4.55±0.055^{c}	1.35±0.03^{a}
T_5	47.13±1.244^{c}	66.36±0.419^{c}	28.78±0.659^{c}	5.97±0.057^{abc}	4.60±0.031^{bc}	1.37±0.027^{a}
T_6	43.51±0.951^{d}	61.36±0.51^{d}	24.73±0.297^{d}	6.15±0.032^{ab}	4.74±0.038^{ab}	1.40±0.019^{a}

T_1= 0.5 ml NSS ; T_2 =Sodium fluoride (SF) only @20mg/kg b.w; T_3= SF + L-Ascorbic acid @200 mg/kg b.w.; T4= SF +AEMO @250 mg/kg b.w; T5= SF +AEMO @500 mg/kg b.w.; T6= SF + AEMO@750 mg/kg b.w.; Values not sharing a common superscript in a column differ significantly (P<0.05).

by 250 mg of *Moringa oleifera* whereas 500 and 750 mg dose could ameliorate the increased levels of SGPT values. The SGOT and alkaline phosphatase values were significantly decreased in treatment groups proving amelioration against sodium fluoride toxicity. Reduction in elevated SGOT, SGPT and alkaline phosphatase by *Moringa* extract in sodium fluoride intoxicated rats was also reported by Ranjan *et al.* (2009). It is concluded from this study that *Moringa oleifera* produced a significant ameliorative effect elevated after sodium fluoride toxicity in rats.

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Research Article

DETERMINATION OF SULFAQUINOXALINE RESIDUES IN MILK BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The study was conducted to monitor the levels of sulfaquinoxaline residues in raw milk samples of bovine in and around U.S.Nagar district, Uttarkhand, India. Residues in animal products play role in development of resistance to antibiotics. Plenty cases of resistance have been demonstrated in farm conditions, making the veterinary practice vulnerable. Subsequently, an endeavour was made to examine "sulfaquinoxaline commonly used antibacterial in Veterinary practice" in milk samples which were collected from local areas of U.S.nagar. An HPLC method with UV detection, at 254 nm, was developed and validated for the determination sulfaquinoxaline in milk. The mobile phase, a mixture of methanol and water in 50:50 v/v and pH adjusted to 4. A linear calibration curve was obtained by plotting area against concentration, with a correlation coefficient of 0.999, while average recoveries were greater than 86.8% with RSD value of 1.85. Out of 60 milk samples that were analyzed 11(18.33%) samples had a perceivable amount of residue levels for Sulfaquinoxaline and one sample was having residue level above the recommended maximum residue limits. The method described in this study would be helpful for routine monitoring of Sulfaquinoxaline residues in milk.

Keywords: Residue, sulfaquinoxaline, Milk samples, HPLC, resistance

INTRODUCTION

Food Safety and Standards Act, 2006 defines veterinary drug residues as "the parent compounds or their metabolites or both in any edible portion of any animal product and include residues of associated impurities of the veterinary drugs concerned" (FSSA, 2006).

After administration, Sulphonamides (SAs) are widely distributed throughout the body and milk depending on several factors, including protein binding and pKa values. The presence of drug residues in milk may lead to allergic reactions in some consumers (Kishida *et al*., 2005) as well as an increase in drug-resistant organisms (Kishida, 2007). Hence, monitoring of such residues in products designated for human consumption is vital for the maintenance of the consumer's health. The European Union has adopted a maximum residue level (MRL) of 100 mg/kg for SAs in foodstuffs of animal origin (EEC, 1990).

Several methods have been employed to determine sulphonamide drugs in milk by reverse-phase high-performance liquid chromatography (HPLC) with UV or DAD (Smedley, 1994), fluorescence (Aerts *et al*., 1988), MS or MS-MS detection (Volmer, 1996).

MATERIALS AND METHODS

The samples of raw cow's milk (n = 60) were obtained from different milk suppliers and local vendors in the U.S.Nagar district. Milk samples were collected from all four quarters of the udder of the cows in sterilized centrifuge tubes. After collection of samples, they were immediately processed in the same day to avoid any contamination.

The standard drug solution of concentration 1mg/ml was first prepared by dissolving 5mg of sulfaquinoxaline in 5 ml of mobile phase. The stock solutions of sulfaquinoxaline were then further diluted with the mobile phase of HPLC to obtain working solutions with different concentrations of 1.0 mg/ml to 1ng/ml range.

10 ml milk was pipetted into the 125ml separatory funnel, and 50 ml of extraction solvent (Chloroform: Acetone, 2:1, v/v) was added. The mixture was allowed to stand for 2-3 minutes after vigorous shaking. Repeated shaking of the funnel was done for 3-4 times and finally allowed to separate into two phases. Acetone extract is filtered through Whatman no.2 filter paper into separate pear shaped flask. With 40 ml of extraction solvent milk was re-extracted two more times and filtered in the pear shaped flask.

The extract was evaporated in the solvent evaporator under nitrogen gas below 40 c. 5ml phosphate buffer was added to the flask and vortexed for 1 minute. 5ml hexane was added to the flask again vortexed for 5 minutes and allowed to separate into two phases.

Sulpeco C-18 cartridges were conditioned with 5ml distilled water followed by 5ml potassium phosphate buffer. Aqueous layer in the flask is passed through SPE cartridges and 5ml potassium buffer is added to the flask and vortexed. Again aqueous layer is passed through SPE cartridge.SPE cartridge was washed with 5ml of potassium buffer and 5ml of distilled water. Sulfaquinoxaline was eluted with 4ml aqueous acetonitrile (20% water). Acetonitrile was evaporated with solvent evaporator under nitrogen gas, and 0.5ml of ammonium acetate buffer was

added to the tube and filtered with 0.22µ millipore filter paper and injected 20ul into the HPLC.

The mobile phase consisted of Methanol: water (50:50 v/v) pH adjusted to 4 using glacial acetic acid. The flow rate was 0.5 ml/min. The injection volume was 20 ìl and the column temperature was 25°C. UV detection was at 254nm.

To validate our procedure, we determined the precision using relative standard deviation (RSD) and percentage recovery. Milk samples which are negative for sulfaquinoxaline were spiked 3 times with 25, 50 and 100 ppm of sulfaquinoxaline standard and subjected to extraction, clean-up and HPLC proce-dures used for the samples. The mean recovery values for the sulfaquinoxaline obtained from raw milk were between 85.5% and 90.8%, while the RSD value was 1.85%. These results showed good linearity and repro-ducibility with the r^2 value of 0.999 obtained from the linear curve.

RESULTS AND DISCUSSION

The results were interpreted based on the standard curve and the standard chromatogram. Standard curves prepared by spiking standards in the range of 0.01-5 µg/ml were linear with a coefficient of correlation () more than 0.99. Based on the peak area versus concentration data, the unknown sample concentration was determined. The RT value of standard Sulfaquinoxaline was 5.75 minutes. The recovery of samples ranged from 85-90%.

The lowest concentration from which it is possible to decide the presence of the analyte with reasonable statistical certainty is the limit of detection (LOD). The limit of quantification (LOQ) is the smallest measured content of the specific analyte in a sample that may be quantified with a specified degree of accuracy and within-laboratory reproducibility. In this study LOD was 0.01 µg/ml (10 PPB) and LOQ was 0.05 µg/ml (50 PPB). The results of this study indicated that out of total 60 milk samples only 11 samples were found to be positive for Sulfaquinoxaline and only one sample was above MRL. The concentration of positive milk samples along with the above MRL concentration is depicted in the Table 1.

Table 1
Distribution of residues of sulfaquinoxaline in different sub divisions of U.S.Nagar district of Uttarakhand.

Sub divisions	No of samples	Positive samples	Conc range (ug/ml)	Above MRL*
Kashipur	10	2	0.015 – 0.030	-
Jaspur	5	1	0.058	-
Rudrapur	10	2	0.034-0.048	-
Gadarpur	10	3	0.034-0.060	-
Sitarghanj	5	1	0.013	-
Kichha	15	2	0.019-0.18	1
Khatima	5	0	0	-
Total	60	11	0.0131-0.0598	

*MRL value for Sulfaquinoxiline in milk (Codex Alimentarius) is 0.01µg/ml

Considering the issue of public health hazards, milk and milk products contaminated with antibiotics and other chemical contaminants beyond a given residue levels, are considered unfit for human consumption (Goffova *et al.*, 2012). Occurrences of veterinary drug residues pose the broad range of health consequences for the consumers. The residues of antibacterials may present pharmacological, toxicological, microbiological and immunopathological health risks for humans (Drackova *et al.*, 2009). Antibacterial agents like tetracyclines, nitrofurans and sulphonamides are used as feed additives in cattle feed which may be excreted in milk and sometimes associated with toxicological effects in human (DeVries, 1997). Apart from the health hazards, antimicrobial residues in milk are responsible for interference with starter culture activity and hence disrupt the manufacturing process of milk products (Katla *et al.*, 2001). Although milk is not regulated at the individual cow basis, use of antibiotic screening tests for individual cow milk is associated with reduced risk of bulk milk residue incidence (McEwen *et al.*, 1997). The increased use of veterinary drugs in dairy herds resulting from the growth in traditional dairy production to meet the increasing population demand for milk and milk products as a major source of pro-tein have become a major global public health con-cern (Nisha, 2008). There are several national and international regulatory control and monitoring efforts to ensure the safety of livestock products meant for human con-sumption. This study determined the prevalence of antibiotic residues in milk and milk products for pub-lic consumption in U.S.Nagar district, Uttarakhand. The results of this study showed a greater prevalence in the samples of antibiotic residues in milk. Overall, antibiotic residue prevalence of 18.33% was obtained in the milk screened from U.S.Nagar district. In the present study, the mean concentration of determined sulphonamides was approximately 8 times lower than the established MRL. According to World Health Organization (WHO) (JECFA, 1995), the accepTable daily intake (ADI) for Sulfadimidine was established at 3 mg for 60 kg BW. The presence of antibiotics resi-dues could have resulted from self-medication by the herdsmen. This result also indicates that consumers of dairy products in U.S.Nagar district are exposed to public health risk associated with consumption of antibi-otic residues. However, there was one sample containing residue levels above MRL.

The present study provides a simple and accessible method for detection of Sulfaquinoxaline in biological samples like milk. This could be applied for other animal products like meat and egg samples to detect the minor quantity of residues using HPLC.

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Research Article

ANTI HYPERGLYCEMIC ACTIVITY OF *FLACOURTIA MONTANA* FRUIT JUICE AND *IN-SILICO* DOCKING OF ISOLATED ACTIVE COMPOUNDS

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ABSTRACT

Flacourtia montana (Family- Flacourtiaceae) was used in this study to evaluate anti-hyperglycemic activity of fruit juice in STZ induced type I diabetes model and analyzed for receptor ligand interaction of active phytoconstituents from n-butanol fraction using Discovery studio® version- 4.0.0.13259. Animals were randomized in to five groups of six each, all the groups except Group I were made diabetic by intraperitoneal injection of streptozotocin (STZ) at the dose rate of 45 mg/kg body weight.The Group I served as the normal control, Group II and III as STZ and glibenclamide control. The group IV and group V were administered with fruit juice @ 1 ml/kg and 2.5 ml/kg body wt. for 14 days. The result revealed an increase in blood glucose, triglycerides, total cholesterol, LDL-C, AST, ALT level and decrease in HDL-C and non-significant decrease in body weight in all groups except normal control on day zero. The group IV and V exhibited reduced blood glucose level and all other parameters in 14 days of treatment. In-silico docking revealed that coumarins and β-sitosterol β- D glucopyranoside exhibited dock score of 160.91 and 118.25 respectively. Further, *in silico* studies clearly offer an economical and efficient way of exploring the problem landscape, thus aid in the formulation of appropriate hypotheses for subsequent testing in (*in vitro* and/or *in vivo*) laboratory studies.

Key words: Anti-hyperglycemic activity, receptor ligand interaction, *in-silico* studies.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by a high blood glucose concentration (hyperglycaemia) caused by [fasting plasma glucose ≥ 7.0mmol/l (126mg/dl) or 2 h post prandial plasma glucose ≥ 11.1mmol/l (200mg/dl) following a 75g oral glucose load or random plasma glucose ≥ 11.1mmol/l (200mg/dl) in the presence of diabetes symptoms] insulin deficiency, often combined with insulin resistance (WHO, 2006). The consequences of diabetes include microangiopathy like nephropathy, neuropathy, retinopathy and macrovascular diseases like accelerated atheroma, stroke, myocardial infarction and cardiovascular disorders. It was known to ancient Indian physicians as 'Madumeha'. (Fakeye *et al.*, 2007).

India is a country with a vast reserve of natural sources with more than 1200 varieties of living species, identified with anti-diabetic property. An ideal oral treatment for diabetes is a drug that controls the glycemic level and prevents development of microangiopathy and macrovascular complications in cardiovascular and renal systems associated with diabetes mellitus. Plants of *Flacourtia sp*. are endemic to Kerala and grows in semi-evergreen and moist deciduous forests of Western Ghats, up to 1000 m -1800 m. There are reports on fruits and barks of *Flacourtia indica*, being used as appetizer, digestive tonic, diuretic, in jaundice, enlarged spleen, pyrexia, nephritic colic and gum in cholera (Nazneen *et al.*, 2009). Pharmacological investigation and characterization of *Flacourtia indica* are available (Amarsinghe *et al.*, 2007; Singh *et al.*, 2010) but literature is scare on *F. Montana* fruit juice. The preset study aims to evaluate phytochemical analysis and anti-hyperglycaemic effect of fruit juice of *F. Montana* in STZ induced diabetes model. Receptor ligand interaction of isolated active phytoconstituents was done by an in-silico approach to study the mechanism of natural compounds using molecular docking studies.

MATERIALS AND METHODS

Plant material

The ripened fruits were collected fresh from their natural stances 0f Wayanad district (coordinates-11.6050 N, 76.0830 E). Fruits of *F. Montana* were authenticated by Department of Botany, University of Calicut, Kerala. Freshly collected fruits were washed with distilled water, deseeded and triturated using mortar and pestle. The fruit juice thus was filtered to remove coarse particles and was used for oral administration. Qualitative phytochemical screening for the presence of secondary plant metabolites was done as per standard procedures mentioned by Harborne (2005).

Experimental animals

The antidiabetic study was conducted in 30 adult albino Wistar rats of either sex weighing 150-180g. The experiment was approved by the Institutional Animal Ethics Committee (IAEC). The albino Wistar rats were procured from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy. All the animals were maintained in well ventilated cages in the laboratory under standard managemental conditions for one week, to get acclimatized with new laboratory environment, before the

commencement of the experimental setup. The experiment was carried out for a period of 20 days.

Acute oral toxicity

Acute oral toxicity testing of fruit juice of *F. montana*was carried out according to the Organization for Economic Co-operation Development [OECD, TG-420 (2001)] guidelines along with the principles and criteria summarized in the Humane Endpoints Guidance Document [OECD, TG-423 (2001)].

Experimental design

Thirty albino Wistar rats of either sex were randomly divided into five groups comprising of six animals each as given below and maintained under standard laboratory conditions. They were injected with streptozotocin (STZ) at the rate of 45 mg/kg body weight intraperitoneally (King, 2012) on day zero and then evaluated for hyperglycemia on day 5. Only animals showing hyperglycemia following STZ treatment were selected for study.

From day 5 of STZ administration, animals were treated orally daily with the test substance. Blood samples were collected on day zero, day 7 and day 14 for estimating blood glucose. Also serum was separated for estimation of TG, TC, HDL-C, LDL-C and serum marker enzymes like AST and ALT. Body weight was also recorded on these days. The animals were sacrificed on day 15, pancreas and liver were collected. Pancreas was preserved in 10 per cent neutral buffered formalin for histopathological examination. Liver tissue was used for the estimation of glycogen content using standard protocols.

Data (Mean ± SD) obtained were analyzed by the following statistical tools to determine the level of significance (Snedecor and Cochran, 1994) and compared ($P < 0.05$) by analysis of variance (ANOVA) followed by Tukey's test using IBM® SPSS® statistics 20 software.

Isolation of active compound using RP-HPLC

Extraction and isolation of the active compound was done using RP-HPLC as per the protocols by Amarasinghe *et al.*, 2007 with slight modifications. Fresh ripe fruits (330 g) of *F. montana*were blended using a mechanical blender. The red colored juice was filtered using a funnel. Filtrate was partitioned with n-butanol and water. Evaporation of the n-butanol gave a dark brown solid. A portion was chromatographed over silica (n hexane–EtOAc–MeOH), followed by Sephadex LH-20 (MeOH) and reverse phase HPLC (STR Prep-ODS 20, 250 mm column; 65% H2O–MeOH, 5 ml/min; UV detection 254 nm) to furnish the active compound at 45 min.

Software models for *in-silico* docking

Screening and profiling of isolated constituents from fruit juice for target identification in preclinical studies was done using Pharmacophore program wherein, Discovery studio® version – 4.0.0.13259 was used. Active compounds isolated were β sitosterol, β sitosterol β D glucopyranoside, ramontoside, β butyrolactone, c butyrolacton, tannins and coumarins which acts as ligand against target receptor – Insulin (PDB-ID1I44). So obtained 3D dock image was analyzed for the docking site and saved for further studies.

RESULTS

Qualitative phytochemical analysis revealed the presence of secondary plant metabolites like alkaloids, flavonoids, glycosides, tannins, phenolic compounds, carbohydrates and saponins, whereas steroids, terpenes, gums and mucilages were not detected.

The results of the acute oral toxicity confirms that the fresh fruit juice possessed no toxicity at doses of 1, 2, 3.5 and 5 ml/kg, respectively. The animals were healthy, feed and water intake was normal. There was no behavioural changes during the entire period of study.

There was no significant difference among the treated groups with regard to body weight. The blood glucose level and serum triglycerides (Table 1) showed a significant difference ($p<0.05$) in group IV and V after 14 days of treatment with fresh fruit juice of *F. montana*but not as prominent as group III which was glibenclamide control.

Considering the lipid profile (Table 2); total cholesterol level and LDL-C was found to be highest in group II (STZ control). All the treatment groups showed significant ($p<0.05$) reduction in serum cholesterol and LDL-C level compared to group III and there was no significant difference among the groups. However group V (fruit juice 2.5 ml/kg) and group III (glibenclamide 5 mg/kg) showed significant ($p<0.05$) reduction in serum cholesterol and LDL-C level. After the induction of diabetes, all the groups except normal control showed a decrease in HDL-C level. Upon oral administration of test substances, there was a dose dependent increase in HDL-C level in all treatment group when compared with the STZ control. There was a significant ($p<0.05$) increase in HDL-C level for group IV and V as compared to the STZ control, though the treatments did not increase the HDL-C level to normal level.

Enzymatic parameters like AST and ALT level (Table 3) showed significant ($P<0.05$) difference wherein, Group II (STZ control) showed the highest level of serum AST and ALT. Serum AST and ALT value of group IV and V reduced significantly ($P<0.05$) after oral administration of test substances. The glycogen amount stored in liver depends on the physical, chemical and biological factors faced by rats (Table 3). Group II (STZ control) showed the lowest liver glycogen at the end of the experiment. There was a significant ($p<0.05$) reduction in liver glycogen levels in group II compared to group I (normal). The fruit juice treated group (group IV and V) and glibenclamide treated group (group III) showed a significant ($P<0.05$) increase in liver glycogen when compared to the group II (STZ

control).

The histopathology slides of the present study revealed that fresh fruit juice at higher doses have better activity than at the lower doses. Glibenclamide control slide found to have restored beta cells but not as prominent as of normal control (Image 1).

Results of *in silico* Docking for Receptor ligand interaction and 3D image of isolated constituents from fruit juice for target identification in preclinical studies was done using Discovery studio® have been shown in Images 2-9. Active compounds isolated were β sitosterol, β-sitosterol β-D glucopyranoside, ramontoside, β-butyrolactone, c butyrolacton, tannins and coumarins which acts as ligand against target receptor insulin (PDB-ID1I44). So obtained 3D dock image was analyzed for the docking site (Images 2-9).

DISCUSSION

Diabetes mellitus is an endocrine disorder involving derangements in the metabolism of carbohydrates, fat and protein. Although many drugs are available for the management of diabetes, they are usually expensive and have adverse effects like hypoglycemia and obesity. Screening of herbs for anti-hyperglycemic activity is of great significance in this context. Hence the present study was conducted to evaluate the antihyperglycemic effect of fruit juice of *Flacourtia montana* once daily for 14 days on streptozotocin (STZ) induced diabetic rats.

Qualitative phytochemical screening revealed the presence of phytoconstituents like alkaloids, flavonoids, saponins, glycosides and tannins which has antidiabetic activities (Kumar *et al.*, 2011). Phenolic compounds provides a positive correlation between their content and

Table 1:
Blood glucose (mg/dl) and serum triglycerides (mg/dl) of treatment groups(Mean ± S.E., n=6).

Gr	Day-0		Day-7		Day-14	
	Blood glucose	Serum triglycerides	Blood glucose	Serum triglycerides	Blood glucose	Serum triglycerides
I	103.35±0.7	107.85±0.74^{ns,D}	103.35±0.77	107.5±0.76^{ns,F}	103.17±0.65	107.67±0.67^{ns,E}
II	256.45±8.42	168.25±1.87^{ns,A,B}	258±7.63	167.67±1.86^{ns,A}	258±7.43	166.5±2.26^{ns,A}
III	267.22±6.42	174.71±2.05^{a,A}	136.33±0.43	125.67±0.56^{b,E}	123.33±0.49	114.67±1.63^{c,D}
IV	247.09±13.6	173.38±2.31^{a,A}	173.33±0.33	165.83±0.79^{b,A}	152.33±0.42	136.33±0.61^{c,B}
V	249.35±6.91	171.84±1.44^{a,A}	167.67±0.42	155±0.89^{b,B}	143.33±0.41	129.67±0.42^{c,C}

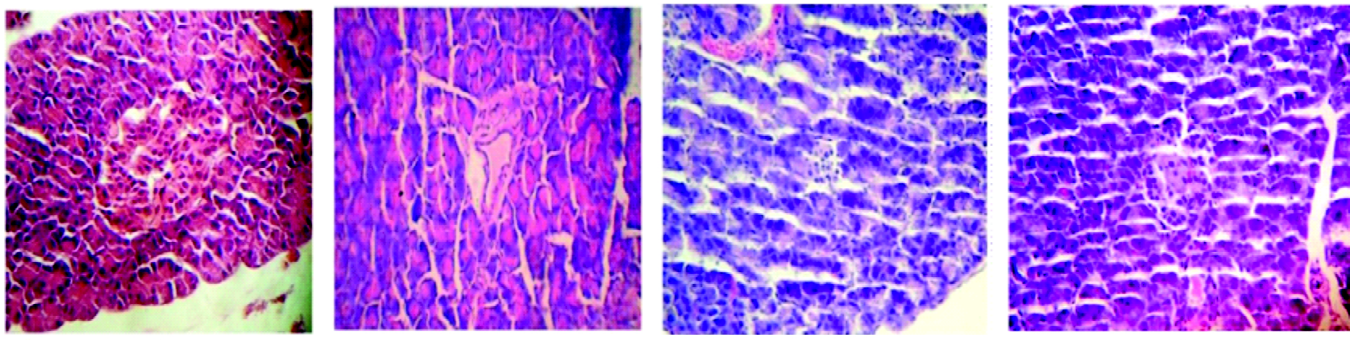
Table 3:
Serum enzymes (IU/L) and liver glycogen (g) of the treatment groups(Mean ± S.E., n=6).

Gr	Day-0			Day-7			Day-14		
	Total Chol	LDL	HDL	Total Chol	LDL	HDL	Total Chol	LDL	HDL
I	87.96± 1.08^{ns,B}	32.10± 0.49^{ns,C}	62.96± 0.63^{ns,A}	87.67± 1.02^{ns,E}	32.03± 0.58^{ns,E}	62.17± 0.6^{ns,A}	88± 1.13^{ns,F}	31.5± 0.67^{ns,F}	62.17± 0.6^{ns,A}
II	141.09± 0.75^{ns,A}	43.76± 0.54^{ns,A,B}	51.36± 0.59^{a,A,B}	141± 0.82^{ns,A}	43.17± 0.31^{ns,A}	42.67± 0.67^{b,D}	140.83± 0.79^{ns,A}	43.75± 0.37^{ns,A}	33.17± 0.6^{c,E}
III	139.6± 0.67^{a,A}	43.36± 0.34^{a,A,B}	53.04± 0.8^{b,A}	112.8± 0.79^{b,D}	37.01± 0.77^{b,D}	51.83± 1.11^{b,C}	93.33± 0.56^{c,E}	33.33± 0.71^{c,E}	59.67± 0.42^{a,B}
IV	139.98± 0.77^{a,A}	44.01± 0.35^{a,A}	52.27± 0.35^{b,A,B}	136.5± 0.43^{b,B}	41.67± 0.49^{b,A,B,D}	54.33± 0.33^{a,B,C}	131.83± 0.54^{c,B}	39.5± 0.34^{c,B}	55.33± 0.33^{a,D}
V	140.69± 0.54^{a,A}	43.17± 0.63^{a,A,B}	50.37± 0.48^{c,B}	133.3± 0.49^{b,B}	39.67± 0.21^{b,B,C}	54.83± 0.7^{b,B}	128.33± 0.21^{c,B}	37.67± 0.21^{c,B}	56.33± 0.49^{a,C,D}

Table 3:
Serum enzymes (IU/L) and liver glycogen (g) of the treatment groups(Mean ± S.E., n=6).

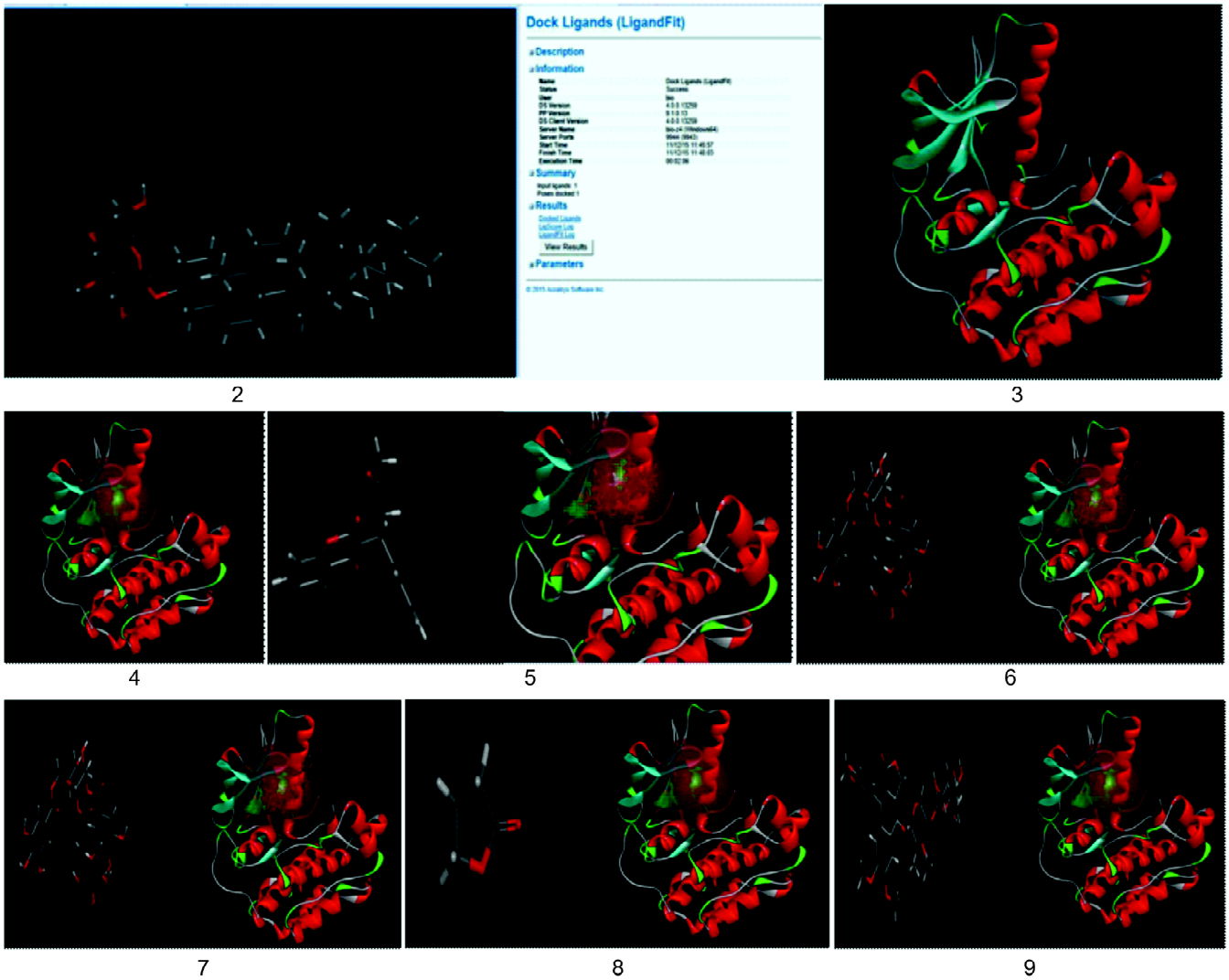
Gr	Day - 0		Day - 7		Day - 14		Day14
	AST	ALT	AST	ALT	AST	ALT	Liver glycogen
I	65.22± 0.56^{a,B}	20.52± 0.27^{ns,D}	54.67± 0.49^{b,E}	20.33± 0.33^{ns,F}	64.83± 0.54^{a,F}	20.67± 0.33^{ns,F}	4.891667±0.71^{ns,A}
II	87.71± 0.46^{ns,A}	43.34± 0.22^{ns,A}	87.83± 0.48^{ns,A}	43.5± 0.22^{ns,A}	87.83± 0.48^{ns,A}	43.5± 0.22^{ns,A}	2.401667±0.68^{a,F}
III	87.01± 0.38^{a,A}	42.57± 0.32^{a,A,B}	70.67± 0.42^{b,D}	31.17± 0.54^{b,D,E}	60.17± 0.48^{c,E}	25± 0.37^{c,E}	3.993333±0.73^{a,B}
IV	87.12± 0.46^{a,A}	42.96± 0.61^{a,A,B}	85± 0.52^{b,B}	37.67± 0.56^{b,B}	83.17± 0.4^{c,B}	35± 0.26^{c,B}	3.708333±0.59^{a,C}
V	86.4± 0.58^{a,A}	42.68± 0.37^{a,A,B}	83.33± 0.49^{b,B}	36.17± 0.31^{b,B,C}	81± 0.37^{c,C,B}	32.17± 0.31^{c,C}	3.961667±0.64^{a,B}

Means having same small letters as superscript indicate treatment are homogenous within rows and means having same capital letters as superscript indicate treatment are homogenous within a column.
Means with atleast one common superscript (a-c) for rows and (A-C) for columns doesn't differ significantly at 5 % level.



1 2 3 4

Image 01:
Microphotograph of pancreas from STZ induced (1), Fruit juice (@ 2.5 ml/kg) of *F. montana* treated (2) normal (3) and glibenclamide treated (4) rats, 400x .
Results of *In-silico* Docking: Receptor ligand interaction and3D image of isolated constituents using Discovery studio®.



2 3

4 5 6

7 8 9

Image 2-9:
2: 3D structure of 6-O-(E) –p-coumaroyl-β-D-glucopyranoside-glycoside; **3:** 3D structure of target receptor –Insulin (PDB-ID1I44); **4:** Eight active sites on target receptor-Insulin; **5:** 3D structure of Coumarins and its binding site with dock score of 160; **6.** 3D structure of â-sitosterol- â-D-glucopyronoside and its binding site with dock score of 112.5; **7:** 3D structure of Tannin and its binding site with dock score of 3; **8:** 3D structure of Ramontoside and its binding site with dock score of 0; **9:** 3D structure of β–butyrolactone and its binding site with dock score of 0.

respective antidiabetic activities and presence of polyphenols directly act on insulin secretion and prevents beta-cell apoptosis (Couman *et al.*, 2012).

Liver is the main effector organ for maintaining serum glucose level. In diabetes, serum markers enzymes such as AST, ALT, ALP and GTP can be co-related with the changes in metabolism or increased activities of transaminases, due to the absence of insulin secretion, increased gluconeogenesis and ketogenesis. In addition abnormal histological sings in structure of the liver were exhibited with prominent changes in diabetes (Gopal *et al.*, 2013). Red colored fruit juice of *F. montana*was partitioned with n-butanol and water. Chromatographic separation of n-butanol extract over silica gel, Sephadex LH-20 and reverse phase HPLC yielded compounds as mentioned in image 02. 3D Structures were established by analysis of spectral data including 2D Nuclear Magnetic Resonance (NMR) and Fast Atom Bombardment Mass Spectrometry (FAB-MS), respectively (Amarasinghe *et al.*, 2007).

Various techniques such as *in-silico* drug design visualization, homology, molecular dynamic, energy minimization molecular docking and QSAR are used in docking studies (Wadood *et al.*, 2013). Hence all the compounds used for molecular docking follows the Lipinski's rule of five – RO5 which specifies - no more than 5 Hydrogen bond donors (N-H and O2-H2 bonds), no more than 10 hydrogen bond acceptors (all N2/O2 atom), a molecular mass less than 500 Daltons and an octanol-water partition co-efficient log p not greater than 5. Hence, *in silico* tools are best applied in herbal drug research with the concept of summarizing and/or visualizing the complex patterns embedded within the output generated through associated 'omics studies.

Thus, the *in vivo* study confirmed the anti-hyperglycemic activity of fresh fruit juice of *Flacourtia montana* in STZ induced diabetes rats. RP-HPLC isolation of active compounds from n-butanol extract of the fruit juice yielded active constituents like 6-O-(E) –p-coumaroyl-β-D-glucopyranoside-glycoside derivate, β–sitosterol, ramontoside, β–butyrolactone, c butyrolacton, tannins and coumarins. Candidate molecule that confirms to the RO5 tends to have lower attrition rates. Thus, *in silico* studies clearly offer an economical and efficient way of exploring the problem landscape, thus aid in the formulation of appropriate hypotheses for subsequent testing in (*in vitro* and/or *in vivo*) laboratory studies.

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Research Paper

EFFECT OF MELOXICAM CO-ADMINISTRATION ON PHARMACOKINETIC OF CEFQUINOME IN COW CALVES

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ABSTRACT

The pharmacokinetic of cefquinome (20 mg kg^{-1}) was studied following intramuscular administration of cefquinome alone, co-administered with meloxicam (0.2 mg kg^{-1}) in cow calves. The concentration of cefquinome in plasma was detected by High Performance Liquid Chromatography. The minimal inhibitory concentration (MIC_{90}) of cefquinome was 0.04, 0.02, 0.6, 0.6, 0.15, and 0.07 µg ml^{-1} for *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Bacillus subtilis, Streptococcus pyogenes, and Proteus mirabilis*, *respectively*. Following Intravenous administration of cefquinome elimination half-life, volume of distribution (Vd_{ss}) and total body clearance (Cl_B) were 1.32 ± 0.03 h, 2.64 ± 0.05 L kg^{-1} and 1.41 ± 0.03 mL min^{-1} kg^{-1}, respectively. Following single dose intramuscular administration of cefquinome alone, peak plasma concentration (9.48 ± 0.23µg mL^{-1}) was observed at 0.75 h. The elimination half-life($t_{1/2\beta}$), volume of distribution (Vd_{area}), total body clearance (Cl_B), area under plasma drug concentration-time curve ($AUC_{(0-\infty)}$) and area under first moment curve (AUMC) of cefquinome were 1.82 ± 0.08 h,1.99 ± 0.18 L kg^{-1}, 0.72 ± 0.05 L h^{-1} kg^{-1}, 28.49 ± 1.70 µg.hmL^{-1} and 87.08 ± 4.01 µg.h^2/mL,respectively.Bioavailability of Cefquinome was good (148.30 ± 15.64 percent) following intramuscular administration in cow calves. Intramuscular co-administration of meloxicam and cefquinomeis advised as it did not altered pharmacokinetics of cefquinome.

Kew words: Cefquinome, meloxicam, pharmackokinetics, cow calves

INTRODUCTION

Antimicrobials and NSAIDs are used most frequently in multiple drug prescription.It is well established that co-administration of several drugs often results in unpredicTable therapeutic outcome. Cefquinome, a new fourth generation aminothiazolyl cephalosporin, has been developed solely for veterinary use. Cefquinome having zwitter-ionic property which facilitates rapid penetration across biological membranes including the porins of the bacterial cell wall resulting in improved bioavailability and extended spectrum of antimicrobial activity compared with the second and third-generation cephalosporins (Thomas *et al*., 2006). It has a broad spectrum of activity and susceptible to various clinically important bacteria such as *Streptococcus spp., Staphylococcus spp., Pseudomonas spp., Moraxella* spp*., Haemophyllus* spp*., Corynebacterium* spp*., Enterococci* spp*., Escherichia coli* and gram positive anaerobes. It is used for treatment of respiratory tract diseases, acute mastitis and foot rot in cattle, calf septicemia, and respiratory diseases in pigs, metritis-mastitis-agalactia syndrome in sows, foal septicemia and respiratory tract diseases in horses (CVMP, 2005). Meloxicam, a novel NSAID of the oxicam class, is one of the most potent inhibitors of inducible cyclooxygenase-2 (COX-2) and have anti-inflammatory, analgesic and antipyretic activitiesPharmacokinetics of cefquinome have been investigated in many species of animals and birds (Dinakaran *et al*., 2013; Shalaby *et al.,* 2015). However, there is no information available on the influence of co-administration of meloxicam on the pharmacokinetics of cefquinome in cow calves, therefore, the study was undertaken to determine effect of meloxicam on pharmacokinetics of cefquinome in cow calves.

MATERIALS AND METHODS

Experimental animals

The study was conducted on six male and female crossbred (Kankrej× H.F) cow calves of 2 to 6 month of age and weighing between 75.0 and 95.0 kilograms. The animals were housed in separate pens andprovided standard ration with *ad libitum* water. Animals were kept under constant observation for two weeks before the commencement of the experiment and subjected to clinical examination to exclude the possibility of any diseases. The experimental was performed as per guideline and approval given by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (F.No. 25/139/2014-CPCSEA dated10/12/2014).

Drug and chemical

Pure cefquinomesulphate powder (99.99%) was obtained from Sigma-Aldrich Pvt. Ltd., Mumbai, India and injectable cefquinomesulphate (25 $mgml^{-1}$; Cobactan 2.5%®Intervet India Pvt. Ltd., Pune, India) was procured from local market. Acetonitrile, trifluoroacetic acid and methanol (HPLC grade) were purchased from Merck India Ltd.

Drug administration and sample collection

The experiment was planned in cross over design

Table 1:
Comparison of pharmacokinetic parameters (Mean ± S.E.) of cefquinome after intravenous and intramuscular administration of cefquinome(20 mg/kg) alone or in combination with meloxicam (0.2 mg/kg) in cow calves (n = 6).

Pharmacokinetic parameter	Unit	Cefquinome alone (Intravenous)	Cefquinome alone (Intramuscular)	Cefquinome and Meloxicam (Intramuscular)
K_a	h^{-1}		1.39 ± 0.22	1.09 ± 0.14
β	h^{-1}	0.53 ± 0.01	0.38 ± 0.02	0.40 ± 0.02
$t_{½Ka}$	h	2.44 ± 0.61	0.56 ± 0.08	0.68 ± 0.08
$t_{½\beta}$	h	1.32 ± 0.03	1.82 ± 0.08	1.75 ± 0.09
C_{max}	$\mu g\ mL^{-1}$	-	9.50 ± 0.22	9.23 ± 0.25
T_{max}	h	-	0.75 ± 0.00	0.75 ± 0.00
$AUC_{(0-\infty)}$	$\mu g.h\ mL^{-1}$	14.26 ± 0.32	28.49 ± 1.70	26.57 ± 0.79
AUMC	$\mu g.h^2 mL^{-1}$	26.84 ± 0.95	87.08 ± 4.01	84.36 ± 3.12
$Vd_{(area)}$	$L\ kg^{-1}$	-	1.99 ± 0.18	1.81 ± 0.06
Vdss	$L\ kg^{-1}$	2.64 ± 0.05	-	-
$Cl_{(B)}$	$mL\ min^{-1}\ kg^{-1}$	1.41 ± 0.03	0.72 ± 0.05	0.75 ± 0.02
MRT	h	1.88 ± 0.03	3.07 ± 0.09	3.17 ± 0.04
F	percent	-	148.30 ± 15.64	-

*Significant at p<0.05, **highly significant at p<0.01.

to receive either an intravenous and intramuscular injection of cefquinome alone at the dose rate of 20 mg kg^{-1} or intramuscular injection of cefquinome (20 mg kg^{-1}) along with meloxicam (0.2 mg kg^{-1}) left jugular vein and left deep gluteal muscle in each six healthy cow calves with a 15 days washout period between administrations. Blood samples (2 mL) collected, before administration and at 5, 10, 15, 30, 45 min and 1, 1.5, 2, 4, 8, 12, 18, 24 and 36 h following intravenous and intramuscular administration of cefquinome were centrifuged (4116 g for 10 minutes) for plasma samples which were transferred to cryo-vials (2 ml) and then stored at –20°C and analyzed within 24h to quantify cefquinome concentration using HPLC.

Analytical assay of cefquinome and pharmacokinetic analysis

Cefquinome was assayed in plasma with modification of procedure described by Uney *et al.* (2011).The HPLC apparatus of Adept Cecil Instrument (England) C18 column was used. Chromatographic separation was performed by using reverse phase (4.6 × 250 mm) at room temperature. The mobile phase was a mixture of 0.1% trifluoroacetic acid and acetronitrile in the ratio of 85:15 with a pH of 3.94. Mobile phase was filtered by 0.2 μ filtersand pumped into column at a flow rate of 1.5 ml min^{-1} at ambient temperature. The effluent was monitored at 268 nm wavelength.

Calibration curve was prepared daily for drug concentration ranging from 0.16 to 166.6 μg ml^{-1} and the assay was found sensitive, reproducible and linear with R^2 = 0.998. Various pharmacokinetic parameters were calculated from plasma concentration of cefquinome using software PK solution (version 2.0).

Minimum inhibitory concentrations

Minimal inhibitory concentrations were determined against bacteria like *Streptococcus pyogenes* (ATCC 8668), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli*(ATCC 25922), *Salmonella typhimurium*(ATCC 23564), *Proteus mirabilis*(NCIM 2241) and *Bacillus subtilis*(ATCC 9372) which were procured from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune. Minimum inhibition concentration of cefquinome was determined by Micro-broth dilution technique (Wielgand *et al*., 2008)

Cefquinomeplasma concentration and pharmacokinetic parameters of different treatment groups were compared by "students' t" test using MS Excel (Version 2007).

RESULTS AND DISCUSSION

Plasma cefquinome concentrations at different time intervals and kinetic perameters collected following intravenous and intramuscular injection alone,cefquinomeco-administered intramuscular with

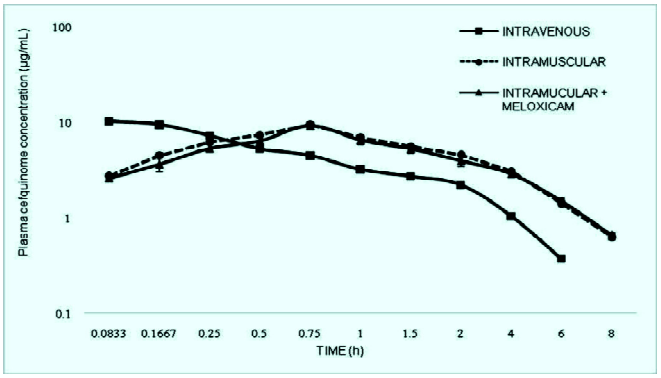

Fig 1.
Semilogarithmic plot of plasma concentrations of cefquinome concentrations following intravenous and intramuscular injection alone, cefquinome co-administered intramuscular with meloxicam in cow calves. Each point represents mean ± S.E of six animals.

meloxicam in cow calves is presented as semi logarithmic plot in Figure-1 AND Table i respectively. Following intravenous and intramuscular administration cefquinome (20 mg kg^{-1}) in cow calves alone and in combination of cefquinome and meloxicam (0.2 mg kg^{-1}) no adverse effects or toxic manifestations were observed. Peak plasma cefquinome concentration (C_{max}) observed in meloxicam co-administrated cow calves was not altered significantly as compared to cow calves given cefquinome alone.Similar findings were observed following concurrent administration of meloxicam and cefquinome in goats (Tiwari *et al*., 2015) and cefepimeand ketoprofenin goats, sheepand cow calves (Patel *et al*. 2012; Patil *et al*., 2012). In contrast, variations in pharmacokinetics of different cephalosporins have been observed following concurrent administration with NSAIDs.

Pharmacokinetic parameters were not altered significantly following intramuscular administration of cefquinome with meloxicam in cow calvesin comparison to cow calves given cefquinome alone. Similar observations were recorded with concurrent administration of meloxicam and cefquinome in goats (Tiwari *et al*., 2015), cefepime and ketoprofen in goats, sheep and cow calves (Patel *et al*., 2012; Patil, 2012) .

Cefquinome is a beta lactam antimicrobial and act as a time dependent bactericidal drug (Thomas *et al.,* 2006). For β-lactam antibiotics. The most important pharmacodynamic/pharmacokinetic parameter for this type of drug is the length of the time during which drug remains above MIC_{90}value (Turnidge,1998; Mckellar *et al*., 2004). It is generally recommended that % T>MIC should be at least 50 to 70% of the dosage interval to ensure an optimal bactericidal effect (Toutain andLees,2004). The minimum inhibitory concentration of cefquinome for a majority of cefquinome sensitive bacteria is in the range of 0.016 to 0.781 µg ml^{-1} (Zonca *et al*., 2011). The minimal inhibitory concentration (MIC_{90}) calculated for cefquinome in present study was 0.04, 0.02, 0.6, 0.6, 0.15, and 0.07 µg ml^{-1} for *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Bacillus subtilis, Streptococcus pyogenes, and Proteus mirabilis*,respectively. The drug levels above the minimum inhibitory concentration (MIC) were detected in plasma up to 8 h following single dose intramuscular administration of cefquinome alone and co-administrated with meloxicam.

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Short Communication

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

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ABSTRACT

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

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

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

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

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

The observations and results of this experiment are reproduced in Table 1. The mean normal rectal temperature among the rats in all the six groups was statistically similar, which was in the range of 99.35 ± 0.06 to 99.60 ± 0.04 ºF. The rectal temperature in the groups (II to VI) which received the Brewer's yeast was elevated (pyrexia temperature) which ranged between 100.68 ± 0.12 and 101.20 ± 0.06 ºF. The difference in rectal temperature between the respective two intervals in all the groups was significant ($P<0.01$). Subsequent to aspirin administration in Group III the temperature was significantly lowered ($P<0.01$) to 99.98 ± 0.04, 99.86 ± 0.04 and ºF at 1, 3, and 6 hr of post treatment, respectively. The temperature in Group IV rats, which received the extract at 100 mg/kg at the three post- treatment intervals ranged between 100.57 ± 0.07 to 100.61 ± 0.06 ºF, which was statistically similar to the pyrexia temperature of 100.80 ± 0.11 ºF. The pyrexic rats in Groups V and VI which received the extract at 300 and 1000 mg/kg respectively, showed the mean temperatures of 100.25 ± 0.11 to 100.45 ± 0.10 ºF and 100.11 ± 0.05 to 100.45 ± 0.05 ºF, respectively,

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

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

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

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

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

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Short Communication

IN VITRO ASSESSMENT OF ANTIVIRAL ACTIVITY OF *ERYTHRINA VARIEGATA* AND *SPONDIAS PINNATA* AGAINST PPR VIRUS

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ABSTRACT

In vitro antiviral activity of hydroalcoholic extract of *Spondias pinnata* (SPBE) and *Erythrina variegata* (EVBE) was assessed against PPR virus using Vero and PK-15 cells lines. Maximum non-toxic concentrations (MNTCs) of EVBE were 1.2mg/ml in Vero as well as PK-15 cells. The MNTCs of SPBE were 39.0625µg/ml (1:256) and 78.125µg/ml in Vero and PK-15 cells, respectively. The antiviral property against PPRV was assessed using selected MNTCs each extract. After visual observation of PPRV-specific CPE on the sixth day, to re-confirm the presence of CPE of PPRV in Vero cells (with or without bark extracts), a cell-ELISA was also performed and optical density was measured at 492 nm (A_{492}) in an ELISA reader. The OD of treatment groups was less than half of the OD set for the antiviral activity. Thus, the result indicated that the extract did not produce antiviral activity against the PPR virus growth.

Key words: Antiviral activity, *Spondias pinnata, Erythrina variegate,* PK-cell line, Vero cell line, PPR virus.

Erythrina variegate (syn. *Erythrina indica,* Common name- Indian coral tree), is a thorny deciduous tree growing upto 60 feet tall. A wide range of chemical compounds have been isolated, mainly alkaloids, flavonoids, triterpenoids, and lectin. Phytochemical analysis of hydroalcoholic extract of *E. variegata* revealed the presence of alkaloids, flavonoids, proteins, sterols, saponins and terpenes (Gupta *et al.* 2015). 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* have antibacterial activity. *E. variegata* exhibit 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). Bark powder of *E. variegata* and *Spondia spinnata* (Common name-Amara) have ameliorative effect on tissues of these organs in cadmium chloride induced toxicity in rats (Pankaj *et al.* 2015).

Vero (African green monkey kidney) cells between 130-150th passage levels were propagated in growth medium (GM) containing EMEM with 10% Fetal Bovine Serum (FBS) [Hyclone, Utah, USA] was used in this study. For maintaining the cells, MM containing EMEM with 2% FBS was used. PK-15 cells propagated in Eagle's minimum essential medium (EMEM) (Sigma-Aldrich, St. Louis, USA) containing 200mM glutamine , 7.5% (w/v) sodium bicarbonate (Sigma-Aldrich, St. Louis, USA) and 100x antibiotic and antimycotic solution (HiMedia, Laboratories Pvt. Ltd) supplemented with 10% horse serum (Cat No. 16050, New Zealand origin, Gibco) was used in this study. For maintaining the cells, maintenance medium (MM) containing EMEM with 2% horse serum was used. PPR virus at passage level 60th in Vero cell obtained from Division of Virology was used. Hydroalcoholic extract of the bark powder of *Erythrina variegata* (L.) and *Spondias pinnata* (L. f.) was prepared for phytochemical analysis and for this study.

To assess the cytotoxicity, 10mg/ml solution was prepared for both plants extracts in PBS. The plant extracts were diluted two fold in maintenance media (EMEM with 2% FCS and 5% DMSO) in a deep well plate. The diluted extracts were added to 24h old Vero cell monolayers in 96-well tissue culture plates. The plates were incubated in an atmosphere of 5% CO_2 at 37æ%C for 4 days. Similarly, the cytotoxicity of extract was determined in PK-15 cells. The plates were observed daily for any change under inverted microscope and compared with cell control. The highest concentration of the extract that showed no cellular morphologic changes was considered as the MNTC (maximum non-toxic concentration).

The selected dilution of each extract was prepared in maintenance media as stock solution (EMEM with 2% Serum and 5% DMSO). A two-fold dilution of each extract from stock solution was prepared. Vero cells were prepared in 96 well microtitration plate; after 24 h, the growth medium was removed and replaced with serial dilutions of extract (@ 50µl/well in maintenance media in duplicate as shown in plate outline below in Table 1.

Then, in first row of each extract, equal volume of pre-titrated PPR vaccine virus having titre of 10^5 $TCID_{50}$/mL was added (Table 1, VE: virus + extract). To ensure that the effect monitored was that of the extract alone, a set of controls like virus control (VC), extract (at different dilutions, E), and appropriate cell controls (CC) were included in the test. The plates were incubated at 37°C in a humidified CO_2 incubator, and maintenance media was changed on alternate day. The cells were observed for CPE regularly under microscope.

After visual observation of PPRV-specific CPE on

Table1:
Plate outline to test the antivirus activity of EVBE and SPBE against PPRV

	Sample	1	2	3	4	5	6	7	8	9	10	11	12
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	CC
A	SPBE	VE	VE	VE	VE	VE	VE	VE	VE	VE	VE	VE	CC
B	SPBE	E	E	E	E	E	E	E	E	E	E	E	CC
C	EVBE	VE	VE	VE	VE	VE	VE	VE	VE	VE	VE	VE	CC
D	EVBE	E	E	E	E	E	E	E	E	E	E	E	CC
E	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC	CC
F	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC	VC	CC

SPBE= hydroalcoholic *S.pinnata* bark extract, hydroalcoholic *E. variegata* bark extract, VE= Virus extract mixture; E= Extract alone; CC= Cell control; VC= Virus control

the sixth day, to re-confirm the presence of CPE of PPRV in Vero cells (with or without bark extracts), a cell-ELISA was also performed following method explained by Sarkar *et al*. (2013). Optical density was measured at 492 nm (A_{492}) in an ELISA reader.

The percent yield (w/w) of hydroalcoholic extracts of *Erythrina variegata* and *Spondias pinnata*, done by rotary vacuum pump extraction evaporator were 6.01% and 8.16%. Extracts of both of the plants (EVBE and SPBE) were subjected to preparation of 10^4µg/ml solution in PBS (pH 7.4) using triple glass distilled water. The dilution 1:8 did not show the toxicity, therefore, calculated MNTC of EVBE were 1.2mg/ml in Vero as well as PK-15 cells. Similarly the MNTC of SPBE was 39.0625µg/ml (1:256) and 78.125µg/ml (1:128, in Vero and PK-15 cells respectively.

There are reports of antiviral activity of bark of plant from the genus Erythrina. Meragelman *et al*. (2001) isolated senegalensein from the stem bark of *Erythrina senegalensis* which was found to exhibit HIV-inhibitory activity (Fomum *et al*., 1987). Tanee *et al*. (2007) reported antiviral and antibacterial activity of the bark of *Erythrina glauca* and *Erythrina lysistemon.* The possible antiviral activity was evaluated vs viral enzymes such as proteases and neuraminidases. Protease is linked to HIV while neuraminidases are related to Influenza. Inhibitors of viral neuraminidase played an important role in the treatment of influenza. *E. senegalensis* showed inhibitory activity on HIV-1 protease, thus letting foresee a prospect in research for treatment against HIV/AIDS infection. The roots ethyl acetate extract of *E. addisoniae* exhibited an antiviral activity against H1N1 and H9N2 neuraminidases.The antiviral activity of extract of plant was evaluated at the dilution ranging from 1:2 to 1:2048 using Vero cell line challenged with PPR virus for 5 days followed by cell ELISA and the optical density was read at 492 nm (A_{492}) in an ELISA reader. A cut-off value with two times the absorbance (A_{492}) compared to uninfected cells were set for declaring a well positive for antiviral activity as mentioned by Sarkar *et al*. (2013). The OD of treatment groups was less than half of the OD set for the antiviral activity. Thus, the result indicated that the extract did not produce antiviral activity against the PPR virus growth. Based upon the standard method for declaration of antiviral activity of an agent, the result indicated that the extracts EVBE and SPBE did not produce any antiviral activity against the PPR virus growth.

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